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**Sedentary aging results in structural impairment of the
neuromuscular junction and may be attenuated by
voluntary endurance exercise**

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List of Abbreviations

Ach	Acetylcholine
Acetylcholinesterase	AChE
AChR	Acetylcholine receptor
ADP	Adenosine Diphosphate
ARC	Animal Resources Centre
BGT	Bungarotoxin
BSA	Bovine Serum Albumin
CSA	Cross Sectional Area
DABCO	Diazabicyclo Octane
EDL	Extensor Digitorum Longus
EOM	Extra Ocular Muscle
EPP	Endplate Potential
F ₀	Tetanic Force
FITC	Fluorescein isothiocyanate
HSP	Heat Shock Protein
GH	Growth Hormone
GHRH	Growth Hormone Releasing Hormone
Hz	Hertz
IGF-1	Insulin Like Growth Factor 1
IP3	Triphosphoinositol
km	Kilometre

LrP4	Lipoprotein Receptor-Related Protein 4
MHC	Myosin Heavy Chains
mtDNA	Mitochondrial DNA
MuSK	Muscle Specific Kinase
NMJ	Neuromuscular Junction
NT	Neurotransmitter
OCT	Optimal Cutting Temperature Compound
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
RO	Reverse Osmosis
ROS	Reactive Oxygen Species
TA	Tibialis Anterior
TPA	Total Perimeter Area
YFP	Yellow Fluorescent Protein

Overview

A striking feature of old age is the decrease in muscle mass and strength, a condition known as sarcopenia. The loss of muscle strength is an important contributor to the frailty of aged individuals and therefore increases the risk of falls and other instability related injuries. One possible cause of sarcopenia is the reduced exercise activity of older individuals.

The neuromuscular junction (NMJ) is the connection by which nerves control muscle movement. This thesis investigates the possibility that alterations in the structures of the neuromuscular junction with ageing might contribute to impaired control and physical maintenance of aging muscle. Pre- and postsynaptic changes to the neuromuscular junction have been reported. These include the loss of nerve terminal from the postsynaptic membrane leading to denervation and fragmentation of postsynaptic clusters of acetylcholine receptors (AChRs) resulting in tiny islands of receptors compared to the healthy pretzel shape and also reduction in the total AChR area.

Exercise in ageing animals and humans have shown to elicit some positive effects on sarcopenic related changes like slowing down the rate of loss in muscle mass and strength. In the neuromuscular junction of old animals and rodents that engaged in exercise the age related changes were reported to be less severe and in some cases the NMJ recovered.

CHAPTER 1

INTRODUCTION

1 Introduction

1.1 What is Sarcopenia?

Sarcopenia refers to the loss of muscle mass and strength (Roubenoff and Hughes 2000).

Older individuals that have developing sarcopenia are much more likely to be disabled in this way compared with healthy individuals with normal muscle mass and strength (Janssen 2006). Sarcopenia tends to be accelerated by the reduced physical activity that accompanies old age, but inactivity may not be the only cause. Other factors that have been linked to the cause of sarcopenia include malnutrition, oxidative stress and impaired regulation of sex steroids and growth hormones. Some of the key anatomical features seen associated with sarcopenia are a decrease in the muscle mass and cross sectional area, which most likely contributes to the decline in muscle strength observed (Kamel 2003), since the cross sectional area of a muscle is proportional to the force developed by the muscle (Brooks and Faulkner 1994). The decrease in muscle cross sectional area and volume is associated with the decrease in muscle fibre numbers and also the atrophy of those muscle fibres that remain (McArdle, Vasilaki et al. 2002). In previous studies of sarcopenia it was shown that the decrease in muscle strength was well correlated with a decrease in the mass of muscles of fast and slow muscles (Brooks and Faulkner 1994).

Muscle fibre atrophy is increased with age where the rate of breakdown of proteins exceeds the rate of protein synthesis (Roubenoff and Hughes 2000). With increasing age the rate of myosin heavy chains (MHC) synthesis begins to decline (Williams, Higgins et al. 2002).

MHC is an important contractile protein in muscle and when remodeling of MHC is impaired it is likely to contribute to the decline in muscle mass with advancing age (Kamel 2003).

Reduction in muscle mass with old age can impact the aerobic capacity of muscle in a negative manner.

Loss of muscle with aging can disrupt other regulatory functions of muscle in the body.

When muscles are used to produce force it actually assists in maintaining bone density and structure. As people age and muscles become less frequently used bone density is also reduced (Goldspink and Harridge 2004). The muscle atrophy causing the decrease in muscle mass and size seen in old people is potentially caused by injuring muscle fibres and an inability of the muscle to regenerate and repair itself. As muscle atrophy and become less used there is also less heat production, heat is produced in muscles during use. The lesser heat production may make it more difficult for elderly people to regulate body temperature (Güneş and Zaybak 2008)Sarcopenia related changes

1.2 Sarcopenia Related changes

1.2.1 Age related alterations to protein synthesis in muscle

Many changes are observed with the onset of sarcopenia. A suggested reason why we see the decrease in the skeletal muscle mass in sarcopenia is due to upset in the balance of protein synthesis to protein breakdown. However it was shown in animal studies that it was unlikely muscle atrophy in ageing is due to changes in protein synthesis (Mosoni, Patureau Mirand et al. 1993).

In studies related to human subjects it was found that the age related reduction in the rate of mixed protein synthesis involved a decrease in synthesis of myofibrillar proteins (Welle, Thornton et al. 1993). The mitochondrial protein synthesis is also reduced with ageing and this may contribute to the decrease in aerobic capacity and muscle performance. In young

human subjects the rate of mitochondrial fraction protein synthesis rates declined rapidly once they reached middle age, but no more changes were seen with further advances in age, which was observed to be accompanied by decline in mitochondrial enzymes (Rooyackers, Adey et al. 1996). The most important period of decrease in fractional protein synthesis protein synthesis was seen to happen rapidly between young and middle age. Thus the decrease in protein synthesis may have an impact on the slow age related decline in muscle mass, but would not completely account for it.

The theory that breakdown of proteins increases with age causing decrease in skeletal muscle mass is poorly understood. This factor is hard to measure as a large amount of proteins in muscle are myofibrillar, which turnover at a very slow rate and the current methods have many imperfections making quantitations unreliable (Attaix, Mosoni et al. 2005). The best known proteolysis pathway takes place through lysosomal proteases (cathepsins) breaking down proteins. However the contribution to overall muscle protein breakdown by cathepsins is not significant and cathepsins are not involved in the breakdown of myofibrillar proteins (Mitch and Goldberg 1996).

The muscle wasting that is seen in sarcopenia is hypothesized to be due to the imbalance in natural protein regulation, but it could also be due to the unresponsiveness of the muscle to external factors. In young rats, 1 hour after feeding (eating normally) there was an increase in protein synthesis rates (Garlick, Fern et al. 1983).

Other studies comparing adult middle aged animals with young animals found that external factors had a much more prominent effect on protein synthesis in the young animals. Fasting was able to reduce the protein synthesis significantly just overnight, while during feeding it

was shown that the young animals had a higher protein synthesis rate than the adult animals (Baillie and Garlick 1992). The absolute and fractional protein synthesis has shown to increase postprandial in young adult animals, but is impaired in old animals. In human subjects the administration of amino acid and glucose mixture nutritional supplement the protein synthesis was increased in young subjects but remain unchanged in old subjects. This supports the idea that during a state of hyperaminoacidemia and hyperinsulinemia muscle anabolism becomes impaired in elderly subjects due to the unresponsiveness of protein synthesis (Volpi, Mittendorfer et al. 2000). The pattern in which protein is supplemented can have an impact on protein anabolism. In elderly women protein supplements taken in pulses allowed higher retention of protein and increased protein synthesis rates. This method of protein intake was shown to be more efficient than taking protein in an even spread (Arnal, Mosoni et al. 1999).

Insulin has shown to potentially work in conjunction with amino acids to induce protein synthesis. When insulin secretion is blocked in rats it reduced protein synthesis regardless of age or if protein supplements were given (Prod'homme, Balage et al. 2005). Hormonal alterations are also known to contribute to developing sarcopenia.

1.2.2 Hormonal changes as causes of sarcopenia

Hormonal changes have also been shown to be involved in the development of sarcopenia. It has been shown that with age there is a decrease in GH (growth hormone) and IGF-1 (Insulin like growth factor 1). These endocrine changes are mainly due to altered hypothalamic factors like somatropin and GHRH (growth hormone releasing hormone) (Karakelides, Nair et al. 2005).

When the subjects were given a biosynthetic human growth hormone it increased their serum IGF-1 levels by 2-3 fold, which is roughly a normal level for young healthy men. Treatment with this biosynthetic hormone was associated with an increase in lean body mass, decrease in adipose tissue and increase in the lumbar vertebral bone density (Rudman, Feller et al. 1990). Although there seem to be positive effects by growth hormone supplementation some adverse effects can be caused by extended periods of this kind of treatment like hyperglycemia, carpal tunnel syndrome and gynaecomastia (growth of breast tissue in males). In much shorter periods of treatment there are no adverse effect detected and positive developments like increase in arm circumference and positive nitrogen balance in malnourished men (Kaiser, Silver et al. 1991). When growth hormone therapy was used in conjunction with diet interventions the walking speeds and the lean body mass of subjects improved (Chu, Lam et al. 2001).

Alterations in the male sex steroid hormone testosterone may also play a role in developing sarcopenia. It has been shown that the level of free serum testosterone is linked to muscle mass and strength. In male subjects it was found that muscle mass was positively correlated with the serum levels of free testosterone and IGF-1 (Baumgartner, Waters et al. 1999).

It has been suggested that testosterone has the capability to increase protein synthesis and IGF-1 so maintaining adequate levels of this hormone can potentially slow down the rate of sarcopenia.

Studies, which examined the effects of intramuscular administration of testosterone showed that in old men it caused a significant increase in the serum GH and IGF-1 levels (Gentili, Mulligan et al. 2002). This stimulation of GH production may have an indirect effect on body mass. In subjects who have low testosterone showed improvements in lean body mass

when testosterone supplements were given (Tenover 1992). With improvements in body mass, testosterone may also contribute to increase in muscle strength.

Male subjects with low testosterone levels showed improvements in hand grip strength after intramuscular administration of testosterone (Morley, Perry et al. 1993; Sih, Morley et al. 1997). Lower limbs have also shown to benefit from testosterone supplements where leg press strength improved dramatically (Wang, Swerdloff et al. 2000).

Overall in studies that involve giving testosterone supplements to old subjects, mainly men who have low testosterone levels can improve muscle mass, strength and decrease body fat.

The menopausal transition involving alterations in female sex steroids may also be a contributing factor in the development of sarcopenia in women. Women who experienced menopause show an increase in fat mass, a decrease in fat free mass and the amount of energy that was burnt during rest and physical activity when compared with women who are pre menopausal (Poehlman, Toth et al. 1995). Similar to testosterone, estrogen also has a stimulatory effect on growth hormone release, which plays role in maintaining body mass and strength throughout a person's lifetime (Veldhuis, Evans et al. 2001). Post menopausal women on hormone replacement therapy did not show any positive association with muscle mass or strength compared to age matched controls (Kamel, Maas et al. 2002), but did show an effect in preventing increase of abdominal body fat and bone loss (Haarbo, Marslew et al. 1991).

1.3 Reactive Oxygen Species

In studies it has been indicated that reactive oxygen species (ROS) can cause oxidative damage to lipids and proteins with aging. The main source of ROS has been hypothesized to be from the mitochondria respiratory electron transport chain. The ROS from here can damage the mitochondrial DNA (mtDNA), which in turn leads production of more ROS and ends in a vicious cycle (McArdle, Vasilaki et al. 2002). Some studies have investigated whether it is possible to suppress the production of ROS by certain compartments of cells so the levels of ROS is reduced (Malcolm J 2009).

With ROS damage to mitochondria leads to reduced density of mitochondria and reduced aerobic capacity in muscles (Kerner, Turkaly et al. 2001). Studies investigating the oxidative capacity of muscle in young and old animals have shown that in old animal tissue the adenosine diphosphate (ADP) concentration is generally higher, suggesting that the phosphorylation potential is lower in old tissue due to a lower oxidative capacity (Taylor, Kemp et al. 1997). In previous studies it was discovered that the reduction in aerobic capacity may be due to the reduction in mitochondrial volume. This may be related to increased levels of oxidative stress (Navarro, Gomez et al. 2004). This is linked to the decreased mitochondrial function of associated enzymes involved. In some cases it was even found that the levels of protein carbonyls are increased, which is a marker of oxidative stress.

1.4 Muscle Adaptations and Damage Susceptibility with Aging

Once a person ages the ability of their muscles to adapt or respond to physical activity and their resistance to muscle damage may potentially change. The muscle CSA (cross sectional

area) and volume begin to decrease with age due to loss of fibres and atrophy in the remaining fibres. This reduction in the muscle size leaves them more susceptible to damage and requires a longer recovery time. It has been long believed that ROS has a role to play in skeletal muscle damage and the reduction in force production capabilities. In current observations and studies it is seen after the most damaging type of muscle contraction (lengthening) small focal lesions appear in muscles causing slight force decline. However after a couple of days a large amount of neutrophils and macrophages appear in the muscle. Presence of neutrophils and macrophages are believed to be the secondary source of ROS and can cause a large decline in force, but recovery from this stage is possible (McArdle, Vasilaki et al. 2002).

Currently most views on the effects of ROS are quite negative, but there is some evidence now which suggests ROS can also be beneficial in the adaptive mechanisms of muscle. Large amounts of contractile activity can lead to oxidation DNA of proteins and lipids, but muscle can adapt by increasing the transcription and production of antioxidant enzymes and HSPs (heat shock protein), which provides protection against potential oxidative damage (Close, Haggan et al. 2007). HSPs (HSP70) have the ability to prevent muscle damage and aid rapid recovery and remodeling. It has been found that production of super oxides and reversible oxidation of proteins causes higher levels HSPs to be synthesized so the role of ROS in muscle damage is controversial at the moment, since it also plays a role in the muscle adaptation mechanisms. In other studies it has been shown in mice following a period of lengthening contractile exercise the overexpression of HSP70 aided muscle repair and prevented loss of force production. However in old mice this process appears to be severely impaired. The protective response that HSPs offer is a preventive mechanism, it

was seen in an experiment where two bouts of lengthening contractile exercise were separated by a period of 4 weeks and the amount of HSPs (HSP27 and HSP70) in the second bout of exercise was much lower than that of the first bout of exercise. So it is suggested that the HSP produced in the first bout of exercise had aided the muscle to develop a tolerance to damage from contraction (Kayani, Morton et al. 2008). Besides oxidative stress other stressors can also elevate HSP levels, like changes in pH, temperature and depletion in glycogen stores.

1.5 Motor unit changes as a cause of sarcopenia

The physical nature of sarcopenia involves the reduction in muscle fibre size (atrophy) and this could be due to a decline in motor unit numbers. In humans it has been observed that the time when muscle fibres begin to atrophy occurs is roughly at 50 years of age, which is the same time that motor unit numbers begin to decrease. This may suggest that the two mechanisms are linked (Faulkner, Larkin et al. 2007). Decrease in number of functioning motor units is also associated with the loss of muscle strength in sarcopenia. In humans it was found that when the extensor digitorum brevis muscle wasting and weakness occurred with ageing (60-96) it was caused by a decrease in the number of functioning motor units (Campbell, McComas et al. 1973).

1.5.1 Contribution of motor unit loss to reduction in muscle strength

The maximum isometric tetanic force in old animals was found to be lower than in young adult animals and this was accompanied by an equivalent decrease in the number of motor units. The degree to which motor units are affected with ageing depends on the motor unit type. In fast fatiguable (type IIa and IIb) motor units the maximum isometric tetanic force

(F_o) declines with age, but increased in slow motor units. This decrease in F_o was due to reduction in the muscle fibre CSA, which leads to decline in muscle mass. The remodeling process of motor units involved a loss of muscle fibres from the fast fatiguable units and increase in fibres in slow units. This also supports the theory where fibres become denervated and reinnervated by sprouting terminals causing fibre conversion as the fibres in fast units was seen to decrease while slow units increased (Kadhiresan, Hassett et al. 1996). However this compensatory mechanism of reinnervating fibres that have become denervated does not last into the advanced stages of ageing and the regions in which the sprouting originates from becomes unstable. The loss in sprouting is hypothesized due to the weaker repair response towards impairment and injury in aged individuals as compared to young.

1.6 Development of the Neuromuscular Junction

The NMJ is the synapse between the motor axon and muscle fibre through which the motor neuron elicits muscle contractions by release of acetylcholine. The main features of the NMJ include the presynaptic nerve terminal where the neurotransmitter acetylcholine is released and the acetylcholine receptors (AChR) on the post synaptic membrane where the neurotransmitter binds and elicits an endplate potential (EPP) that trigger muscle action potentials and muscle contractions (Conti-Fine, Milani et al. 2006)(Fig 1.1). Recent evidence outlined below suggests that NMJ loss is correlated well with whether they are innervated by motor neurons of the spinal cord or brainstem (Valdez, Tapia et al. 2012). Thus it is important to consider the factors that might influence the formation, maintenance and regeneration of the neuromuscular junction, and what might make it vulnerable in aging.

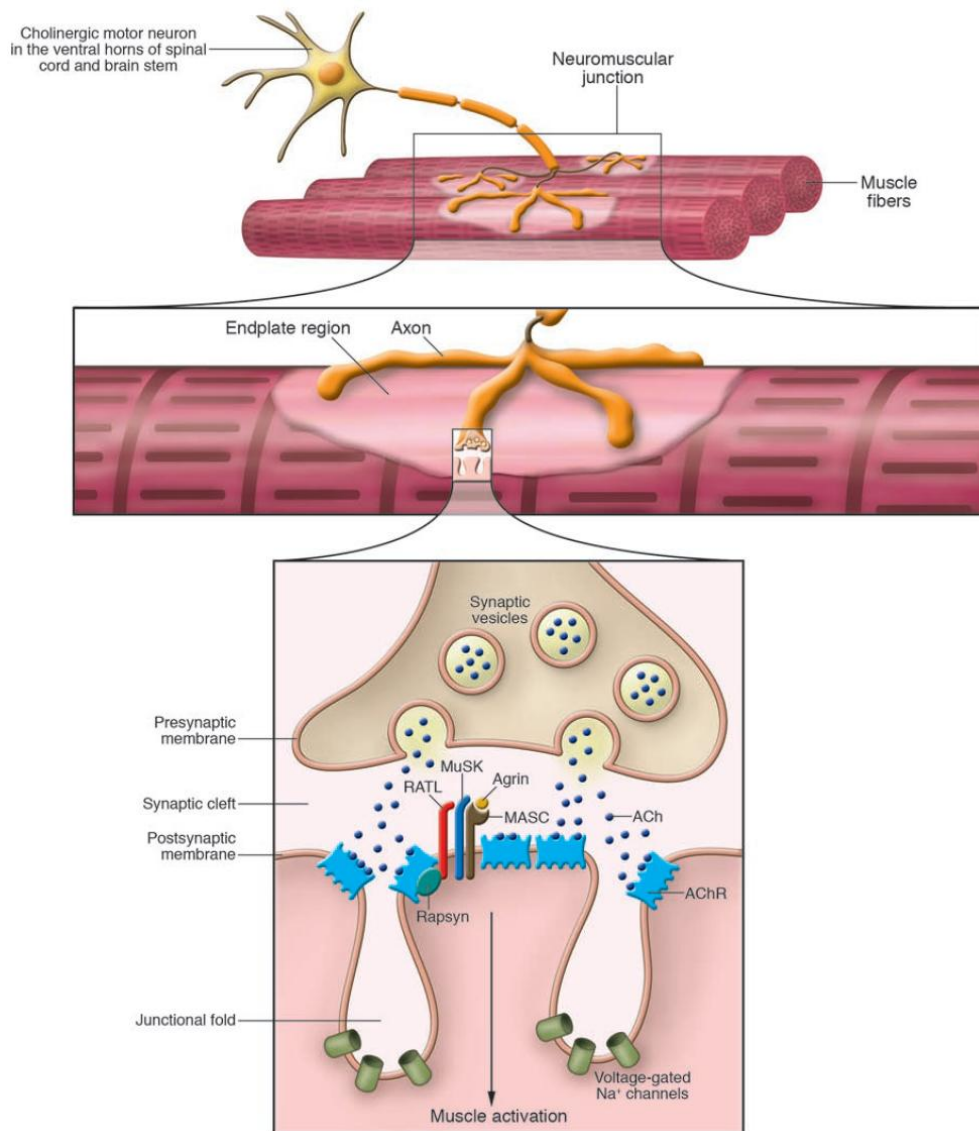


Figure 1-1 Structure of the neuromuscular junction. The motor neuron extends down to the muscle fibre where it releases synaptic vesicles containing acetylcholine. The acetylcholine travels across the synaptic cleft and interacts with the AChRs on the postsynaptic membrane where it causes depolarization of the membrane by opening up the voltage gated sodium channels inside the junctional folds of the membrane resulting in influx of sodium ions. The influx of sodium ions allows the generation of an endplate potential and once it reaches a threshold it results in an action potential to propagate down the muscle fibre eliciting muscle contractions. (Figure modified from (Conti-Fine, Milani et al. 2006)

The development of the NMJ begins with appearance of the premature motor neurons. The neurons will begin to extend an axon from the spinal cord and discover their own paths which lead them to the muscles they are supposed to innervate. Once the axon makes contact with the muscle, it will start sprouting. However this process may be guided by pre patterned aneural AChR clusters that had already formed on the muscle fibre (Gordon, Gribble et al. 2012). In mutant mice that lack Muscle Specific Kinase (MuSK) and aneural clusters displayed nerve terminals that were stray from the muscle fibre centre region, suggesting that the pre patterned aneural AChR clusters plays a role in guiding nerve terminals (Jing, Lefebvre et al. 2009).

1.6.1 The Agrin-MuSK-Rapsyn pathway in NMJ formation

One important pathway that is involved in postsynaptic differentiation of the NMJ is the agrin-MuSK-rapsyn pathway. A question asked about the agrin-Musk pathway is how agrin transmits its signals to MuSK. It has been reported that the transmembrane protein low-density lipoprotein receptor-related protein 4 (Lrp4) is needed for development of the NMJ (Weatherbee, Anderson et al. 2006). Lrp4 is an agrin co receptor, which agrin binds to and is needed for MuSK internalisation upon activation and AChR clustering. In the NMJ agrin is a heparin sulfate proteoglycan synthesized by motor neurons and released from the presynaptic nerve terminals where it then becomes associated with the basal lamina. It is known that agrin is secreted from the motor neuron and causes postsynaptic differentiation as it causes aggregation of many postsynaptic components and most importantly clustering of AChRs (Wallace 1989).

Agrin alone is not the only growth factor needed for clustering of AChRs. AChRs clusters can be formed in the absence of nerve or can be induced by nerve, but in animals which lack MuSK the aneural AChRs cluster never form, which suggests MuSK is critical for NMJ development (Lin, Burgess et al. 2001). Agrin released from the nerve alone was not able to induce AChR clusters, but the sensitivity to agrin was restored when MuSK was expressed. This implies that for the formation of aneural pre patterned AChR clusters only MuSK is required, but for neural induced AChR clusters both agrin and MuSK is needed (Herbst and Burden 2000). It is also observed that when MuSK is absent the presynaptic nerve terminal innervated a much broader region (Yang, Arber et al. 2001). A third protein involved in this pathway is rapsyn, which causes AChR clustering. It has been shown that rapsyn interacts with α -actinin and β -catenin and when agrin stimulates rapsyn it interacts with AChR and with α -actinin, which causes clustering (Dobbins, Luo et al. 2008).

The Wnt ligand is hypothesized to be presented to the motor axons to trigger NMJ formation. Studies have reported that before innervation occurs and in the absence of neural agrin Wnt and MuSK signaling was able to regulate axonal guidance and cause aneural clustering of AChRs. After innervation the Wnt-Musk complex interacts with the agrin/Lrp4/Musk pathway to regulate agrin-induced AChR clustering (Wu, Xiong et al. 2010). This suggests that before innervation Wnt is a MuSK ligand, but afterwards play a role in nerve induced AChR clustering.

1.6.2 Role of IP3 receptors in the Agrin-MuSK-rapsyn pathway

IP3 receptors also play a role in regulation of AChRs during NMJ development. It was discovered that in myoblasts the stimulation of AChR caused a transient increase in

cytoplasmic calcium and dispersal of AChR, which requires the activation of a calcium activated protease (calpain). It is hypothesized that the source of calcium originates from IP₃ receptors in skeletal muscle. When IP₃ receptors were silenced it prevented the dispersal of AChRs during NMJ development (Zhu, Bhattacharyya et al. 2011). During NMJ development opposing Lrp4, MuSK and rapsyn, which act to form pre patterned AChRs, acetylcholine is able to activate a pathway involving subsynaptic AChRs, IP₃ receptors, calcium and calpain to dismantle developing AChR clusters (Morsch, Reddel et al. 2013). In development of the NMJ in the event of agonist induced AChR dispersal requires IP₃ receptors for calcium release and calpain activation.

1.6.3 Maturation of axons in the developing NMJ

In rodents it is seen a few weeks after birth the extramuscular sections of the axon becomes myelinated but the parts of the axon still stay unmyelinated. Over several postnatal weeks synapse elimination will cause many of the number of multiple innervated NMJs to decline (Lichtman and Colman 2000). This is due to during development the innervations of junctions will favour one particular axon, so the other axons will then retract, but this does not happen instantaneously but rather in a stepwise fashion. The remaining axon will broaden its contact on the muscle (Fig 1.2).

1.6.4 Neurotransmission and synaptic elimination

Axons can release the neurotransmitter acetylcholine (ACh) even when they are not in contact with muscle, but contact with muscle increases the amount of quantal content release (Xie and Poo 1986). Quantal content, which is the amount of vesicles containing acetylcholine released per nerve impulse is correlated with size of the synapse and this act

as competition as to which synapse is ultimately withdrawn and it is usually the smaller ones that are eliminated. It is relatively important during this process that the motor neuron innervates muscle fibres with similar properties. However an alternative method that may be employed is through a molecular recognition system where there is activity dependent expression of surface molecules so that only the most compatible pairs will be retained. The postnatal changes also improve the NMJ function since there is an increase in the accumulation of synaptic vesicles therefore increasing quantal content, which will cause the NMJ less likely to fatigue (Fox 2009).

The immature NMJ has high input resistance meaning that only small amounts of Ach is needed to elicit an action potential, but once the muscle grows the input resistance is lowered and a larger amount of Ach is required to elicit an action potential. At birth AChRs and acetylcholinesterases (AChE) occupy plaques on the muscle which define the NMJ and once synaptic elimination is complete the nerve terminals will expand and the regions with high density of AChRs and AChE become associated with the nerve (Slater 2009). There are also areas of the postsynaptic membrane containing synaptic folds (synaptic gutters) where the voltage gated sodium channels are believed to be located. Many components of the immature NMJ become converted to the mature form when during development. The expression of the immature AChRs as well as its subunits and voltage gated sodium channels become suppressed when muscle activity is present and the mature genes of AChRs and voltage gated channels become expressed, which do not get suppressed from muscle activity. During regeneration in the ageing NMJ it is likely that the same mechanisms involved in embryonic development also play a role.

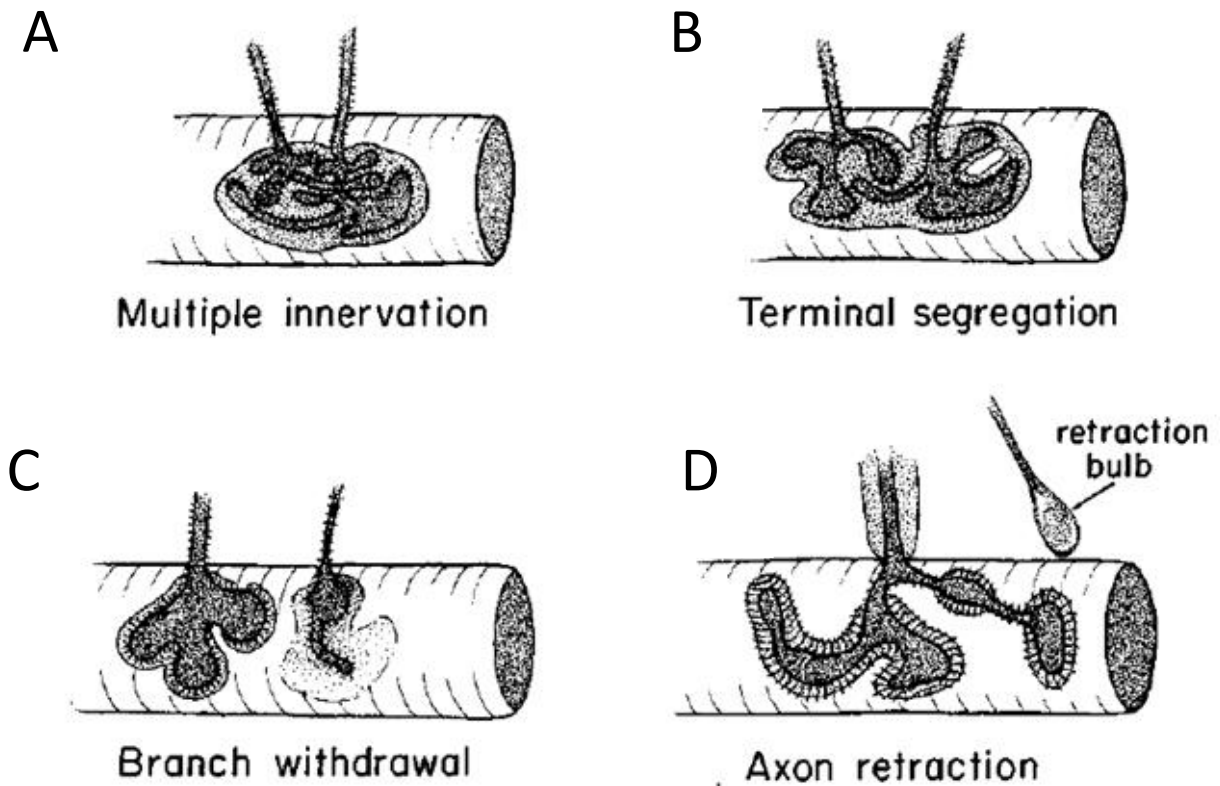


Figure 1-2 Synaptic elimination in NMJ development (A) Immature NMJ with multiple axons innervating the same muscle fibre. **(B)** Synapse elimination in the NMJ causes the competing nerve terminals of the multiple axons to begin separating. **(C)** Only 1 axon starts to become favoured and other axons begin to withdraw from the synapse. **(D)** Other axons have become withdrawn from the muscle fibre leaving only 1 axon to innervate it, allowing expansion of the nerve terminal and growth of the NMJ. Modified from (Sanes and Lichtman 1999)

1.7 Rodent models of ageing

Rodent models of ageing offer several advantages. In particular the animals are relatively short lived so the onset of ageing can be observed quicker. However, the main issue is the similarity in age related changes during the onset of sarcopenia. During ageing loss of motor units in fast muscle fibres is something that is observed both in humans and rats ageing studies (Jang and Van Remmen 2011).

Another feature in which the rodent sarcopenia resembles human sarcopenia is that both rodents and humans experience denervation of muscle fibres (Wang, Zheng et al. 2005).

Rodent studies make it possible to collect the muscles of interest from multiple animals for studies of the effect of age upon the motor neurons and muscle. The survival curve (Jackson Lab) indicates that 3-6 months is considered young adult, which is equivalent to 20-30 years old in humans, while 10-14 months is middle aged, which is equivalent to 38-47 years old in humans. The final comparison is late old age, which is 18-24 months and 56-69 years old in humans (Flurkey, Curren et al. 2007).

1.8 Age-associated impairment to the NMJ

Comparison of NMJs from rodents with those of young adults has revealed several age-associated changes in structure. Repeated live imaging of 15 month old mice showed a reduction in stability of the aged NMJ from about 20 months of age. When mice were observed at older ages it was found that the area covered by the nerve terminal branches and the underlying AChR rich regions were reduced. AChR clusters were found to disappear before the loss of the overlying nerve terminal (Balice-Gordon and Lichtman 1993). The

function of the endplate was also reported to deteriorate with age where low frequency stimulation of nerves failed over half the times tested to cause a muscle contraction and using high frequency failed to cause a muscle contraction at any of the times tested (Balice-Gordon 1997).

1.8.1 Pre and postsynaptic changes with age in rodent NMJs

Although age related changes are observed in the NMJ, these changes varied depending on the muscles that are studied and activity levels as well. In Fischer 344 rats it has been reported in the EDL (elonedated digitorum longus) and soleus that the amount of sprouting decreased with age, but in the diaphragm was maintained. The proliferation of nerve terminal branches was seen to increase with age in the diaphragm, but decreased in hind limb muscle. The presynaptic nerve terminal branch number appeared to fluctuate with age, where a sudden increase was seen at the age of 28 months, but this was followed subsequent decrease by 31 months (Jang and Van Remmen 2011).

In older rats the branching complexity and branch length of nerve terminals had decreased when compared with middle aged. The ability of nerves to respond to injury was also reduced in old rats where the repair and regeneration of nerves progressed at a much slower rate (Pestronk, Drachman et al. 1980). In aged mice it was found that their adaptive response to denervation was still present, but was less effective when compared to young mice. In old mice post denervation they exhibited smaller nerve terminal area, shorter perimeter and less sprouting (Fahim 1993).

One important change seen in ageing NMJs is the retraction and withdrawal of nerve from the postsynaptic membrane. Chai and colleagues reported that in old mice aged 29 months

there appeared to be enlarged nerve endings and extensive sprouting, the amount of completely denervated endplates were seen to increase by 2.5 fold compared with young endplates. However this was only seen in the TA muscle and not the soleus, which suggests that age related changes are only seen in some muscles or are less severe in other muscles (Chai, Vukovic et al. 2011). Accompanying the changes in the nerve terminal there also appeared to be granular fragmentation on the postsynaptic membrane.

When the same NMJs in mice are observed throughout their lifetime it appears once they become old they start to develop changes like varicose nerve terminals and fragmentation of the endplate (Li, Lee et al. 2011). However in the old muscles it appeared that some of the NMJs remained healthy and young looking, denervation was not seen in this particular case. Although there are age related changes in the NMJ it appeared that once these changes had onset there were no much further changes. This suggests that the stability of the endplate is maintained even in old animals. There appeared to be a uniform reduction in the intensity of the stained AChRs indicating there is a uniform turnover of the AChRs with age.

With age the endplate in rats were observed to increase in complexity where the average branch length and total branch length was higher. In some cases there was age related expansion of the endplate where the total perimeter area and stained endplate area was greater in the aged animals when compared to young (Deschenes, Roby et al. 2010).

1.8.2 Effects of Aging varies with different muscles

Studies involving the mouse tibialis anterior have shown that with progressing age the postsynaptic AChRs can begin to lose contact with the presynaptic nerve terminal resulting in partial denervation. The motor axons of the NMJ would at times appeared to intersect or

meet at the same place on the postsynaptic membrane resulting in multiple innervation, which was due to sprouting of nerve in response to denervation. The postsynaptic AChRs also appeared fragmented and in some cases there were regions with low visibility indicating some areas with reduced AChR density (Valdez, Tapia et al. 2010).

Subsequent studies by Valdez and colleagues showed that that the age related changes in the NMJ varied greatly from muscle to muscle in mice. There was no one factor that could predict which muscles would suffer NMJ changes in old age. NMJs in the extra ocular muscle (EOM) and the external anal sphincter muscle appeared to be extremely resistant to ageing and did not show any signs of age related changes in the NMJ. However in the sternomastoid, soleus and extensor digitorum longus muscles there appeared to be fragmentation of endplates, partial or complete denervation of endplates, and also multiple sprouts of the nerve terminals accompanied by thinning of axons. A factor that appeared to play a role and correlate well with age related changes in this study was the size of the motor units where the EOM muscle was quite small with only 5 fibres per unit. The smaller sized motor units appeared to be more resistant to ageing, but are not a necessity. A second factor that showed a good correlation to the muscles susceptibility to age related changes is the origin of nerves which innervate the muscle. Muscles that were innervated by brain stem cranial nerves showed good resistance to age related changes, but muscles innervated by spinal nerves were susceptible to age related changes (Valdez, Tapia et al. 2012).

1.9 Potential effects of exercise and Muscle Adaptive ability

For a long time it has been speculated that the main reason that sarcopenia occurs is due to the decline in physical activity which follows when people age (Morley, Baumgartner et al.

2001). With exercise some effects of aging might be attenuated, stopped or in some cases even reversed. In old animals that were encouraged to exercise the NMJ structure was more regular and homogenous. The nerve terminal area was also smaller than the old control mice suggesting that exercise prevents age related expansion of the NMJ (Fahim 1997).

Changes in the NMJ becomes more prevalent as age wears on, but studies have shown that these changes can be dampened with the help of exercise provided from an external stimulus like a running wheel. Animals that were engaged in exercise showed reduced denervation of endplates and lesser fragmentation (Valdez, Tapia et al. 2010). However the positive effects of exercise in this case only appeared to be in muscles, which were directly involved in exercising.

Studies in young adult rats showed that either increase or decrease in physical activity can elicit specific changes in the NMJ (Deschenes, Tenny et al. 2006). Increase in physical activity in old Sprague Dawley rats mainly caused changes in the presynaptic side of the NMJ, the branch length and the amount of branching was observed to increase, but no changes were seen in the postsynaptic side. In contrast in animals with decreased physical activity by hind limb suspension did not cause any changes in the presynaptic region, but caused the total perimeter area and stained endplate area to decrease.

When muscles were just made to do more work chronically and not necessarily exercise it was found that it did not cause a change in NMJ structural morphology. The gastrocnemius was partially removed to overload the soleus and plantaris muscles in young and aged rats, but this did not elicit any change in the NMJ in both muscles and ageing also did not show an effect on the NMJ morphology (Deschenes, Tenny et al. 2007).

Rats that were made to do resistance exercise showed some promising results of NMJ development. It was found that resistance exercise was able to cause significant enlargement of the NMJ of the rats and an increase in perimeter length. The dispersion of endplates (percentage of the AChR cluster area as the whole endplate area) also improved significantly (Deschenes, Judelson et al. 2000). Although some positive effects are seen upon the NMJ from resistance exercise, but the same effects are not seen on muscle morphology. From the control or trained subjects the cross sectional area of muscle fibres were similar and fibre type composition was similar. The cross sectional areas of type I and II fibres in the trained and control rats were also similar.

Resistance exercise in middle aged rats showed increase in the endplate area and AChR dispersion, which has functional benefits in the NMJ and is seen as reduction in fatigue during high intensity contractions. This improvement in the NMJ however did not cause muscle hypertrophy, which suggests that exercise uncouples NMJ and muscle development (Deschenes, Judelson et al. 2000).

1.10 Hypothesis: Sedentary aging results in impairment in NMJ size and this may be inhibited by voluntary endurance exercise

The neuromuscular junction as a whole begins to experience age related changes when a person becomes old. Presynaptic nerve terminals can retract and withdraw from the postsynaptic membrane resulting in denervation of muscle fibres, which can potentially cause muscle weakness experienced by elderly individuals. My purpose in this thesis is to test whether sedentary aging in mice really does lead to the loss of NMJs and if so at what stage of life. Moreover I will test whether exercise begun late in life has the capacity to prevent such changes.

CHAPTER 2

EXPERIMENTAL DESIGN

2 Experimental Design

The study that was carried in this project consisted of:

- i) Monitoring the activity pattern of mice,
- ii) Confocal and morphometric analysis of the structure of the mouse NMJ at different ages and
- iii) Measuring the impact of exercise on the structure and integrity of the NMJ.

2.1 Animal care, monitoring and the need to exclude some animals from analysis

The mice that were used in this study were C57B16/J female ex-breeder mice that had been ordered from the Animal Resources Centre (ARC) in Perth at 8 months of age. Mice of older ages are not routinely available in Australia. The mice were group housed together from 8 months of age till the time of sacrifice in order to minimize the chances of stress and/or conflict within the group. Once the mice reached the ages of interest then some of the mice would be culled and their tibialis anterior (TA) muscle would be collected. This project was approved by the University of Sydney Animal Care and Ethics committee (Protocol # K22/10-2011/3/5602). However this study also involved mice aged 2 months, which were ordered in at this age and culled immediately and required no long term housing or care.

2.1.1 Animal housing and welfare

The mice were group housed (initially 4 per box) in transparent plastic filter top cages with dimensions 17 x 34 x 13cm (width × length × height), which consisted of YSF branded mouse and rat chow and water was provided *ad libitum*.

This study involved the use of aged animals, so monitoring them in a careful manner was an important issue since as animals begin to age abnormalities can develop. Under the terms of the ethics protocol I or another member of the lab inspected the mice twice weekly from when they arrive in the Blackburn animal house until they were killed for analysis. Given that the mice are allowed to live out nearly a full (or near-full) life span, mice were occasionally found dead in the box of natural causes. Some mice also needed to be euthanased due to sickness. Most commonly this involved a mouse developing an obvious external swelling that did not resolve (putative tumor) or a mouse displaying sores that did not heal within a week or so. The frequency of such events is recorded in chapter 3 together with a composite survival curve for our aging mice.

In addition some mice needed to be excluded from analysis at the time of sacrifice because they displayed what seemed to be an unhealthy condition when dissected. We used published guidelines for age research in rodents, which suggest excluding animals with a markedly enlarged spleen or internal tumours (Miller and Nadon 2000). Records of all cohorts of mice (each cohort comprised of 12 mice received from ARC Perth on successive months) were kept on a spreadsheet, saved under 'Groups' on the Physiology Server under directory 'phillipslab', 'Lab stocks'.

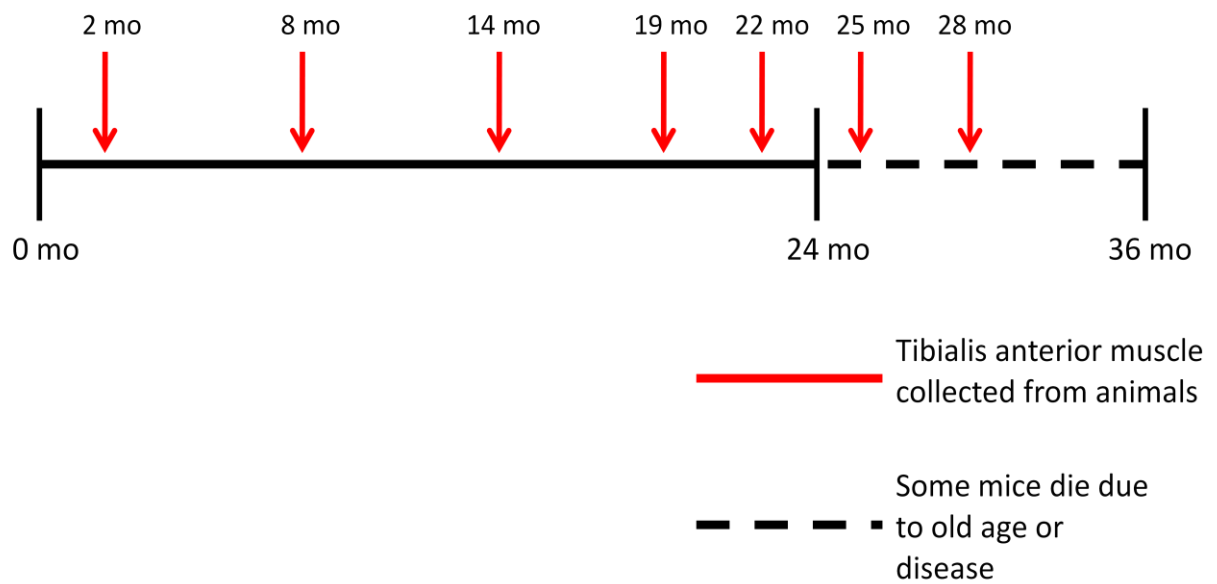
2.1.2 Exclusion of animals due to unhealthy symptoms

The initial data and analysis for effects on the NMJ of wheel addition compared with controls was done on in cohort 2 that had been housed in Animal House 27 aged 27 months. There were a total of 12 mice in this cohort, but 4 had to be culled earlier than the desired date due to sores and one more had to be culled due to a neurological problem. Out of the

remaining mice 5 had access to a running wheel, which they could use whenever they desired, however there was one more mouse in this experimental group that had to be excluded from use and had to be culled due to an enlarged spleen. In the end only 7 mice remained healthy and suitable to be used for histological immunofluorescence and analysis. The mice in this cohort were aged 27 months when culled.

In the second set of experiments comparing effects of exercise with controls and observing the changes that happen with aging, mice from Blackburn animal house were used. The animals used for histological immunofluorescence and analysis in this experiment came from cohorts 4, 5 and 6. The mice came from more cohorts in this experiment as there were more unhealthy mice that had to be excluded. From cohort 4 there were mice that had tumours, enlarged spleen indicating improper immune function or just died on their own naturally. In cohort 5 there were also some mice, which died, but there were some mice with sores that did not heal and therefore needed to be culled.

In this study it was desired for us to investigate the changes that occur with aging, but we also wanted to observe when the changes occur and not just if they occur. To do this we collected tissue from animals at the ages of 2, 8, 14, 19, 22, 25 and 28 months as seen in figure 2.1.



2-1 Aging timeline of C57Bl/6J mouse. The red arrows represent the time points at which the animals were culled and had their TA muscle collected. Dotted lines indicates the time period where some mice died or had to be culled due disease, which arose from aging.

2.2 Running wheel intervention

Boxes of mice were assigned to two experimental groups: control group and exercise group. The boxes assigned to the exercise group received a wireless running wheel (Fig 2.2). The running wheel signaled to a HUB, which was attached to a laptop computer and the number of rotations were recorded from all running wheels every 30 seconds. The data collected was exported to an Excel spreadsheet, which was used for subsequent analysis to depict the usage of the running wheels by the mice. The environmental conditions of the room in which the mice were kept were monitored and recorded by an environmental monitor on the mouse rack: specifically temperature, humidity and light-dark cycle. The animal house lights were on a 12hr day/night cycle.



Figure 2-2 Housing for our mice. Mice placed in a cage along with a running wheel, which they have free access to. The bottom of the cage is lined with recycled paper, which is used as bedding material for the mice. The running wheels used were wirelessly connected to a USB-HUB and the amount of rotations that the wheel turns is recorded every 30 seconds.

2.2.1 Analysis of Running Wheel and Environmental Data

The second part of this study was to investigate the effects of exercise and this was carried out by supplying the mice with a wireless running wheel (MED ASSOCIATES INC). The data that had been collected on how much each cohort of mice had run was exported into an excel spreadsheet with each box of mice on a different spreadsheet. The amount of running done by each box was recorded by the running wheel, which counts the number of revolutions every hour and was outputted into a computer. The time and date of each record was also recorded and inputted into the spreadsheet.

After the data had been collected the “sum” function in excel was used to subsequently calculate the amount of running each cohort of mice had done in each week. This was calculated as the total revolutions that the running wheel had recorded in a week. This was then converted into the distance that the mice had run by multiplying the revolutions that had been recorded by pi and the diameter of the running wheel, in this case it was 10.9 millimetres (circumference = $2\pi \times r$). The average distance run per day was calculated on a week-by-week basis by dividing the total distance run in a week by 7.

We were also interested in the average running that each mouse did in each box, so the weekly averages of each box was divided by the number of animals in the same box during that week. This value was adjusted accordingly when and if animals in the box died or needed to be culled. However we did not track the activity of individual animals so this method of calculation would not reveal if specific mice ran more than others.

The activity of each box was also monitored to identify if any unusual patterns appeared during the time course of the study. If some unusual patterns were seen like sudden drops in

the average running done by the animals then the data from the time that the unusual pattern appeared would be looked at individually on a daily timescale. Once the time at which the unusual running had been identified it was noted down and were analysed in conjunction with environmental factors to see if there were potential effects the environment could have had.

Environmental factors were also considered in the analysis as it is important to create a comfortable environment for the animals. The temperature and humidity of the room that the mice were kept in was recorded every hour. For both factors the mean values every week was found using the “average” function in Excel.

2.3 Dissection and storage of muscles and other tissues

On the day of planned sacrifice, mice were transferred from the animal house to the lab where they were killed by overdose of pentobarbitone. After the mouse had no pain reflex to pinching at the leg and no blink response indicating certainty of death the TA (tibialis anterior), EDL and the gastrocnemius muscles were dissected out from both hind limbs by Dr Marco Morsch and myself. Each muscle was then placed in an optimal cutting temperature compound (OCT) along with a small square of paper with line rules to indicate the orientation of the muscle. The mould containing the OCT and muscle was made of aluminium foil and was rapidly frozen by immersion in a shallow pool of liquid nitrogen in a metal cylinder. The frozen muscle blocks were each individually placed into a 1.5ml Eppendorf tube that had been labelled with indelible Sharpie pen and which contained a drop of RO (reverse osmosis) water at the bottom preventing the block from drying out in the freezer over time. The Eppendorf tubes were then placed inside a 50ml screw cap Falcon

tube and were stored in a -80°C freezer in a box labeled with a specific alphabet and number that was recorded in an excel spreadsheet named “freezer stocks”. The following labeling system was used: TA indicated tibialis anterior, EDL indicated extensor digitorum longus and Gastroc indicated gastrocnemius. Each muscle was also labeled with “left” or “right” to indicate which side of the hind limb it had come originated from.

2.4 Immunostaining for NMJs

The frozen muscle blocks were transferred from the -80C freezer to the cryostat in a thermos flask of liquid nitrogen to avoid thawing. Each muscle block was aligned on the chuck so as to be cut in a longitudinal orientation at a thickness of 20 µm. Cryosections were collected directly onto Polysine microscope adhesion slides (Thermo Scientific; Menzel-Gläser Polysine Slides), 4 sections per slide. The cutting of the sections was done in the chamber of a cryostat (Leica CM3050S, Leica Microsystems) at an objective temperature of -20°C and a cabinet temperature of -22°C.

2.4.1 Preparation of tissue for immunofluorescence

After the sections of tissue had adhered firmly to the slide at room temperature they were processed as follows: 1) slides were fixed with 2% paraformaldehyde (PFA) for 15 minutes. The PFA was made from diluting 8% stock with PBS to prepare 2%; 2) the slides were then washed in 1× Phosphate Buffered Saline (PBS) for 30 minutes, changing the PBS every 10 minutes; 3) After 30 minutes the slides were immersed in 0.1M glycine dissolved in PBS for another 30 minutes ; 4) slides were given a single wash in PBS for 10 minutes; 5) The slides were then immersed in -20°C methanol to extract lipids; 6) The slides were again washed in PBS for 20 minutes, changing the PBS every 10 minutes; 7) sections were preincubated for

1 hour with 0.2% Triton-X100 (ICN Biochemicals, Ohio), 2% Bovine Serum Albumin (BSA, 30% aseptically filled, Sigma) dissolved in PBS to block non-specific binding; 8) after 1 hour any excess blocking solution remaining on the slides was wiped off with Kimwipes (Kimberly-Clark Professional, 21cm X 12cm, ProSciTech).

Immunostaining was then conducted as follows. Sections were layered over with 12 micro litres of the same blocking solution containing primary antibody: rabbit anti-synaptophysin (dilution factor 1:200) and were incubated overnight at 4°C. On the following day the sections were again washed in PBS for 30 minutes with 10 minute changes. The sections were then incubated with a mixture consisting of blocking solution, Alexa 555 α -bungarotoxin (BGT, 1:200, Invitrogen) and donkey anti rabbit fluoresceine isothiocyanate (FITC)- conjugated secondary antibody (1:250, Jackson Lab) for 1 hour. During the time of incubation the slides were placed in a humid chamber to prevent them drying out. The chamber was covered with aluminum foil due to the light sensitivity of the antibodies. The slides were once again washed with PBS and were mounted in polyvinyl alcohol mounting medium with 1,4-1,4-diazabicyclo octane (DABCO; Sigma), by pipetting some onto each individual piece of tissue just enough so the tissue is covered to minimize fading under fluorescence illumination. Coverslips were then placed gently over the DABCO covered tissues and pressed down upon, any excess DABCO was wiped away with a tissue. The coverslips were held in place by applying clear nail polish on the edges of the coverslip. The slides were stored in plastic boxes at -20°C until use. Imaging of the slides was usually completed within 2 weeks.

To ensure that the binding of antibodies was specific, 3 control slides were prepared with every staining run. The first control had no anti synaptophysin primary antibody. The second control was without FITC secondary antibody and the third control was without α -bungarotoxin (α -BGT).

2.5 Optimization of NMJ confocal imaging

The main experimental techniques used in this study were immunohistochemistry and confocal microscopy where Z focal plane stack images were taken. When establishing a protocol for the collection of images certain variables had to be considered. 1) Each image had to meet a reasonable quality in order for structural analysis of endplates to be carried out, 2) the time that each endplate image required to be collected must not exceed a period of 4 – 5 minutes since a reasonable number of images must be collected in order for potential statistical significance to be obtained. Moreover time on the confocal microscope was limited so the time that each image took to collect had to be considered.

2.5.1 Confocal microscope settings for best possible quality images

For the quality of the image to be reasonable the settings on the microscope were the key variables that had to be considered. When deciding the settings to use on the confocal microscope it was understood that there would always be a trade-off, when each setting was altered to give better image quality the time needed to take each image would also increase. The main settings experimented with were: 1) Choice of Objective lens; 2) Resolution, meaning the density the pixels in field of the image; 3) Scan Speed; 4) Averaging of repeated scans; 5) Spacing of Z dimension intervals.

Several trials were done at the outset using different settings attempting to yield the best quality images within a reasonable period of time for each image (4-5minutes). The 63× oil immersion objective was determined to yield better quality images than other objectives.

Resolution refers to the density of pixels within the image. This means that using a higher resolution will improve the potential quality of the image, but will also increase the imaging time. Using the preset resolution options it was found that 1024 x 1024 was able to yield a reasonably sharp image. Scan speed is the time that the laser spends to scan each point in the field of view of the tissue section. Using a lower scan speed will enhance the quality of the image since more information can be collected per scan. I discovered that with a resolution of 1024 x 1024 and a scan speed of 600Hz I was able to produce an adequately good quality image.

Averaging of scans improves quality image by enhancing signal to noise ratio. I found that averaging of 4 scans was able to remove most of the electronic 'noise' in the background of the image and also avoided the pixilated edges of the endplate that were seen using less averaging. The zooming option is able to exclude part of the field which does not contain the endplate. A zoom factor of 2 was able to omit most of the field that did not contain parts of the endplate while all of the endplate remained well within view.

The interval thickness is the distance between each scan level in the Z dimension, when creating a Z-stack. This option was taken from previously established protocol by Honours student Yui Murata who found that 0.7 μm was the maximum thickness, which could be used while still retaining all information in the Z stack since the slices would just overlap each other.

2.5.2 Adjusting the confocal parameters for each endplate

Individual endplates can vary substantially in intensity. While the parameters outlined in Table 2.1 were retained for each image, the gain and offset for each image was optimized so as to avoid compressing the tonal information. This yielded more detailed images but precluded direct comparison of the intensity of staining between endplates collected in different images.

To further limit the chances of subjective bias during the imaging process, the slides were coded by another laboratory member without me having any knowledge of the code. The images were taken blind to treatment group and the code was not broken until quantitative analysis of the images from the staining experiment in question had been completed.

2.6 Rules and procedures for sampling endplate images

The sections were visualized in the Z plane orientation with a Leica confocal microscope (SPeII Leica). To first identify endplates, the visual field of the tissue was observed through the microscope eyepiece using both eyes and endplates were recognized by rich rhodamine- α -bungarotoxin stained- AChR clusters. When searching for endplates on each section, so as to minimize the chances of missing endplates the objective of the microscope was moved across and down the piece of tissue in a zigzag orientation (Fig 2.3).

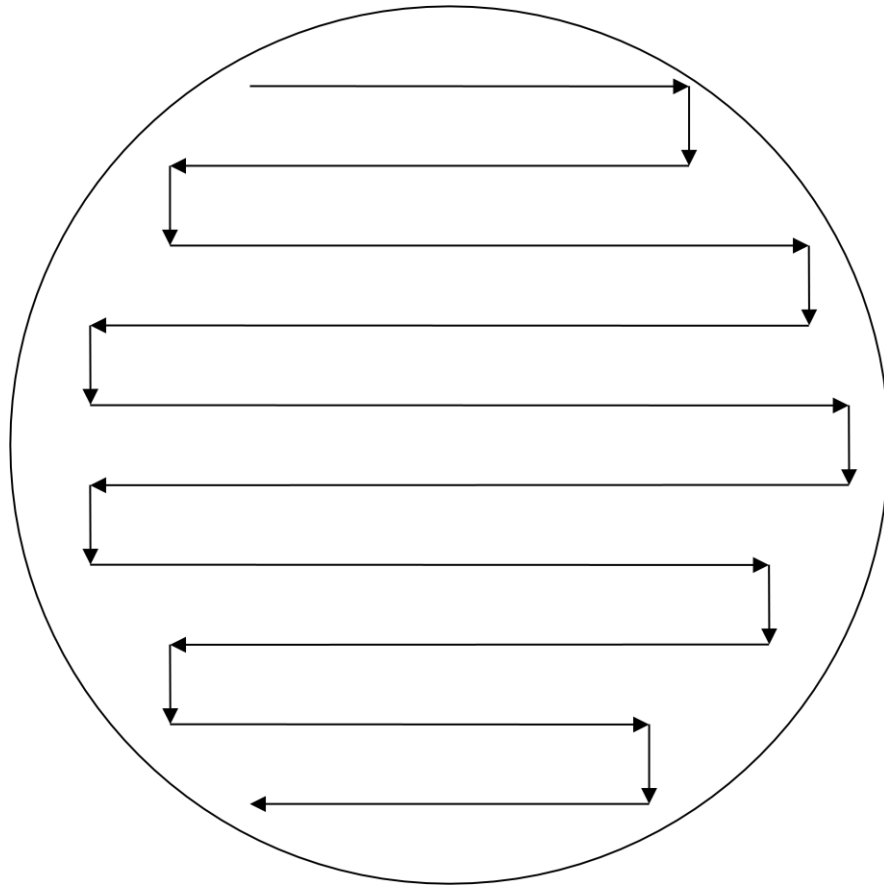


Figure 2-3 Method in searching for endplates. Arrows indicate the direction and position on the muscle fibre that I used for searching endplates while looking down the microscope lens. This method minimizes the chances of missing endplates or reimaging the same endplate while searching.

Before beginning to collect the images that yielded the quantitative data in this thesis I established a set of rules to try to avoid subjectivity in the sampling of NMJs within the sections. When a structure suspected of being an endplate suitable for analysis was found it must fulfill set criteria for it to be imaged:

1) The AChR cluster had to be “enface” to the objective, meaning that the endplate must resemble a pretzel shape, 2) the AChR cluster had to be no more than 15 microns deep in the Z dimension. The test I used to efficiently realize whether the endplate was 15 microns thick or not was that it should be contained within approximately one quarter turn of the fine focus knob on the microscope (this equates to about 15 microns).

Table 2.1

Optimized confocal Z-stack parameters for imaging endplates

Resolution	1024 × 1024
Zoom Factor	2
Objective	63× oil immersion
Pin Hole	1 Airy Unit
Scan Speed	600 Hz
Averaging	4
Z-stack thickness	0.7µm
Gain and Offset	Optimised for each individual image

2.7 Analysis of NMJ Images

The analysis of the images was done using ImageJ software (<http://rsb.info.nih.gov/nih-image/>). The parameters that were analysed included: 1) the total endplate perimeter area; 2) Total stained area of endplate acetylcholine receptors; 3) Anti-synaptophysin-stained nerve terminal area; 4) area of overlap between the anti-synaptophysin stained area and the AChR clusters; 5) Percentage of AChR cluster area covered by nerve terminal staining, and 6) Percentage of nerve terminal area with AChR cluster underneath. The optical Z slices of the endplate were stacked on top of each other retaining the maximum intensity of the image and it was this maximum intensity image that was used for analysis.

2.7.1 Measuring areas in postsynaptic region

The total perimeter area was measured by first converting the image to 8 bit black and white image and a line was drawn around all the fragments of the endplate, figure 2.4A. The scale of the image was first removed, by using the option Analyze >> Set Scale >> Click To Remove Scale and using the measure tool it will give us the area in pixels within the line.

This parameter includes the spaces between the fragments as well as the area of the fragments themselves. The area that was measured in pixels was then multiplied by 0.00728 to convert it into microns; the same conversion factor was applied to all other measurements to convert pixels into microns. This number was obtained from the Leica software used to operate the Leica SPE II confocal microscope.

The total acetylcholine receptor area was measured by first converting the image to an 8 bit black and white image. A line was drawn around the endplate fragments and a threshold was

then applied the image as shown in figure 2.4B, using the threshold tool in ImageJ. When the AChR cluster was thresholded I made sure that all parts of the AChR were coloured red, meaning that all the necessary areas were thresholded. The analyze particles tool was then used for measuring the size of each individual AChR cluster fragment within the encircled area and the number of fragments present, but fragments smaller than 15 pixels were excluded. The size of each individual AChR cluster fragment was then added together to calculate the total AChR cluster area of the endplate.

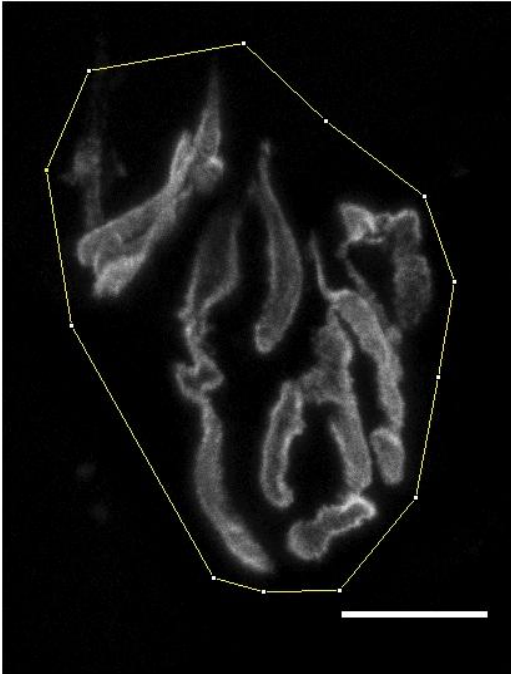
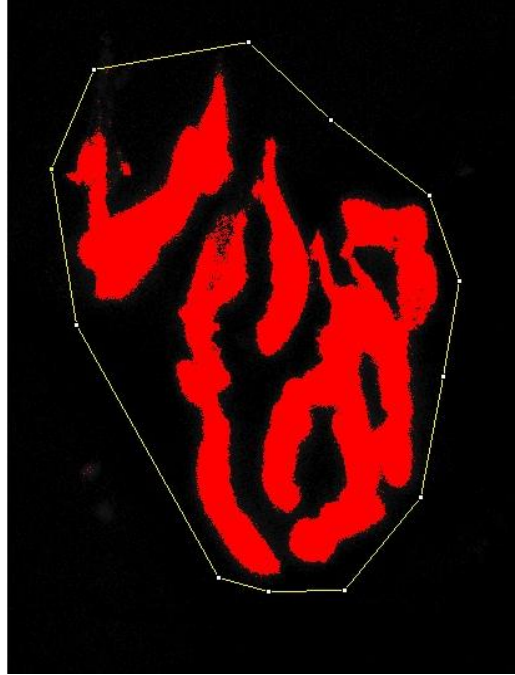
A**B**

Figure 2-4 Measurement of endplate total perimeter area and AChR area. (A) Line drawn around all endplate fragments of an 8 bit tonal image for measurement of the endplate total perimeter area. Scale bar=10 μ m **(B)** Line drawn around an 8 bit thresholded image for measurement of the acetylcholine receptor area. Measurements are limited to only the thresholded regions, which appear in red.

2.7.2 Measuring areas in presynaptic region

Nerve terminal area was measured in a similar way that the AChR area was measured. The image was first converted to an 8 bit black and white image. The image was then converted to an 8 bit black white image and was thresholded (Fig 2.5B). A line was drawn around the nerve terminal (Fig 2.5A). However since nerve terminal is not as well defined in old animals as compared to young making it more difficult to set the correct threshold so when a condition like this was encountered then the threshold would be lowered till some noise could be seen in the background where there is certain to be no anti synaptophysin staining. The analyze particles tool was not used since we were not measuring the degree of fragmentation in the nerve as we were in the AChRs. Instead the measure tool was used to directly yield the nerve terminal area in pixels. This value was again multiplied by 0.00728 to convert to microns.

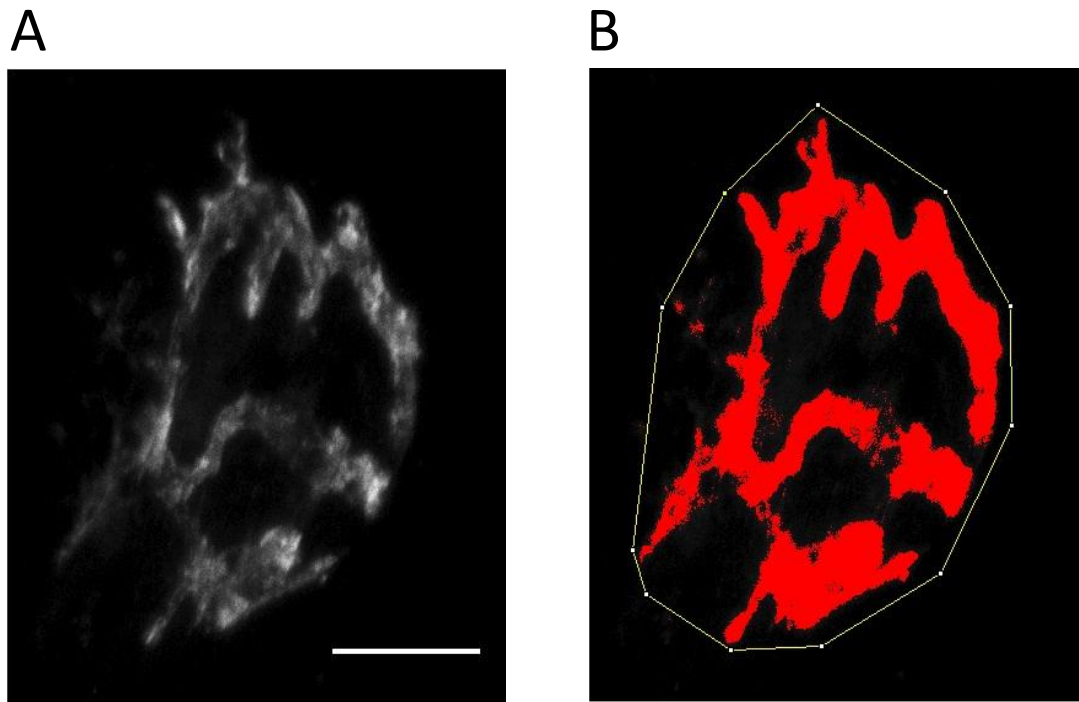


Figure 2-5 Measurement of presynaptic terminal area. (A) 8 tonal image of the presynaptic nerve terminal. Scale bar=10 μ m **(B)** 8 bit tonal image of the presynaptic nerve terminal with a threshold applied allowing the area of the nerve terminal to be measured.

2.7.3 Measuring pre and postsynaptic overlapping regions

The area of overlap between the α -BGT stained AChR cluster area and anti-synaptophysin stained nerve terminal area was measured by using the colocalization plug-in in ImageJ. This tool utilizes the threshold levels that as set above for the anti-synaptophysin stained nerve terminal and α -BGT stained AChR images. The Plug-in creates an overlay of the two images. The region of overlap between the two images can be seen as an area of white pixels (Fig 2.6A). The area of overlap was measured by converting the image to 8 bit and setting the threshold to include up to 255 so that it included only the region of overlap (Fig 2.6B). A line was then drawn around the image and the threshold was raised to 255 to include all the regions of overlap then the measure tool was executed so the area of overlap within the specified region was obtained in pixels. This measurement was then multiplied by 0.00728 to express it in microns.

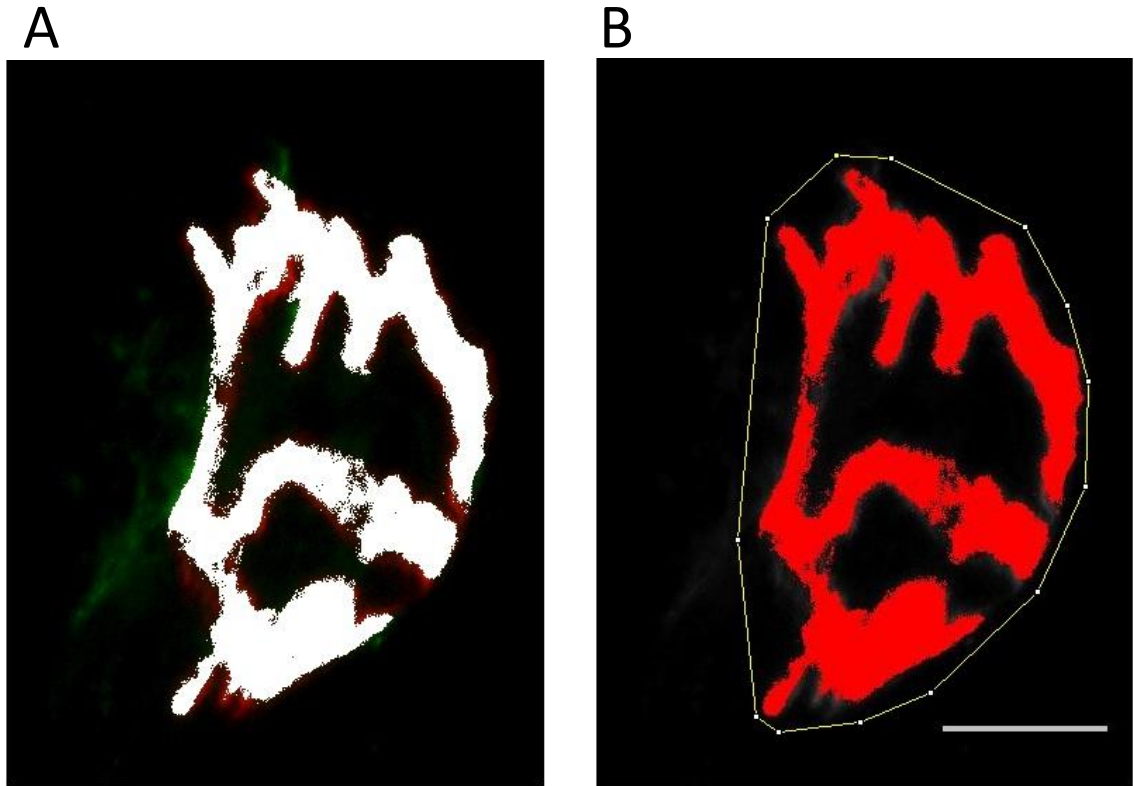


Figure 2-6 Measurement of area of overlap between AChRs and presynaptic nerve terminal. (A) Colocalization image of AChRs and presynaptic nerve terminal, area of colocalization appears as a region of white pixels. **(B)** Thresholded colocalization image with line drawn around the areas of colocalization. This allows the area the colocalization to be measured. Scale bar=10µm

The percentage of AChRs covered by nerve was calculated by first measuring the total AChR cluster area of the endplate. Then once the overlap area between AChRs and presynaptic nerve terminal was also measured, the area of overlap was divided by the total AChR area and this was multiplied by 100. This will give a value that expresses the percentage of AChRs covered by nerve staining. The percentage of nerve which had AChRs underneath was measured by dividing the presynaptic nerve terminal area by the overlap area and then this value was multiplied by 100.

2.8 Investigation of muscle fibre cross sectional area with increasing age

To assess the cross sectional area of muscle fibres in the tibialis anterior muscle transverse sections were stained for laminin.

2.9 Immunostaining for Laminin

Each frozen tissue block was aligned on the cryostat chuck so that transverse sections could be cut with a thickness of 12 μm . After the sections were cut they were collected onto a Polysine (Thermo Scientific) slide where they adhered firmly. The conditions of the cryostat chamber were set as the same as previously described in section 2.4.

After the sections had set firmly on the slides they were prepared in the following procedure:

1) the sections were fixed with 4% PFA for 15 minutes. This PFA was made from diluting out an 8% stock down to 4% with $1 \times \text{PBS}$ 2). The sections were then washed in $1 \times \text{PBS}$ for 30 minutes, changing the PBS every 10 minutes. 3) To block non-specific binding the sections were incubated for 1 hour in PBS containing 0.2% Triton-X100 (ICN Biochemicals, Ohio) and 2% (w/v) bovine serum albumin (BSA, diluted from 30% aseptically filled; Sigma) in PBS. 4) After 1 hour remaining blocking solution on the slides was wiped off with Kimwipes (Kimberly-Clark Professional, 21cm X 12cm, ProSciTech).

Immunostaining was carried out by layering the sections with the same blocking solution containing primary antibody rabbit anti-laminin (dilution factor 1:100, Sigma) overnight at 4°C . On the next day the sections were again washed in $1 \times \text{PBS}$ for 30 minutes. The sections were then incubated for 1 hour with blocking solution containing fluoresceine isothiocyanate (FITC) - conjugated donkey anti-rabbit IgG (1:250, Jackson Labs). During the incubation period the sections were placed into a humid chamber to avoid them drying out and the

chamber was covered in aluminum foil to avoid bleaching the light sensitive secondary antibody. After 1 hour the sections were again washed with PBS and mounted in polyvinyl alcohol mounting medium with 1,4-1,4-diazabicyclo octane (DABCO; Sigma).

2.10 Optimization of muscle fibre confocal imaging

The imaging protocol for muscle fibres was optimized to obtain the best quality images within the time constraints of the standard imaging session. In this case I wanted to collect as many muscle fibres within each image as possible so I used the 10 × objective and 1024 × 1024 pixels over the whole field of view. In this experiment only the muscle fibre area was being measured and not the whole structure so Z- dimensional stacks were not required. This permitted averaging of more scans and a lower scan speed to improve quality of the single optical section images. A scan speed of 400 Hz was used and 20 scans were averaged for every field. All microscope settings used for imaging muscle fibre transverse sections are listed in table 2.2

Table 2.2

Resolution	1024 × 1024
Zoom Factor	2
Objective	10 ×
Pin Hole	1 airy unit
Scan Speed	400 Hz
Averaging	20
Gain and Offset	Adjusted for each image

When deciding where to sample muscle fibres on the section a vast number of fibres had to be imaged so if there were any variations in fibre size a good number of muscle fibres imaged would allow for most fibres of varying sizes to be sampled. 3 images were taken from each animal using the 10 × objective and in each image roughly 70-80 fibres were sampled.

The parameter I was interested in this experiment was the CSA (cross sectional area) of the muscle fibre. This analysis was also done with the Image J software. Figure 2.7A depicts an image of muscle fibres all in cross sectional orientation. The cross sectional area was measured by converting the image into an 8 bit tonal image then inverting the image so the surrounding laminin-stained basement membrane sheath around each muscle fibre appeared as a dark ring demarcating the light-coloured fibre. A minimum threshold was then applied to the image making sure that the muscle fibre would be suprathreshold without including the laminin surrounding the fibres (Fig 2.7B). The *analyze particles* tool of NIH Image J was then used to measure the area of each individual muscle fibre. This command numbered the measured fibres and outlined each measurement (Fig 2.7C). Each individual measurement was inspected by eye to make sure that two adjoining fibres had not been inadvertently been joined and counted as a single (oversized) fibre. In instances where two muscle fibres appeared to have been mistakenly taken as being one fibre by the Image J software I would manually measure the areas of each fibre by drawing a perimeter around the muscle fibre and used the *measure* function in Image J to derive a cross sectional area.

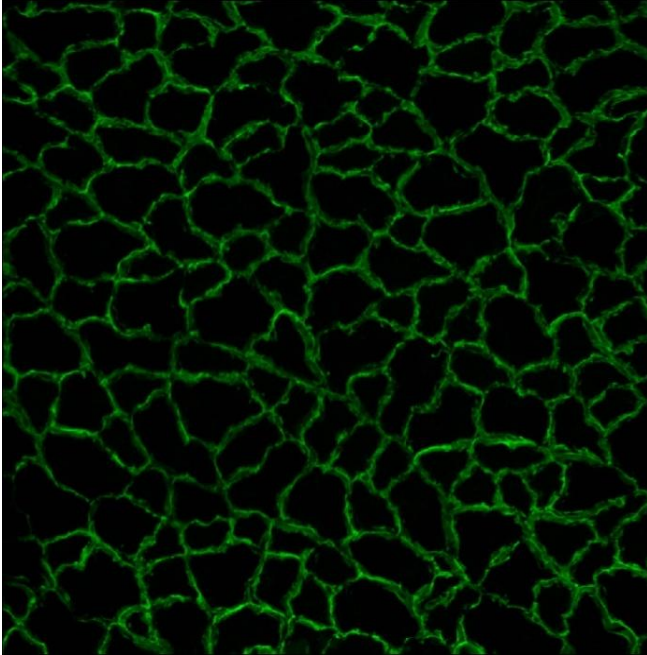
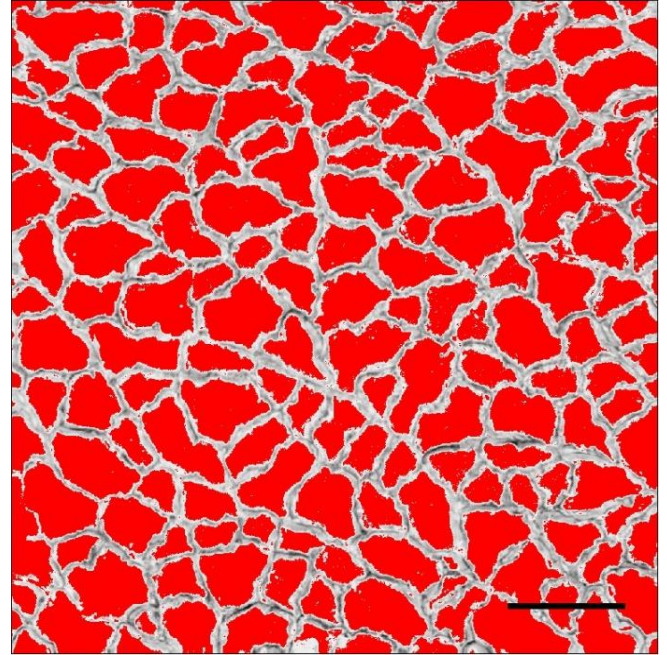
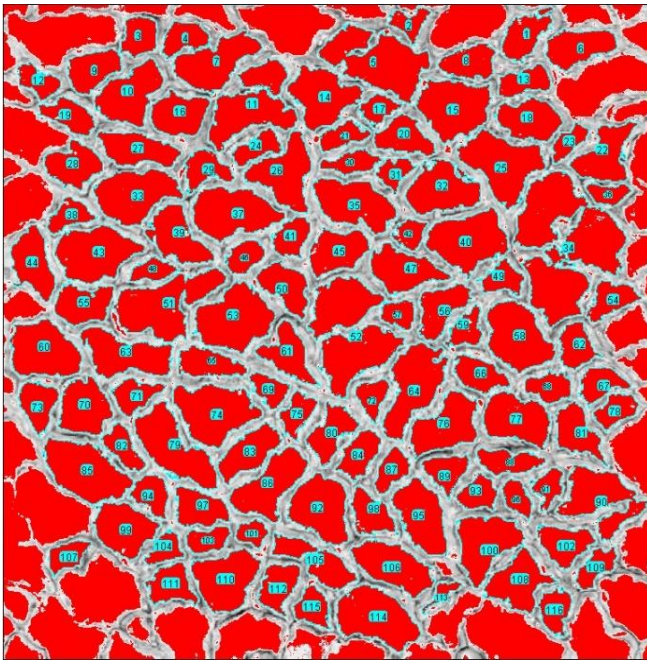
A**B****C**

Figure 2-7 Muscle fibre CSA measurement. (A) Muscle fibre image in cross section stained with a primary rabbit antibody anti laminin and secondary donkey anti-rabbit antibody fluorescein isothiocyanate (FITC) **(B)** Thresholded 8 bit tonal image, laminin surrounds the muscle fibres, which appear red. Scale bar=100 μ m **(C)** Muscle fibres cross sectional areas are measured and numbered illustrating the number of fibres that were measured.

CHAPTER 3

AGE RELATED CHANGES IN THE NMJ

3 ALTERATIONS IN THE NEUROMUSCULAR JUNCTION WITH AGING

3.1 Introduction

In this chapter I describe age-associated changes in the structure of neuromuscular junctions from C57Bl6J female mice. I have chosen to focus my studies on NMJs in the tibialis anterior muscle because it has been documented in literature that during aging there is atrophy or loss in cross sectional area of type II fast fibres and during the process of denervation can cause a net conversion of type II fibres to type I (Lang, Streeper et al. 2010). In previous studies it has been shown that in the TA when standardized to how much the tibial bone length had decreased with age, with the decrease in weight this was accompanied by a large decrease in fast type IIA fibres, but increase in type IIX fibres. The CSA (cross sectional area) in all fibres was unchanged or decreased with ageing (Chai, Vukovic et al. 2011). Changes appear to occur in muscle fibres with age, I decided to investigate if there are any age related alterations in the NMJ of the TA with age, by comparing the pre-synaptic and postsynaptic changes with age and muscle fibre cross sectional areas. I seek to clarify the period of life in which impairment of nerve-muscle control should be investigated.

3.2 SURVIVAL AND MORBIDITY OF OUR AGEING MICE

This study required the use of aging and aged mice so therefore there would be some possible complications that could arise in the health of the animals. It is important to explain how these were handled as it affects the sampling of animals in any study of aging. In this study we monitored mice from 8 months through 28 months in an animal facility at the University of Sydney. However we were also interested in ages younger than 8 months so we ordered 2 month old mice from ARC (Animal Research Centre) in Perth, which were culled and used for tissue collection instantly when they arrived at the University of Sydney.

3.2.1 Exclusion of mice with unhealthy symptoms

When the ageing animals became sick we needed to cull them and exclude them from the study. Some animals died before the desired age for analysis. Even the ones that reach the intended age would sometimes show signs of disease like tumours in organs or an enlarged spleen. In this case they would be excluded from further analysis. The criteria for judging a spleen to be enlarged was by comparing the weight of the spleen to the body weight of the animal. If the ratio of spleen weight to body weight was larger than 0.01, (as spleen roughly 3 times larger than the healthy size (Fig 3.1) then it was classified as being enlarged and unhealthy. In this study we had to exclude a total of 34 animals due to one of the previously described abnormalities. A total of 66 animals were judged fit to use (Fig 3.2).

Figure 3.3A compares survival curves for our aging mice with results published online by the Dr DE Harrison at Jackson Laboratories. Our survival rates compare favorably with those of Jackson Labs. Figure 3.3B also from Jackson Laboratories portrays that from 18

months of age onwards in C57Bl6J mice would be considered old age, which is why we have chosen to study this time period in more detail for our exercise protocol.

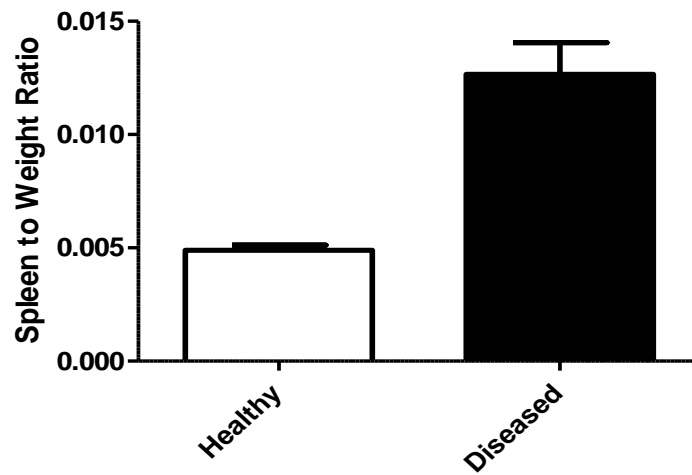


Figure 3-1 Difference in spleen to weight ratio in healthy and diseased mice. Column graph comparing the spleen weight to body weight ratio, illustrating that the diseased mice exhibit a spleen to weight ratio of about 3 fold higher than the mice that are classified as healthy. The healthy group included n=51 animals and the diseased group had n=13 animals.

Healthy	Died due to natural causes	Culled due to Sickness	Excluded due to abnormalities
66	16	5	13

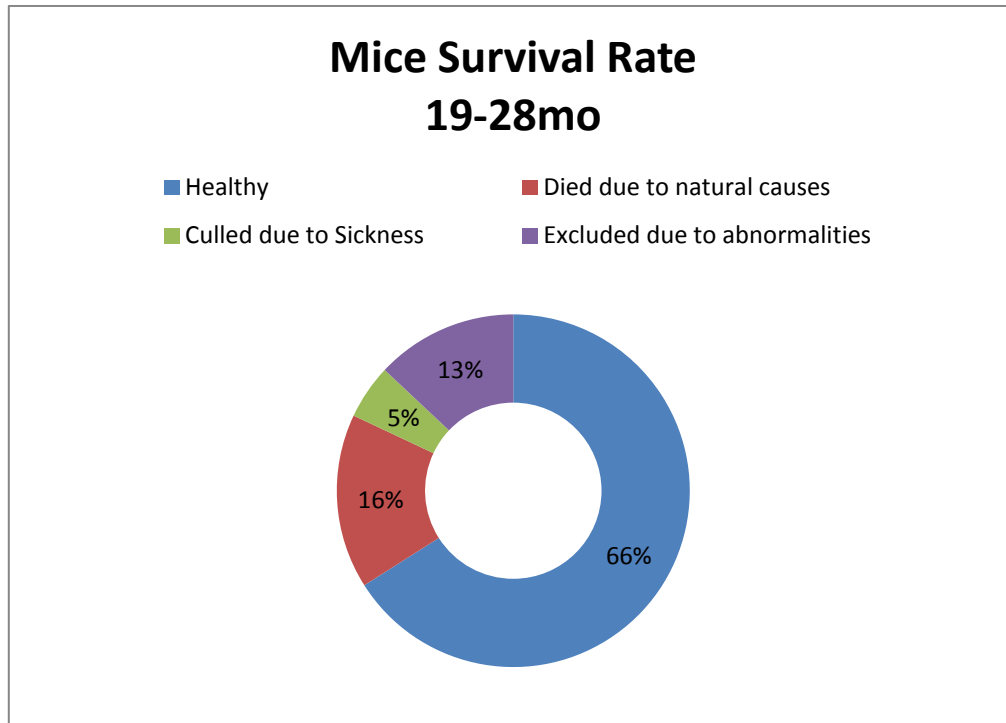


Figure 3-2 Doughnut graph representing the percentage of healthy and unhealthy animals. A large portion of the animals were considered healthy and were included in the study. Animals which were considered unsuitable either from having died at a premature age from natural causes or having to be culled as they appeared they were in a unhealthy state due to disease or may have been excluded due to abnormalities like an enlarged spleen were not used.

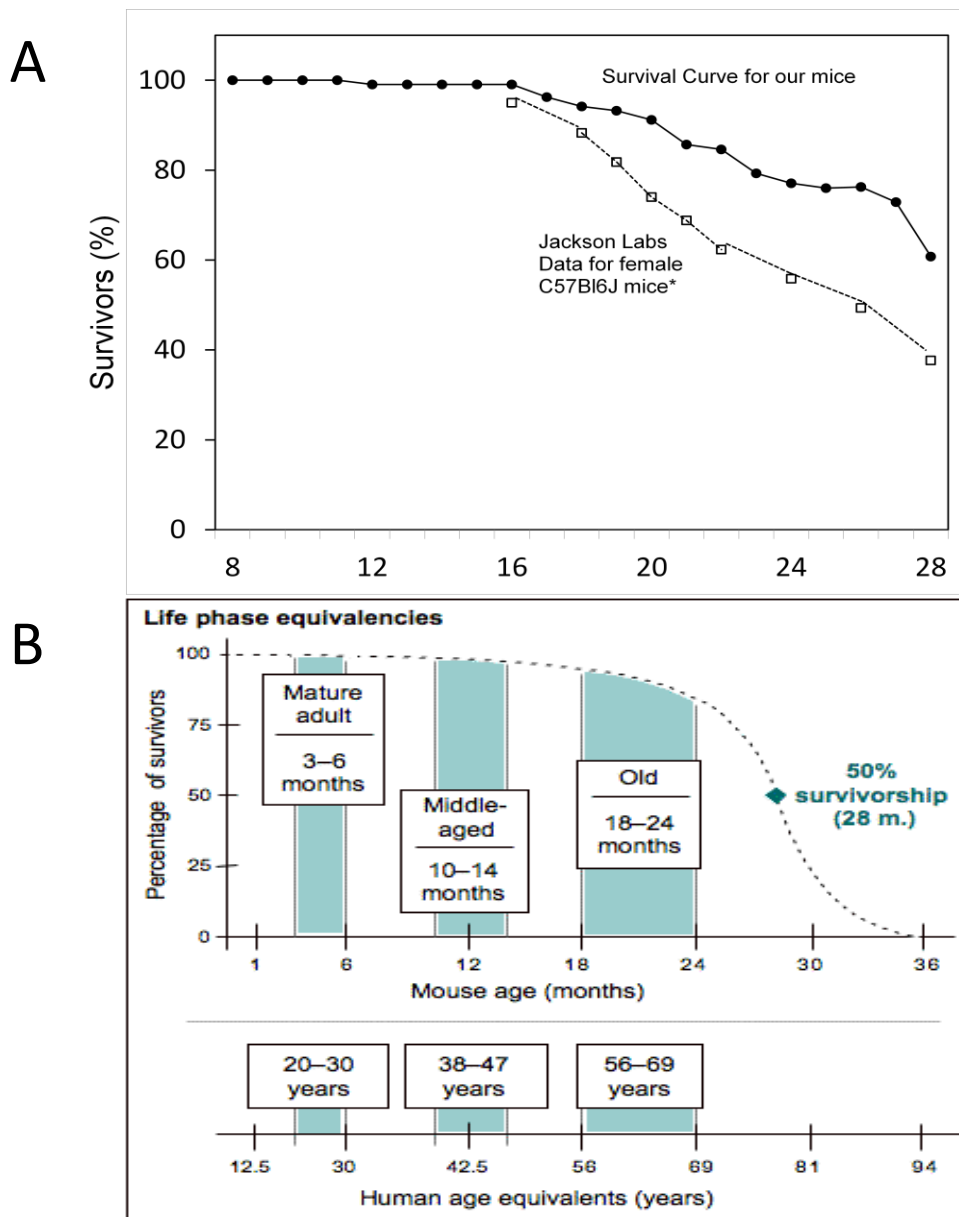


Figure 3-3 Survival curves for female C57Bl6J mice. (A) Solid line: under our care from 8 months (curve constructed from survival data for a total of 107 mice). Included in our calculations are mice that we culled for ethical reasons and those that were found dead. Dotted line: results reproduced from Jackson Laboratories (http://research.jax.org/faculty/harrison/ger1vi_LifeStudy2.html ©2012 The Jackson Laboratory) (B) Life phase equivalency graph comparing the ages and lifespan of C57Bl6J mice with humans (<http://research.jax.org/faculty/harrison/ger1vLifespan1.html>)

3.3 Changes to the NMJ with Age

Longitudinal cryosections from the tibialis anterior muscle were stained for presynaptic nerve terminals and postsynaptic AChRs and maximum-projection confocal images were collected as detailed in the Methods chapter. Figure 3.4 illustrates a typical neuromuscular junction from a young (2 month old) mouse. As can be seen in figure 3.4A the highly branched presynaptic nerve terminal labeled here with a mixture of anti-synaptophysin and anti-neurofilament forms an uninterrupted structure resembling the shape of a pretzel. The preterminal motor axon enters from the bottom of the panel. The cluster of AChRs in figure 3.4B (labeled with α -bungarotoxin) form a continuous matching pretzel shape. In figure 3.4C depicts an overlay of the presynaptic nerve terminal and postsynaptic AChRs staining, illustrating the close alignment and a well innervated healthy young adult NMJ. The results I found for NMJs in young mice were similar to those of (Jang and Van Remmen 2011).

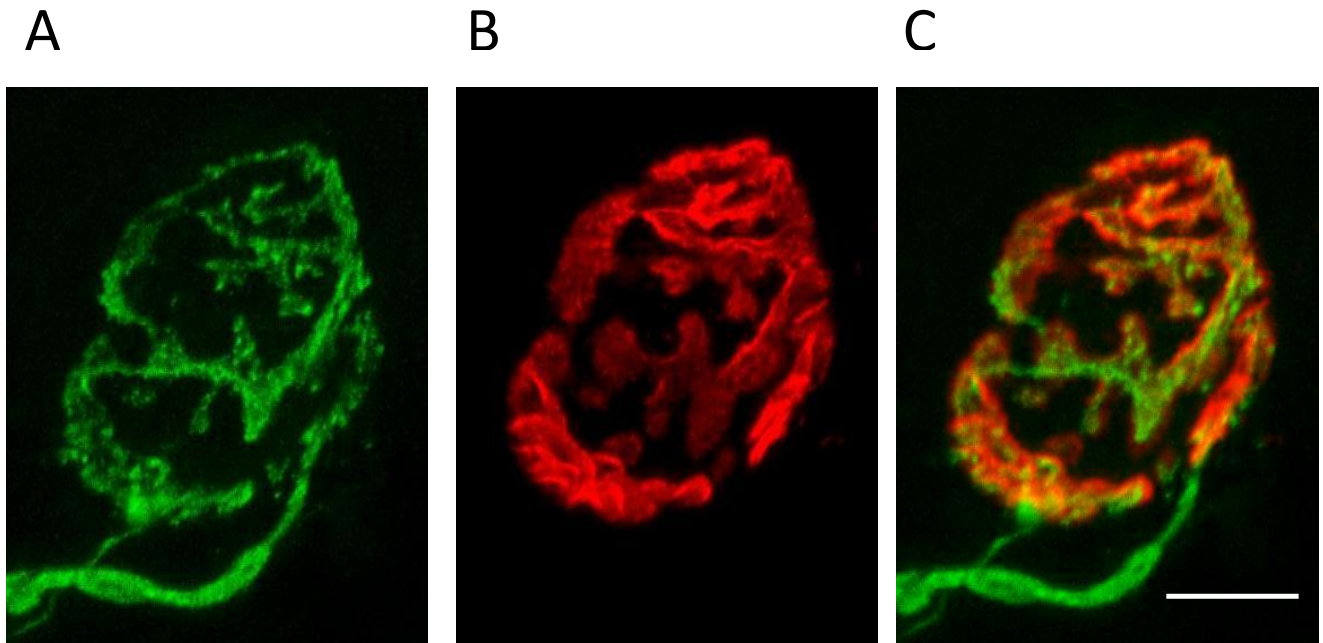


Figure 3-4 Healthy neuromuscular junction from a 1.5 month old mature aged animal from the TA. (A) Presynaptic nerve terminal stained with anti-synaptophysin primary antibody and FITC (Fluorescein isothiocyanate) secondary antibody. A motor axon is attached at the bottom stained with anti-neurofilament primary antibody and FITC (Fluorescein isothiocyanate) secondary antibody. **(B)** Postsynaptic AChRs stained with rhodamine conjugated -bungarotoxin. **(C)** Overlay of nerve and AChR showing good innervation of the AChR by the nerve. Scale bar in panel C =10 μ m.

In the aging NMJ several differences were often found. The first difference that may often be seen is fragmentation of the endplate. What was usually a continuous postsynaptic endplate AChR plaque in young animals was often broken up into several AChR cluster fragments. An example of this fragmented endplate can be seen in figure 3.5 panel B. However not all AChR clusters from ageing animals were fragmented.

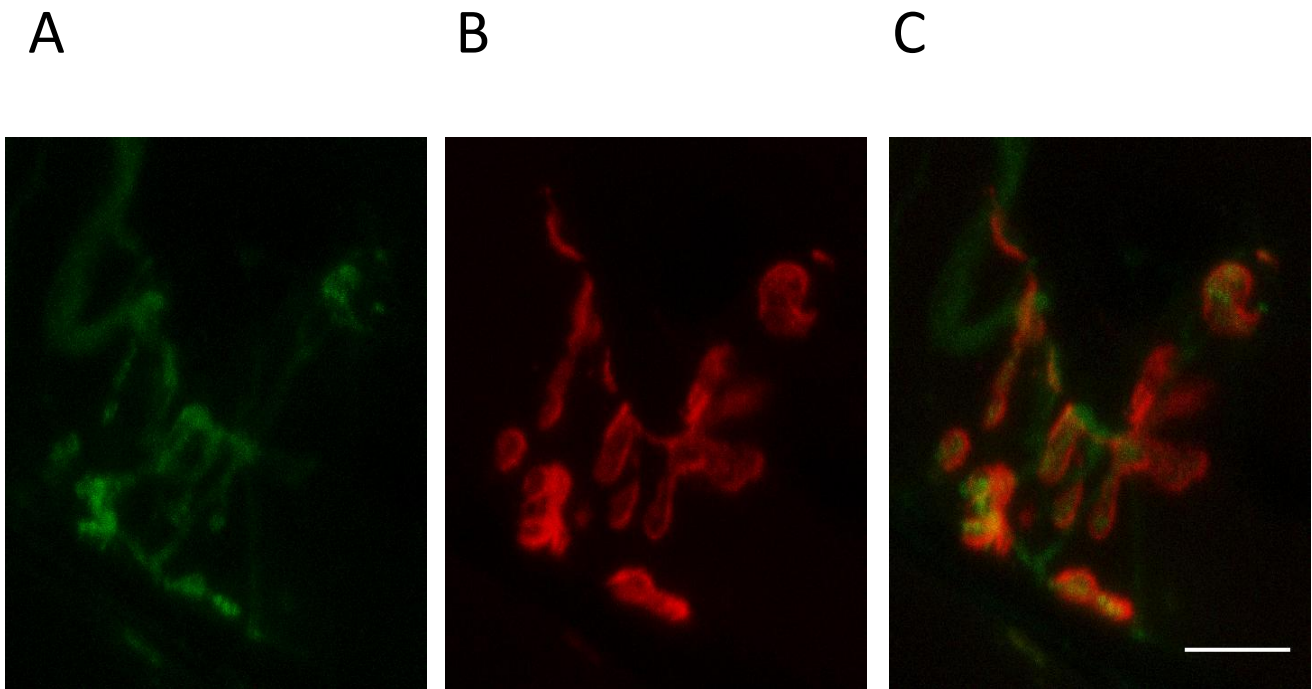


Figure 3-5 Old neuromuscular junction of a 28 month elderly mouse TA muscle with clear signs of fragmentation of the endplate. (A) Presynaptic nerve terminal still intact and present at the endplate. **(B)** Postsynaptic AChRs with signs of fragmentation into several smaller islands of the same endplate. **(C)** Overlay of nerve and AChR showing although fragmentation has occurred due to age the endplate are still quite well innervated. Scale bar in C = 10um.

A second difference observed at many aged NMJs was that the presynaptic nerve terminal apparently occupied only part of the endplate if at all. An example is shown in figure 3.6. This resulted in many areas where the endplate AChR cluster was not fully covered by the presynaptic nerve terminal as can be seen in the overlay figure 3.6C where this AChR cluster is partially denervated.

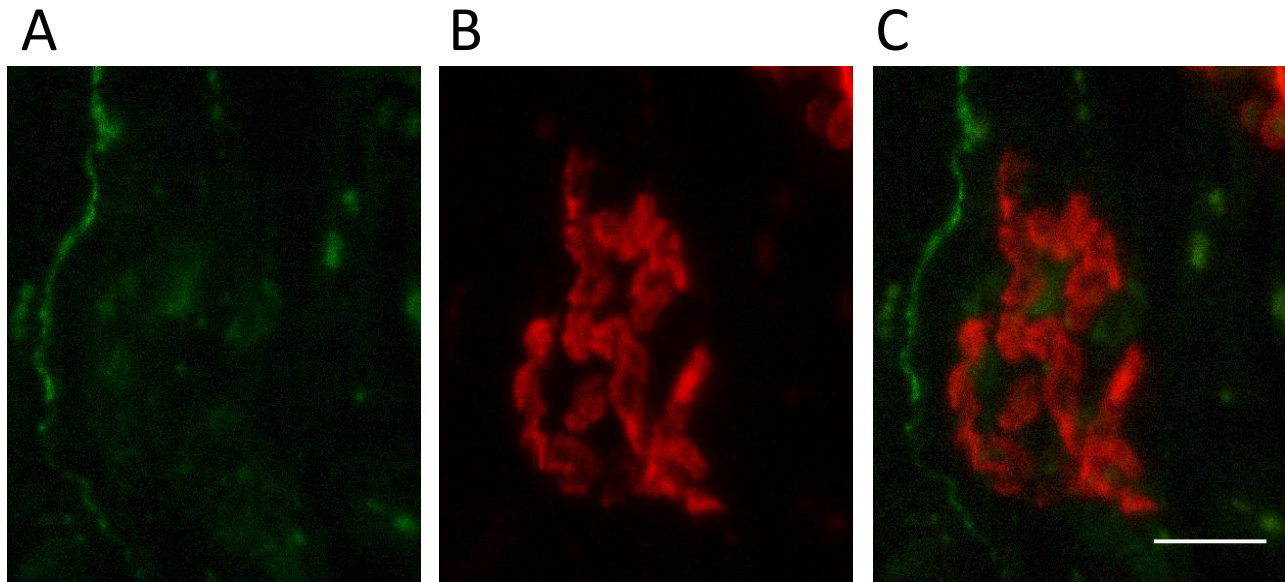


Figure 3-6 Showing and old neuromuscular junction from a 28 month old elderly animal TA muscle. (A) Presynaptic nerve terminal of the endplate showing clear absence of nerve staining suggesting retraction and withdrawal of the nerve from the endplate. **(B)** Postsynaptic AChR has maintained its integrity and has remained in one piece without signs of fragmentation. **(C)** Overlay of the nerve and AChR showing clear signs of the nerve being absent from the endplate showing poor innervation. Scale bar in panel C = 10 μ m

3.4 Morphometric Analysis of Endplates Images

Extended focus confocal images of the kind shown in Fig 3A, B and C were used to quantitate the areas of the synaptophysin-stained nerve terminal, endplate AChR clusters and the area of overlap/alignment between the two. During the investigation of changes that happen in the aging NMJ other parameters that may have been susceptible to the effects of aging were also investigated. These parameters include the total area occupied by the endplate (including the areas between the AChR clusters; total perimeter area) and the number of AChR fragments comprising the endplate. The animals, used were aged 2, 8 14, 19, 22, 25 and 28 months and all the animals in each age group were compared to every other age group. There were 3 animals sampled in each age group with the 22, 25 and 28 month groups being the only exception having 4 animals sampled. Tests for significant differences among the means were conducted using one-way ANOVA with a Bonferroni post test, all the error bars indicate \pm standard error of the means. The significance level was taken to be $P < 0.05$

3.4.1 Investigation of postsynaptic changes with age

The total area of the postsynaptic AChR was investigated first. The mean area of the endplate AChR plaque at 2 months of age was $226.4 \pm 9.1 \text{ um}^2$. The largest decline in AChR area was between 14 months and 28 months. A significant decrease in AChR area was also seen to occur between 2 months and 22 months and also 2 months and 28 months (Fig 3.7A). With the decrease in the mean AChR area with age it can be seen that the number of individual endplates with small AChR areas progressively increasing, this can be seen as a skewing in the frequency distribution to the left (Fig 3.7B).

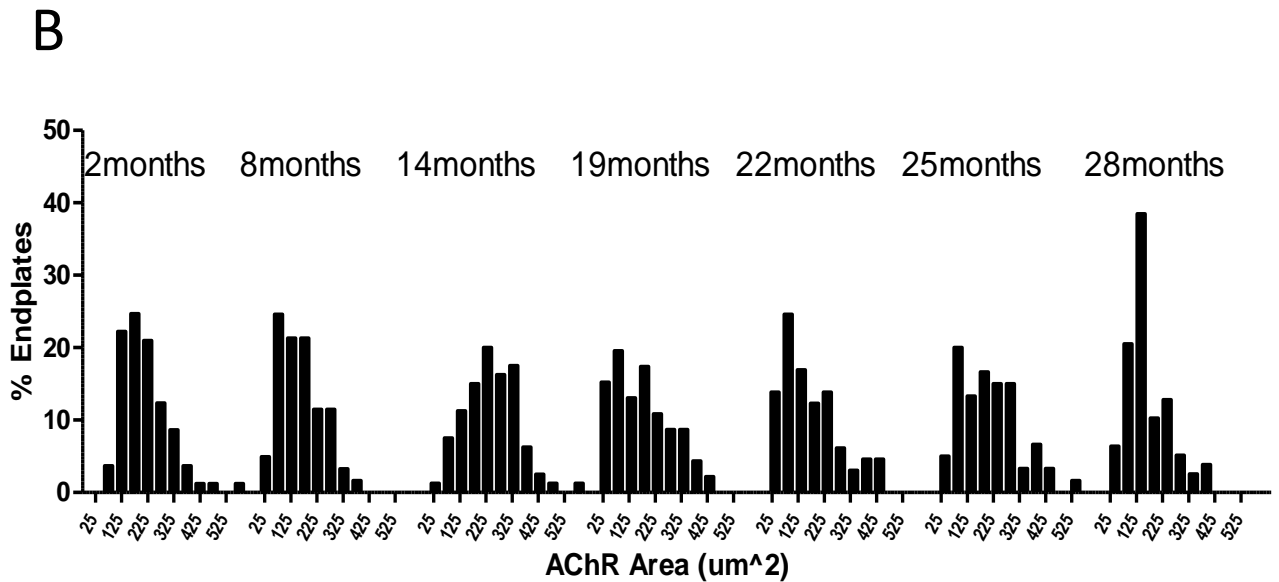
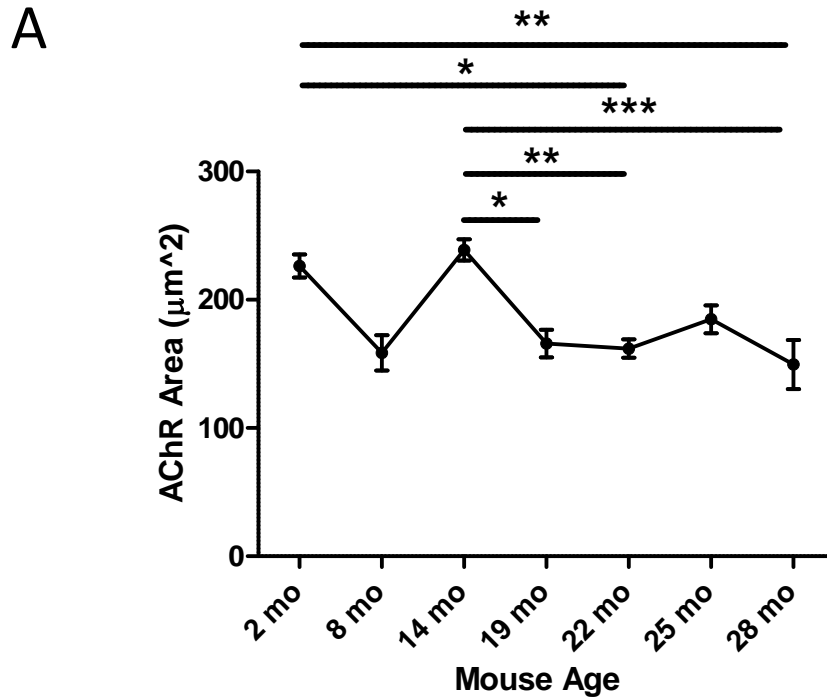


Figure 3-7 AChR areas for animals in aging time course. (A) Line graph of AChR means \pm SEMs ($n=3-4$ mice as described in the text). **(B)** Frequency distributions for AChR cluster areas at the ages studied in individual endplates (data pooled from 3-4 mice). Each bar represents the percentage of endplates that were measured to be within the specified BIN value shown on the x-axis

The second parameter investigated is the total perimeter area (TPA) including all unoccupied spaces within the endplate. The highest peak was seen at 14 months of age with a TPA of 527.2 ± 14 , but there was a not a big difference in this case among the studied ages (Fig 3.8). However there was a trend towards a lower TPA at the oldest ages of 25 and 28 months.

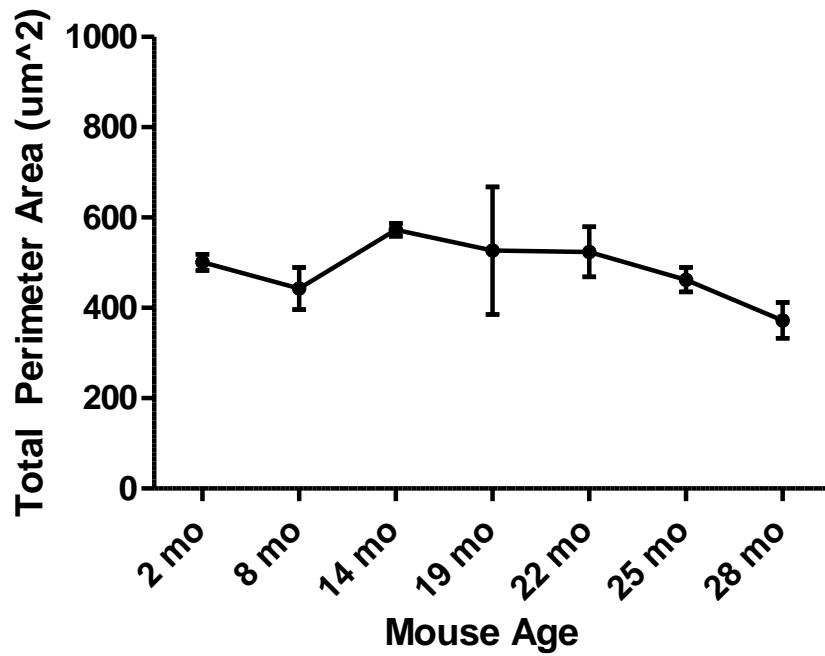


Figure 3-8 Mean total perimeter areas in aging time course. Line graph of mean total perimeter area in all age groups (mean +/- SEM, n=3-4 mice). Highest peak at 14 months and gradually decreasing till 28 months of age, but decline was not significant.

The third parameter investigated was the number of discrete postsynaptic AChR clusters comprising each endplate. Fragmentation of the ageing endplate AChR plaque into multiple smaller AChR clusters has often been reported (Valdez, Tapia et al. 2010; Li, Lee et al. 2011; Valdez, Tapia et al. 2012). At 2 months of age the mean number of discrete AChR clusters per endplate was 3.8 ± 0.7 . Surprisingly I detected no significant differences in the number of endplate fragments among the ages from 2 to 28 months (Fig 3.9A). However it is important to note that there was a high degree of variation among the mice within each age group in terms of the number of AChR cluster fragments per endplate. This animal to animal variability may have prevented the demonstration of statistical significance of possible changes with age. In particular one 19 month old animal (mouse #116) had substantially more fragmented endplates than the other 2 animals in the same age group (Fig 3.9B). This highlights the potential importance of individual differences in studies of the aging neuromuscular junction.

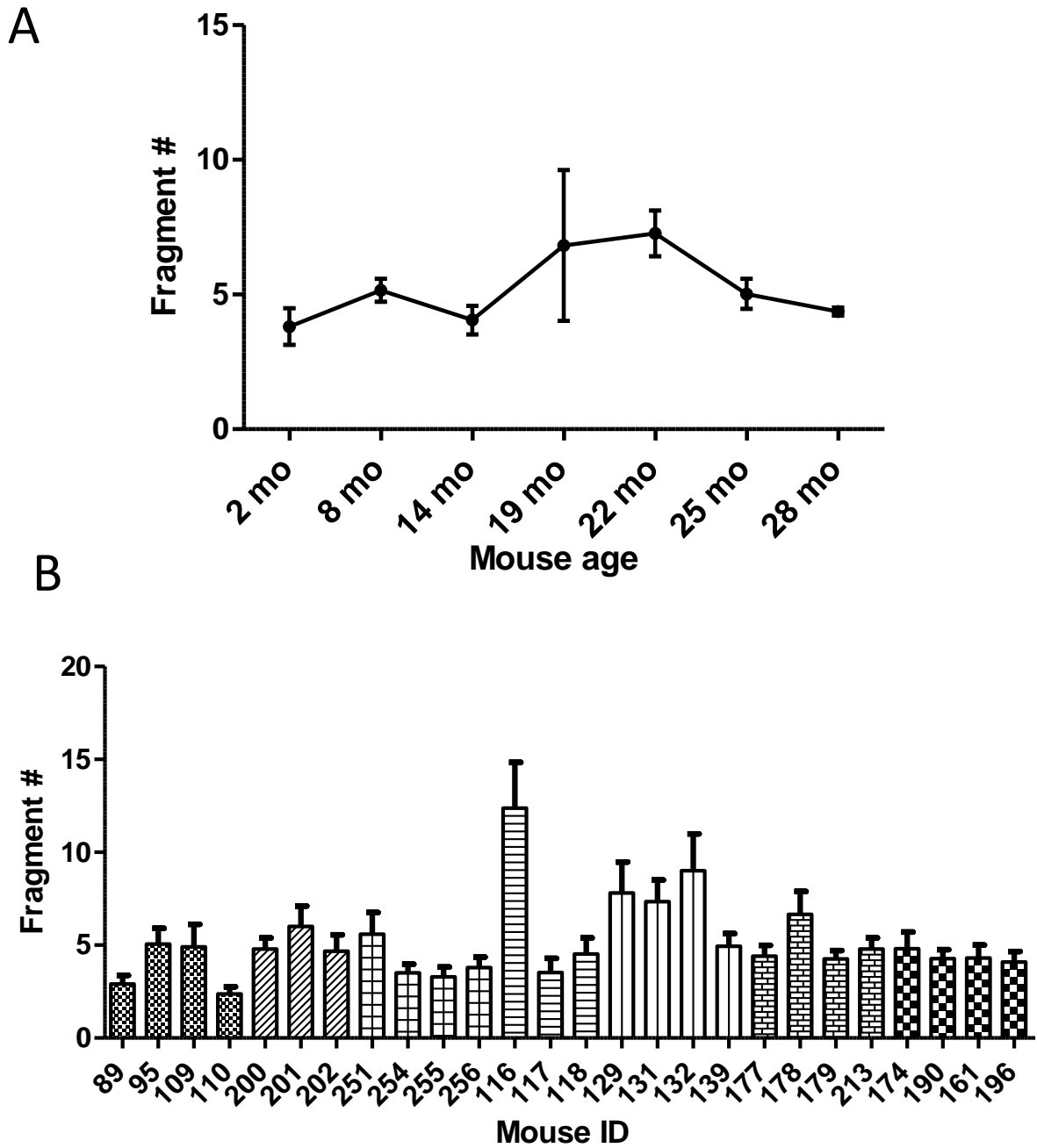


Figure 3-9 AChR fragment counts in aging time course. (A) Line graph of mean endplate fragments present per endplate in each age group of animals. No significant difference found between the age groups. **(B)** Column graph of average number of fragments found per endplate in each animal. Columns with the same pattern represent animals from same age group.

3.4.2 Presynaptic changes with increasing age

The fourth parameter was the area occupied by anti-synaptophysin positive presynaptic nerve terminal staining. There was a significant decline in the nerve area between 2 months and 25 months and between 2 months and 28 months of age. The biggest change can be seen to happen between the ages of 14 months and 28 months ($P < 0.001$) as illustrated in figure 3.10A. The mean nerve area at 28 months was slightly less than at 25 months, but this was not significant. Figure 3.10B shows frequency distributions for the nerve area of endplates at each age. While the nerve terminal area showed a roughly normal distribution at 2, 14 and 19 months, at later ages there was a marked skewing to the left for individual endplates, indicating that the endplates with very small nerve terminals were more abundant in older animals. The smallest bin ($0-50\mu m^2$) contained close to half of the endplates in 28 month old mice and this compares to the modal nerve terminal of $200-250\mu m^2$ in 19 month mice. These results suggest that ageing endplates in the TA may become partially or completely denervated.

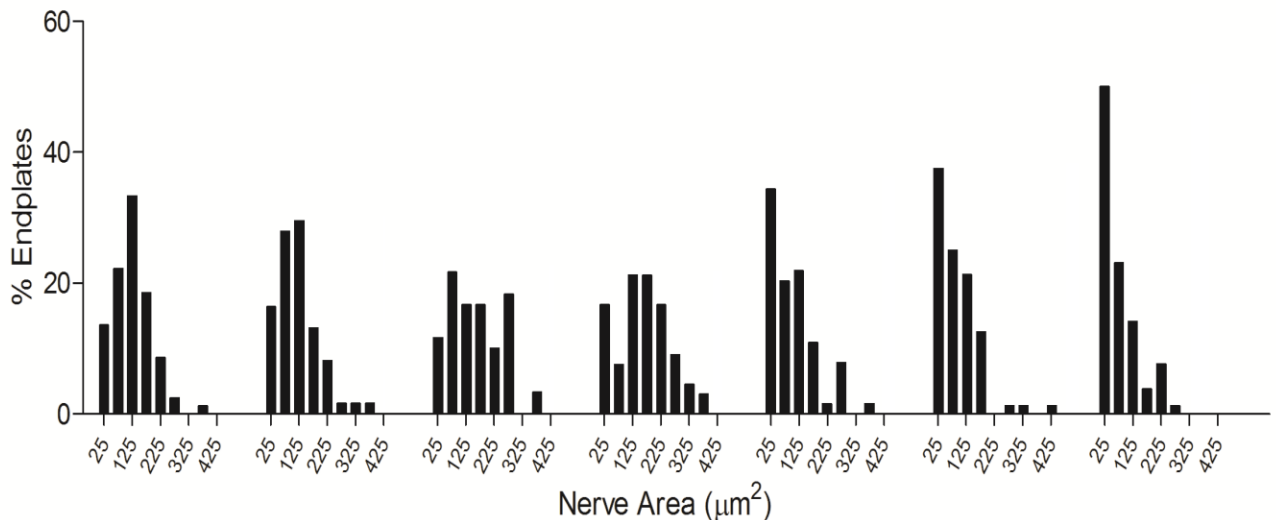
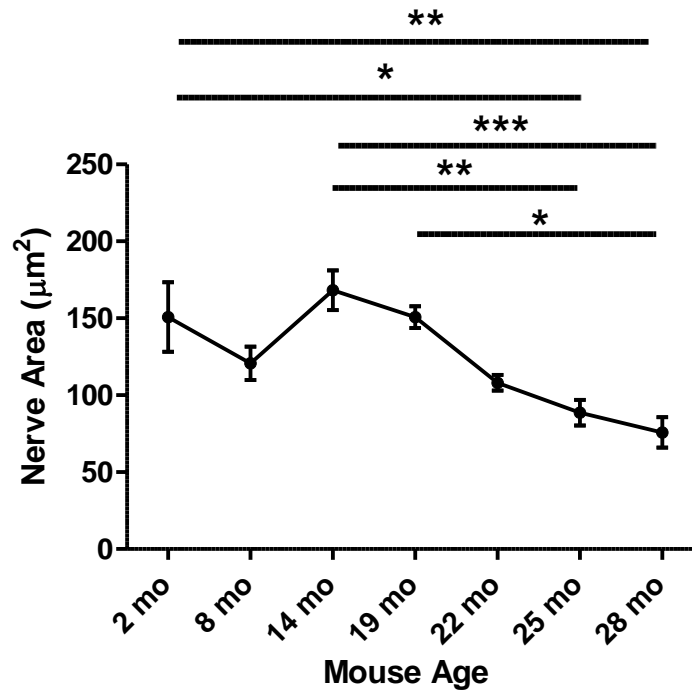


Figure 3-10 Presynaptic nerve terminal area of aging animals. (A) Line graph of the mean presynaptic nerve terminal areas of animals the ages indicated (mean \pm SEM, $n=3-4$ mice). There was a significant difference in nerve area between 14 months to 25 and months and also between 8 to 25 and 28 months. The largest difference was seen to be between 14 and 28 months ($P<0.001$). **(B)** Frequency distribution of the same animals 2, 8, 14 and 19 month animals showed a normal distribution in nerve areas. However 22 month animals showed a skew to the left due to larger number of small nerve area endplates. 25 month animals showed an even more prominent skew to the left.

3.4.3 Alignment of presynaptic nerve terminal and postsynaptic AChRs

The interaction of the presynaptic nerve terminal and postsynaptic AChR were also studied. To do this I measured the area of overlap between the anti-synaptophysin nerve terminal and the postsynaptic AChR cluster in extended focus projection images of the kind shown in 3.4C and 3.5C. At 14 months (middle aged) the mean area of overlap was $116.2 \pm 8.4 \mu\text{m}^2$. However the area of overlap significantly decreased as the animals got older. The biggest change in the overlap area was seen to happen between the ages of 14 months and 28 months as shown in figure 3.11A ($P < 0.01$). This change was due to the fact that a larger proportion of endplates, displayed only a small overlap area ($0-50 \mu\text{m}^2$) in 28 month old muscles compared with 14 month old muscles (Fig 3.10B note skew to left at 28 months). Thus the age related changes seemed to involve an increase in the proportion of endplates with very small areas of overlap (Fig 3.11B). These poorly innervated endplates represented about 60% of endplates in 28 month old muscles. To facilitate comparison, the changes in AChR cluster area, nerve terminal area and overlap area are re-plotted together in Figure 3.12. This shows that the overlap area, which presumably represents the function portion of the NMJ, declined over a similar time-course as the decline in nerve terminal area, that is between 14- and 28 months. There was one feature of the aging trend, which was unexpected in the parameters measured, this being the reduction in the AChR, nerve and overlap area at the 8 month age point. This was a transient dip in area returning to the 2-month level by 14 months of age. The reason for this dip at 8-months is not certain. One possibility is that it may be due to our animals being ex-breeders, which retire from breeding at around 8 months of age. Up to till this age the animals would have been pregnant several times and

are recently postpartum. Perhaps the hormonal changes may have affected the size in their NMJs.

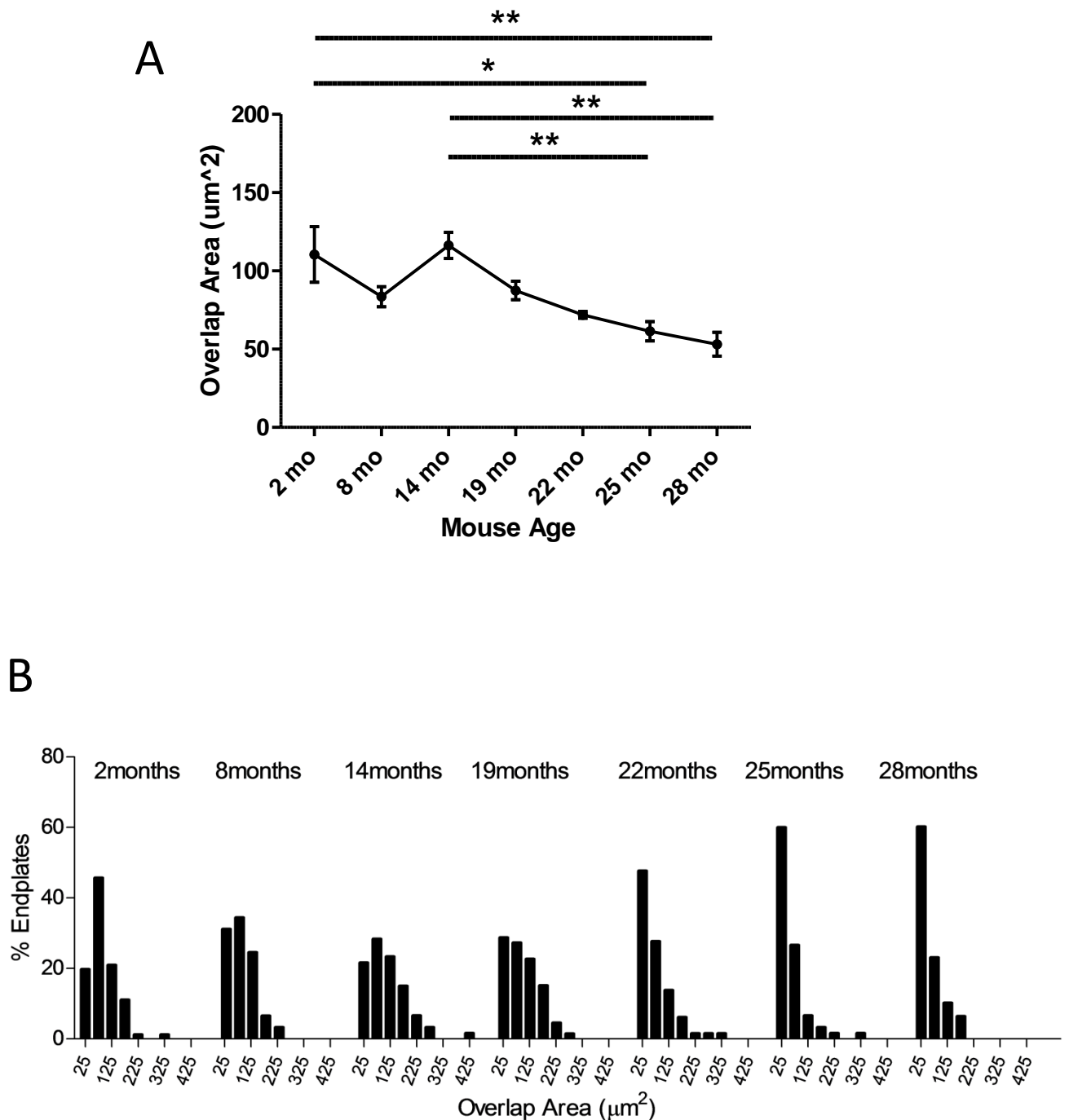


Figure 3-11 Mean area of overlap between the presynaptic nerve terminal and AChRs. (A) Line graph of the average area of overlap between nerve and AChR in unexercised animals of 2, 8, 14, 19, 22, 25 and 28 months of age (means \pm SEM, $n=3-4$ mice). There was a significant difference between the 2 month to 25 and 28 month groups and also between the 14 month to 25 and 28 month groups ($P<0.01$). **(B)** Frequency distribution of individual endplate overlap areas. 2 and 14 month animals showed a normal distribution whereas the 22, 25 and 28 month groups showed skewing to the left most prominent in the 25 and 28 month groups.

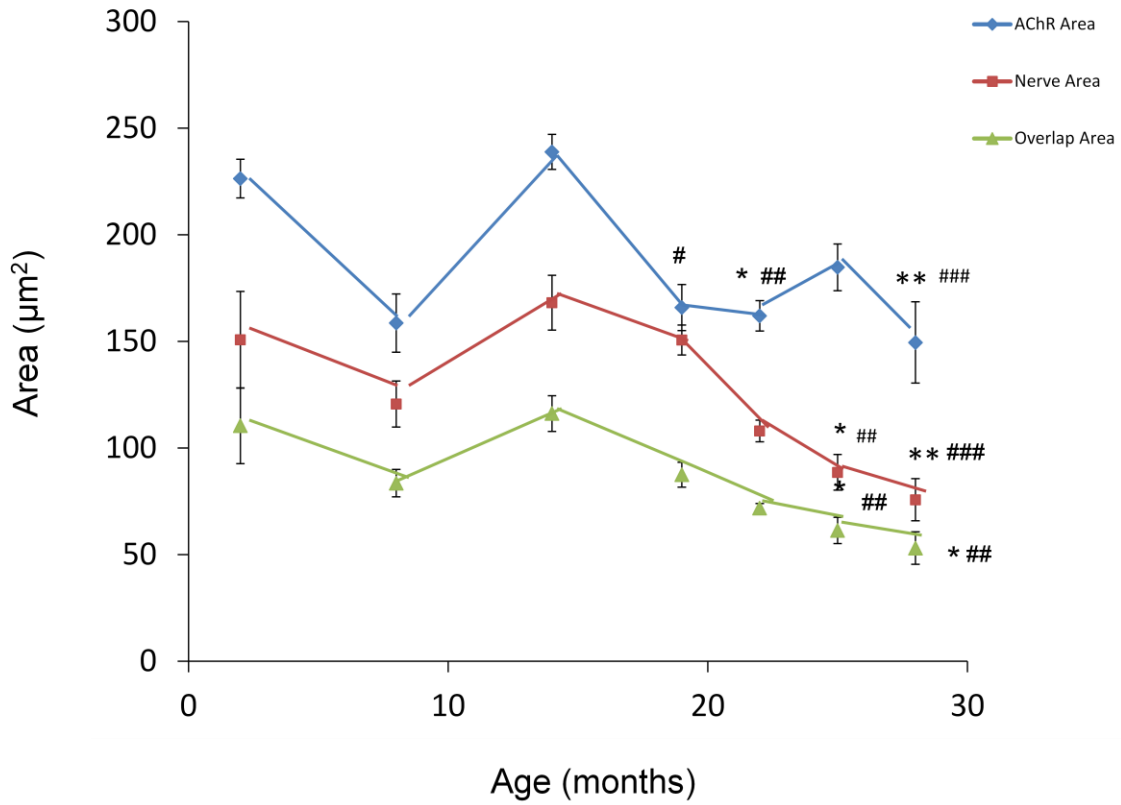


Figure 3-12 Data from figures 3.7A, 3.9A and 3.10A replotted for comparison purposes. Line graph of the AChR, Nerve and the overlap area of endplates from animals at various time points in a mouse's lifetime. The * symbol represents that point is statistically significant from the 2 month data point. The # represents that point is statistically significant from the 14 month data point.

Can the age-associated reduction in nerve terminal-AChR overlap be caused by the reduction in nerve terminal area or AChR area or does it involve a misalignment of the nerve terminal from the AChR cluster? To address this question I first calculated the percentage of endplate AChRs that were covered by nerve terminal. This was done by expressing the overlap area of AChR (α -bungarotoxin staining with nerve anti-synaptophysin) as a percentage of the AChR staining area. At 19 months of age the mean percentage of AChRs innervated by nerve was $54.9 \pm 1.8\%$. There was a significant decline by 25 and 28 months of age ($P < 0.05$). This decline may be due to the increase in the proportion of endplates, which had very small amounts of innervation. The number of endplates, which had low innervation was much higher in the 28 month old animals than the animals aged 19 months. The age related changes illustrate that the nerve, which is present at the endplate innervating AChRs decreases. The mean percentage of AChRs of an endplate with innervation had decreased by 20% by the age of 28 months when compared to 19 months (Fig 3.12).

To see if the nerve terminal might have become displaced from the AChR clusters I examined the proportion of the nerve terminal that had AChRs clustered underneath it. This parameter did not change much with age. At 19 months $59.3 \pm 4.7\%$ of the nerve terminal had AChRs clustered beneath it. There was a significant difference between 2 and 19 months of age as seen in Figure 3.10. No significant changes were found after 14 months of age (Fig 3.13). Together these results suggest that the loss of nerve terminal-AChR overlap area can be largely attributed to withdrawal of nerve terminals from endplate AChR clusters.

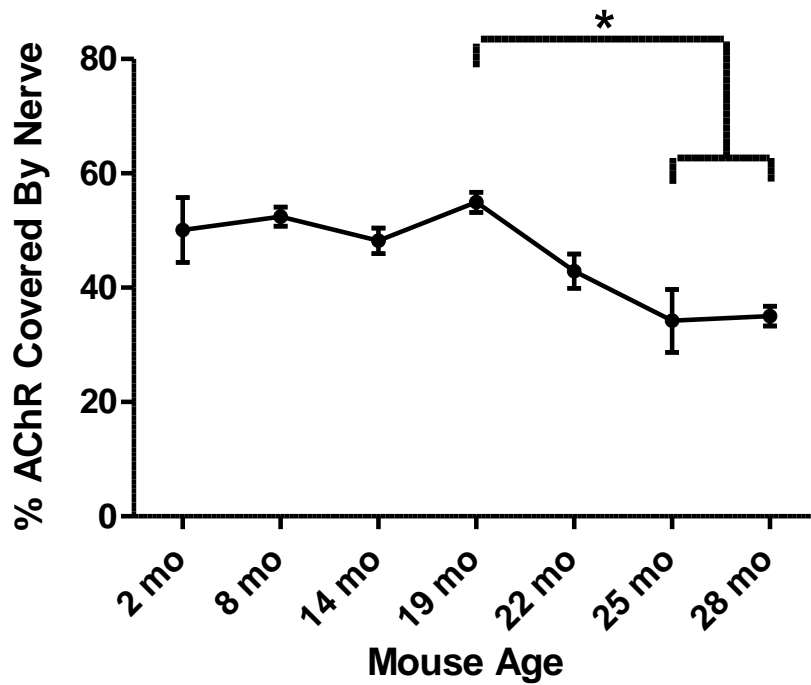


Figure 3-13 Age related difference in mean percentage of AChR covered by the presynaptic nerve . Line graph of average percentage of AChR that is innervated by Nerve (mean +/-SEM, n=3-4 mice). Significant difference seen between the age groups of 19 months to 25 and 28 months ($P < 0.05$).

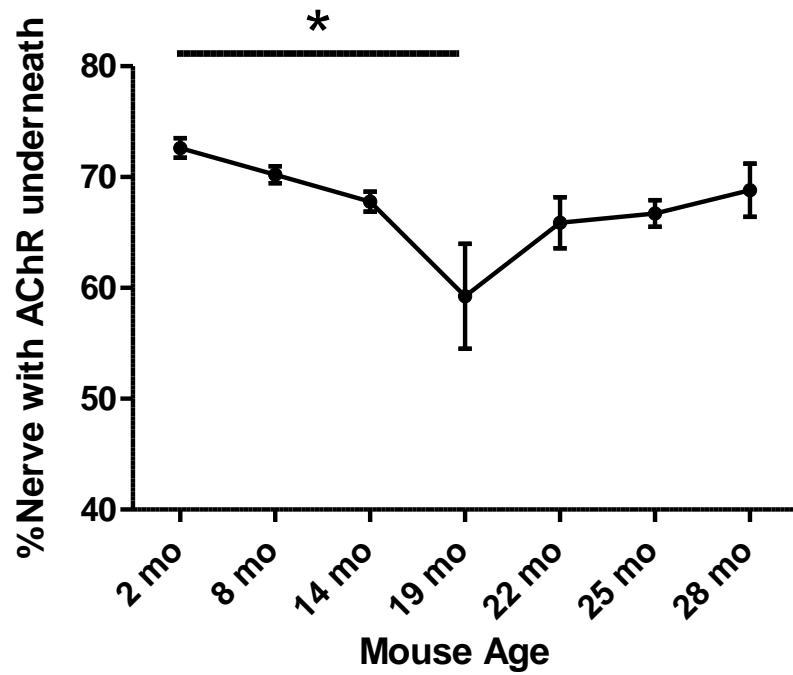


Figure 3-14 Changes in mean percentage of nerve terminal with AChRs underneath. Line graph of mean percentage of nerve with AChR present underneath (mean \pm SEM, $n=3-4$ mice). Significant difference between 2 month and 19 months ($P<0.05$).

3.5 Chapter Discussion

The effects that I found of age on the NMJ appeared to be mainly originating from the presynaptic side. In the aging animals that I studied the nerve terminal area appeared to become smaller in size. On the postsynaptic side significant differences were only seen in the AChR area and not fragmentation of the endplate AChR cluster, reported by others (Valdez, Tapia et al. 2010; Li, Lee et al. 2011; Valdez, Tapia et al. 2012) and could not be confirmed. The reduction of nerve area could largely explain a significant decline in the nerve terminal-AChR cluster overlap area as shown by my analysis of the percentage of AChRs that had innervation.

In this study I used anti-synaptophysin as my major marker of the nerve terminal.

Synaptophysin is a synaptic vesicle transmembrane glycoprotein shown to be present at the mouse NMJ (Colasante, Brouard et al. 1993). Synaptophysin has been proposed to be involved with neurotransmitter secretion, by affecting the probability of vesicular exocytosis and the number synaptic vesicles initially docked at the active zones (Alder, Kanki et al. 1995). Being mainly involved with the release of NT (neurotransmitter), may have an important role at the aging neuromuscular junction, where the loss of this protein can cause inadequate activation of AChRs. Synaptophysin has been widely used as a marker for studies of innervation of the NMJ (Bartolome, Zuluaga et al. 2009; Ling, Gibbs et al. 2011). Certainly it should provide a consistent marker for where synaptic vesicles become clustered in the vicinity of sites of transmitter release. Nevertheless, my results would not be expected to reveal any remaining nerve terminal that was no longer specialized for transmitter release.

Thus it is possible that authors who use other nerve markers (eg cytoplasmic YFP) might measure larger nerve areas than would be revealed by my anti-synaptophysin staining.

Although I found some significant changes of synaptophysin staining in the presynaptic nerve terminal, the motor axon is much larger than the terminal. The motor axon reaches down to the muscle fibre and eventually forms the presynaptic nerve terminal. If there are impairments in the motor neuron or the motor axon it may also cause some functional problems at the NMJ, but changes in the preterminal axon is an area that we have not addressed in this study

CHAPTER 4

EFFECTS OF EXERCISE ON THE AGING

NMJ

4 Effects of voluntary exercise upon the aging neuromuscular junction

4.1 Preamble

The second part of my investigation was to test whether the age-associated changes to the NMJ described in the previous chapter can be prevented or dampened if animals are allowed to exercise. We can see from the results in the previous chapter that most changes mostly occurred between 19 months and 28 months of age so it was assumed that 19 months is when the degenerative effects of aging begins. In our investigation we wanted test the effects of either longer (10 months) or shorter (4 months) periods of exercise and also different starting points so in our protocol we decided to give the animals a running wheel for either ten months starting at 18 months or for 4 months starting at 21 months of age. A running wheel was provided, which they were free to use whenever they wanted and for as long as they wanted. Animals were then analyzed at 25 and 28 months of age and compared to control mice that did not have access to a running wheel.

4.2 Assessing the use of the running wheel and voluntary exercise

In part of the study a wireless running wheel was placed into the mouse box as an intervention. The use of the wheel was assessed from records of wheel rotations during subsequent months. Animals aged 25 months began running at 21 months of age and animals at 28 months began running at 18 months of age. This allows for a long period of assessment of running wheel usage starting from the mice were aged 21 months which they were allowed to use till the age of 25 months and also mice that were aged 18 months, which exercised till 28 months. Analysis of running wheel activity discussed in methods. Regular

monitoring of running wheel activity revealed that the mice did a large amount of running once they became familiar with the wheel. Fig 4.1A shows an example of the circadian pattern of use of the wheel when the mice were aged 18 months. The distance ran peaked at roughly 1 kilometre (km). Fig 4.1B shows another example of circadian pattern of use of the wheel, but the mice here were only 12 months old and in this case we can see the running distance peaking at 1.4km. Wheel use occurred predominantly between the hours of 10 pm and 3 am in both age groups consistent with the natural nocturnal pattern of mouse activity. Fig 4.2 shows the average wheel activity over a period of 10 months from when the wheel was first placed into the boxes. It was observed that when the wheel was first introduced the amount of usage varied by quite a large amount. After 1-2 months the variability of the usage in the wheel had become much smaller. When the mice were 18-22 months old the amount of running that was done by the mice was about 1.1 km/day/mouse. As the mice progressed into the very old ages the amount of running began to drop off. However with the introduction of the wheel it seems likely that the running activity of the mice was greater than for control mice without a wheel, given the small dimensions of the box without a wheel. Even at 26-28months of age (when approximately half the original population had died off) the mice were still running approximately 0.5 km/day/mouse.

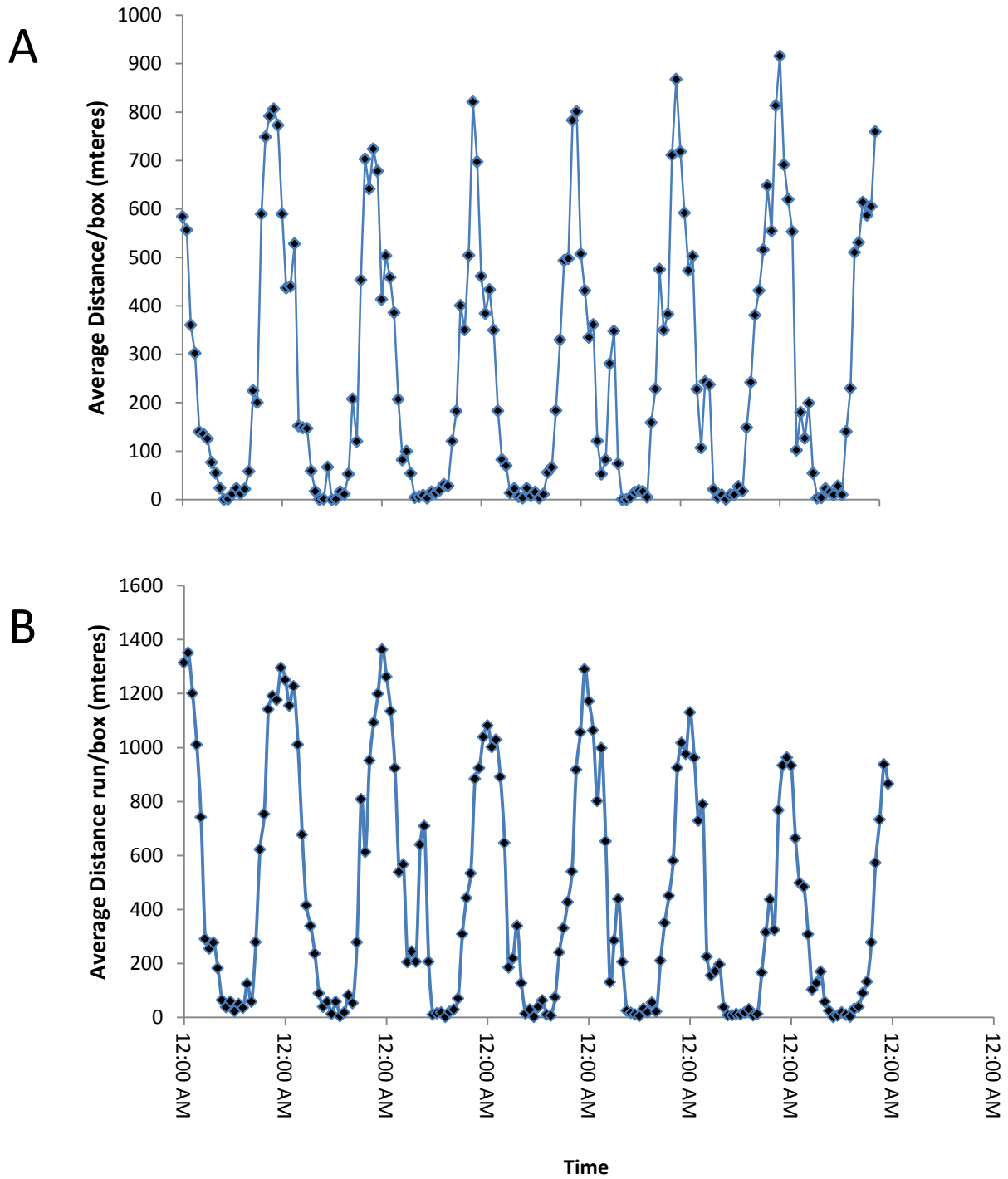


Figure 4-1 Circadian rhythm of running wheel animals. (A) Line graph showing the circadian rhythm of wheel activity over 1 week in mice that were 18 months old. **(B)** Circadian rhythm of mice aged 12 months over 1 week period.

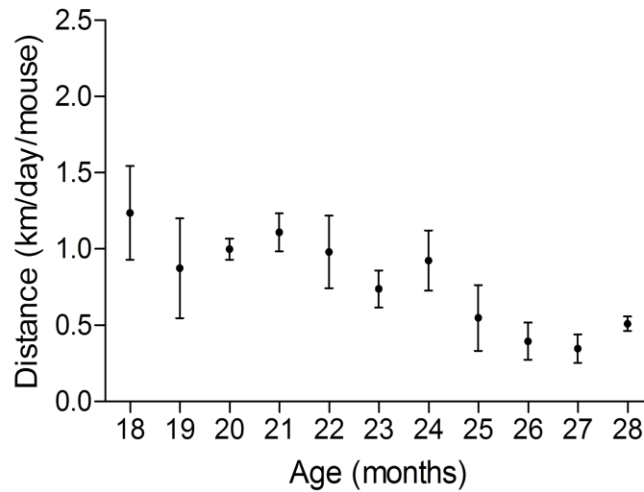
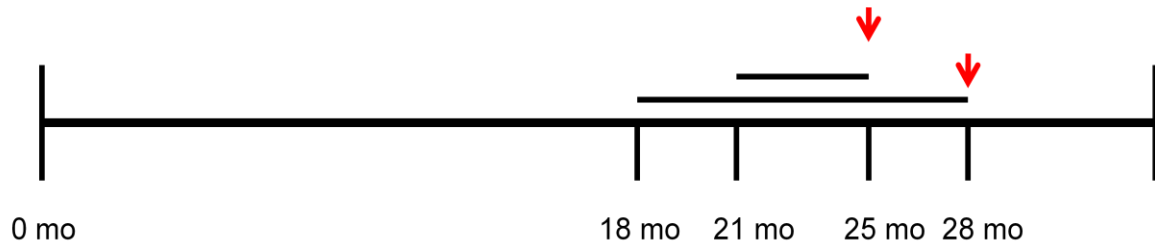


Figure 4-2 Wheel running by aging mice. Bars at top show the period during which mice were provided with a running wheel, while arrows indicate the age at which their NMJs were analysed. The graph shows average distance run for mice provided with a wheel from 18 months of age. Data points show the mean running distance per mouse per day over the time course from when they had the running wheel to the day they were culled. Averages were calculated by dividing the total distance run by the number of mice in the box. Error bars reflect SEM for n=5 boxes (Each box contained 3-4 mice)

4.3 Effect of running wheel access upon the NMJ

I compared the effect of running wheel access upon the parameters described in the previous chapter. Comparison of results for wheel access and control (no wheel) was made using a Student T test. Statistical significance was taken as $P < 0.05$. All the error bars in the graphs depict standard error of the means.

Fig 4.4A depicts an example of an endplate that was imaged from a 25 month old control mouse where it appears to nearly completely denervated. Fig 4.3B delineates an endplate imaged from a 25 month old wheel mouse where the endplate has retained a good amount of innervations and appears to be relatively healthy.

A

B

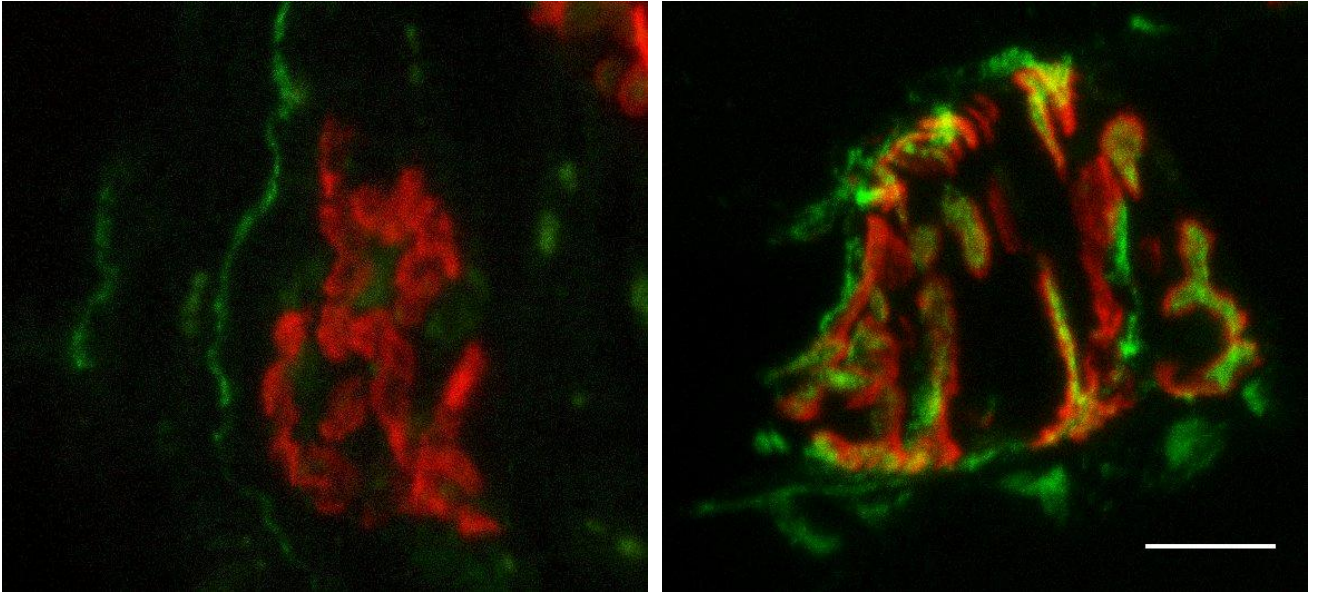


Figure 4-3 Aging control and exercised endplates from TA. (A) NMJ overlay of AChRs and presynaptic nerve terminal from 25 month old sedentary control mouse. **(B)** NMJ overlay of AChRs and presynaptic nerve terminal from 25 month old wheel treatment mouse. Scale Bar in panel = 10 μ m

4.3.1 Effects of exercise on age related postsynaptic alterations

Twenty-five month old mice provided with a wheel from 21 months of age (25month wheel mice) showed a significantly larger AChR area, compared to age matched mice that did not have access to a wheel (Fig 4.4). Twenty eight month old mice provided with a wheel from 18months of age (28month wheel mice) showed a similar trend but the difference was not significant compared to 28-month mice without a wheel increasing from 149.5 ± 19.1 to 209.8 ± 19.09 (mean +/- SEM, Student T-test). There was no significant difference in average number of AChR cluster fragments per endplate for wheel mice compared to no-wheel control mice at either 25 or 28 months (Fig 4.5).

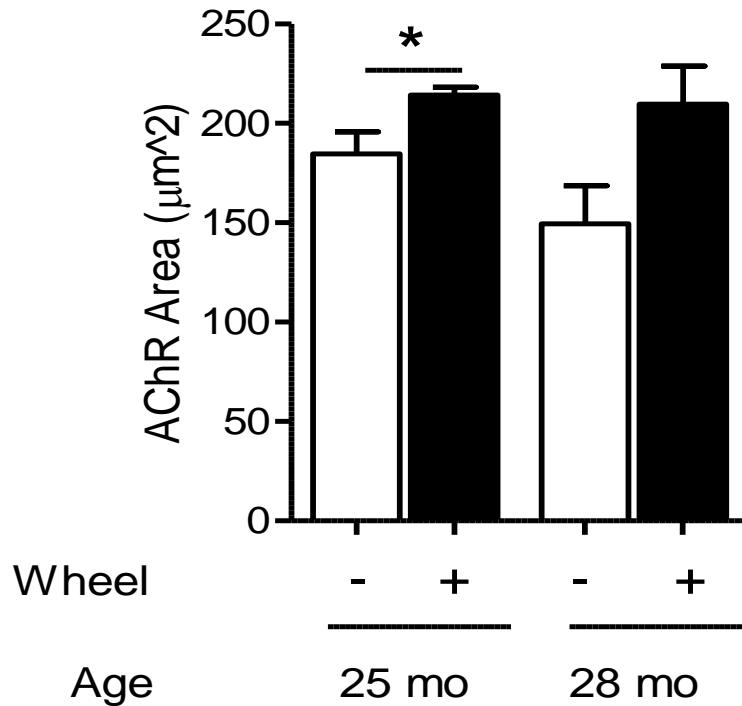


Figure 4-4 Effects of exercise on AChR area. Column graph of average AChR areas between the no exercise and exercise treatment groups. A significant difference was observed between controls animals and exercised animals aged 25 months ($P < 0.05$). The + symbol represents the animals that received a running wheel and the - symbol represents the age matched controls. In the 28 month age group the animals with exercise also showed a sizeable increase in AChR area, but was not significant in this case ($P = 0.06$). Shown are means \pm SEMs ($n = 4-5$ animals, Student T-test).

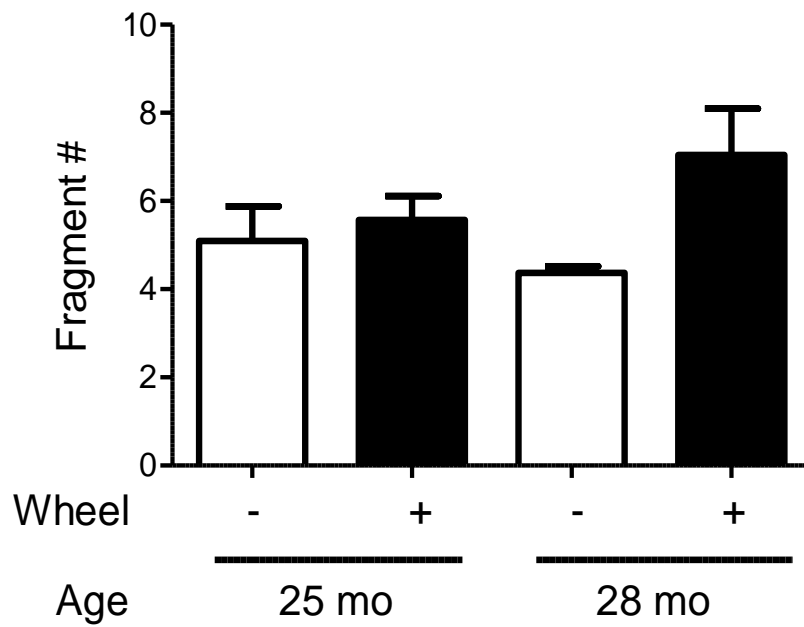


Figure 4-5 Effects of exercise on endplate fragmentation. Average fragment numbers found in no exercise and exercise treatment groups. There was an increase in fragment number with exercise in animals aged 25 months, but did not show significance ($P=0.63$). The increase with exercise was more prominent in animals aged 28 months, but was also not significant ($P=0.06$). Shown are means \pm SEMs ($n=4-5$, Student T-test).

There was no significant difference in the Total Perimeter Area (TPA) between the control and wheel group in both 25 and 28 age groups. There was a trend toward a higher TPA in the wheel treatment group. This trend was particularly prominent in the 28 month age group increasing from 372.4 ± 39.43 to 525.3 ± 51.67 , but was not significant (Fig 4.6).

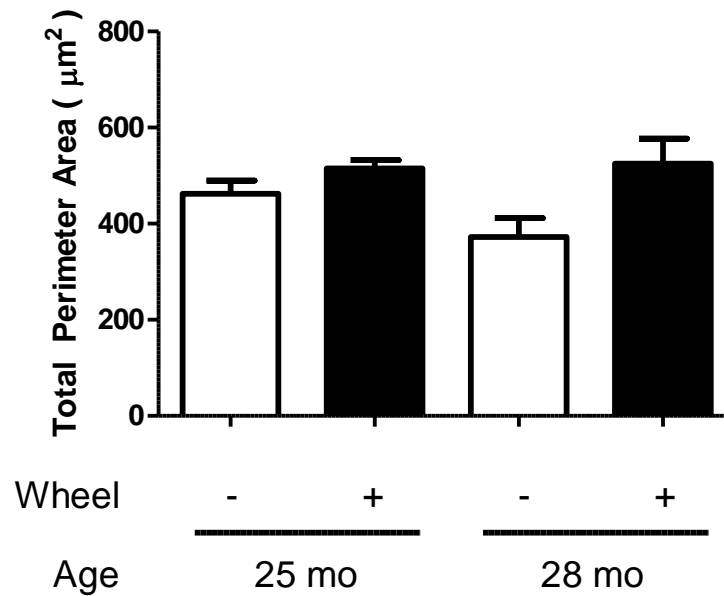


Figure 4-6 Effects of exercise on TPA. There were no significant changes in the total perimeter area between control and wheel treatment groups in both 25 and 28 month age groups. There was an insignificant increase in TPA with exercise in animals aged 25 months ($P=0.13$). Increase in TPA with exercise was also observed in animals aged 28 months, but was also not significant ($P=0.06$). Shown are means \pm SEMs ($n=4-5$, Student T-test).

4.3.2 Effect of exercise upon nerve terminals

The area of synaptophysin stained nerve terminal was increased by about 1.5 fold ($P < 0.05$) in the 25 month wheel mice compared to age matched controls increasing from 88.6 ± 8.35 as seen in figure 4.7A. A similar increase was seen in the 28 month wheel mice when compared to aged sedentary controls increasing from 75.78 ± 9.91 to 122.2 ± 12.82 .

Frequency distributions of pooled data confirmed that for the wheel groups fewer of endplates were innervated by very small nerve terminals compared to aged matched mice without access to a wheel (Fig 4.7B). The skewing of the graph is more obvious for the control groups. These results suggest that exercise may be able to slow the age-associated loss of nerve terminal area.

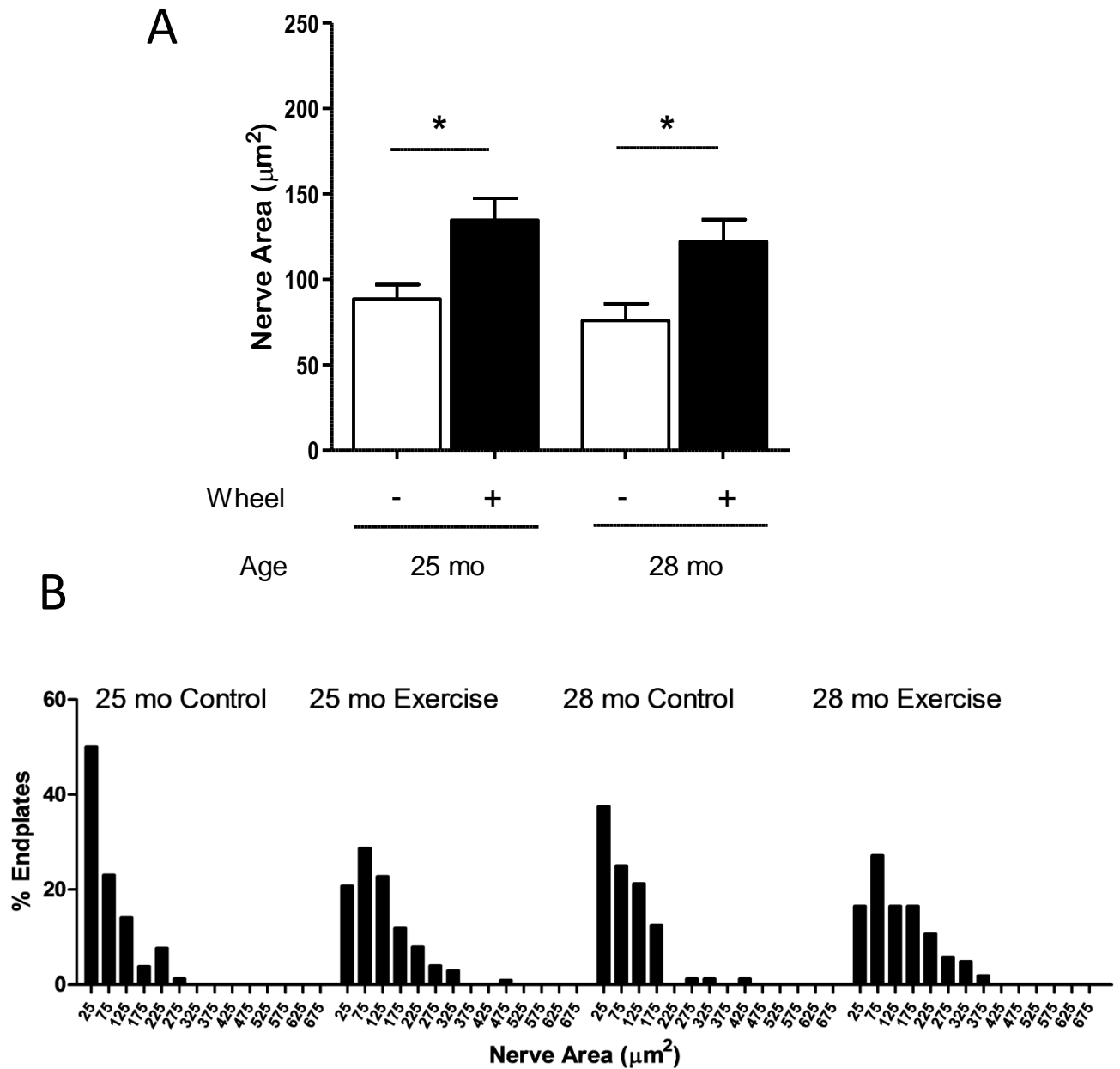


Figure 4-7 Effect of wheel access upon nerve terminal area in aging mice. (A) Column graph of nerve terminal areas in the 25month and 28 month wheel mice compared to age-matched control mice. In the exercise treatment group the nerve area was significantly larger in at both ages. Data represent the mean +/- SEM for n=4 mice in each control group and n=5 in each wheel group ($P < 0.05$). **(B)** Frequency distribution of nerve terminal areas (data for no-wheel controls is reproduced from Chapter 3). The skewing to the left not so marked in the exercise treatment groups.

4.3.3 Effects of exercise on pre and postsynaptic overlapping regions

The area of overlap between the presynaptic nerve terminal and postsynaptic AChR clusters was also significantly higher in the wheel treatment groups ($P < 0.05$) as shown in figure 4.8A. This was true at 25 and 28 months. The number of endplates with small overlap area endplates was reduced in the exercise treatment groups (Fig 4.8B).

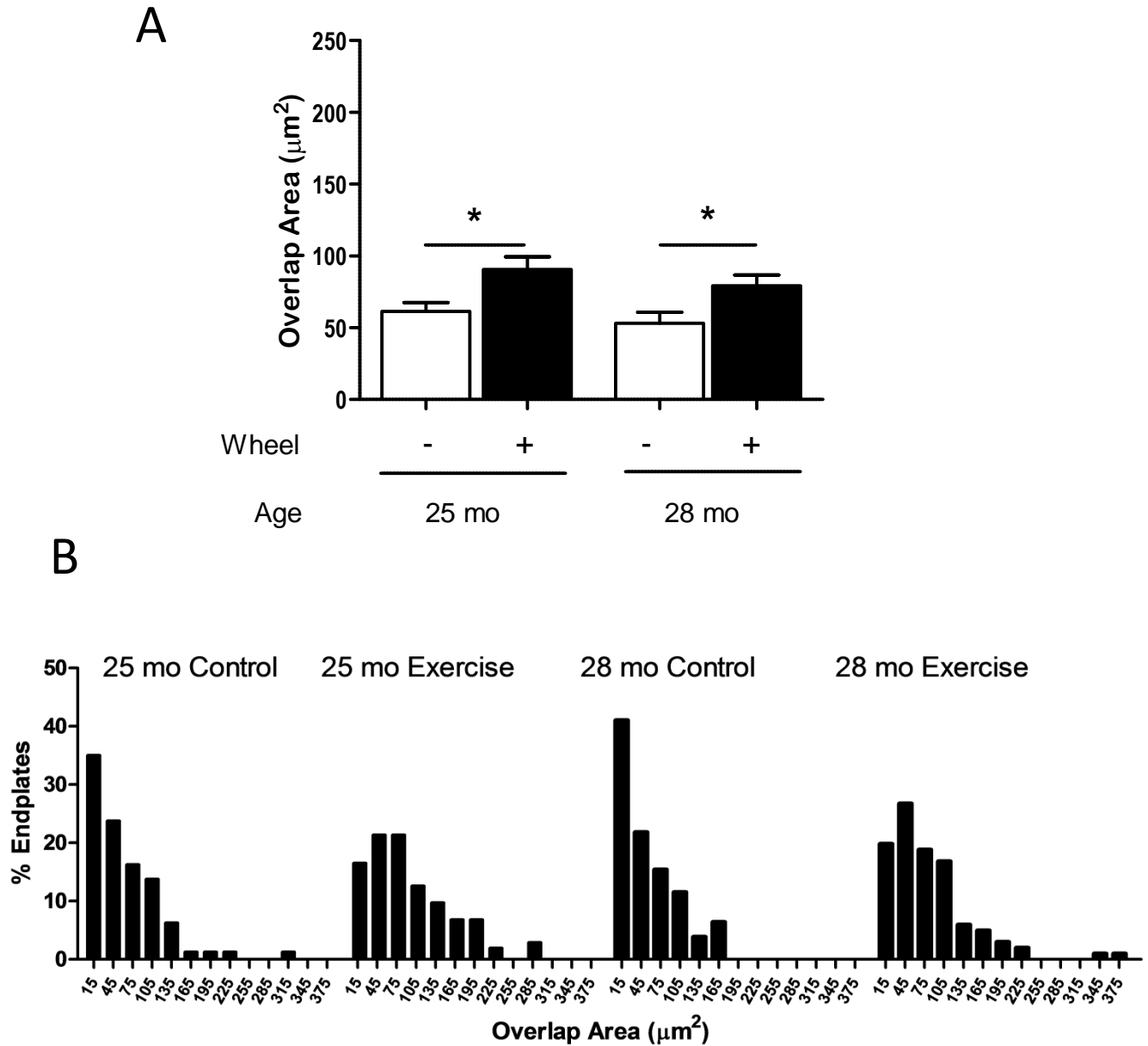


Figure 4-8 Nerve terminal-AChR overlap for endplates of mice with and without access to a running wheel. (A) Column graph of 25- and 28- month wheel mice are compared overlap areas for age-matched control data (replotted from Chapter 3). The Overlap area was significantly larger in the exercise group when compared to no exercise group ($P < 0.05$). Data represent mean \pm SEM for $n=4$ mice in each control group and $n=5$ in each wheel group. **(B)** Frequency distribution of overlap areas, the exercise treatment group exhibit less small overlap areas than the no exercise treatment groups therefore showing a less prominent skew to the left. Data for endplates was pooled. Less small overlap areas than the no exercise treatment groups therefore showing a less prominent skew to the left. Data for endplates was pooled.

There was no significant change in the proportion of the postsynaptic AChR cluster that was covered by nerve terminal staining (Fig 4.9), nor in the proportion of the nerve with AChR clustered underneath between the wheel access mice and aged matched sedentary control mice (Fig 4.10).

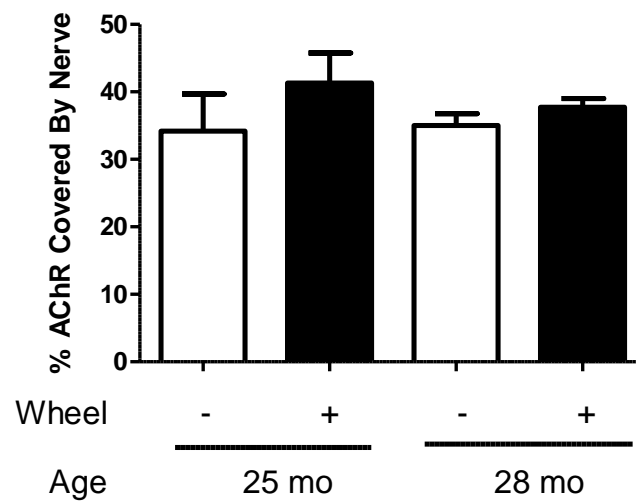


Figure 4-9 Effect of exercise on AChR percentage innervation. Column graph of the average percentage of AChRS that is innervated by nerve. There was an insignificant increase in %AChRs covered by nerve with exercise in animals aged 25 months ($P=0.34$). This increase with exercise was also observed in 28 month animals, but was also insignificant ($P=0.24$). The columns show means \pm SEMs ($n=4-5$, Student T-test).

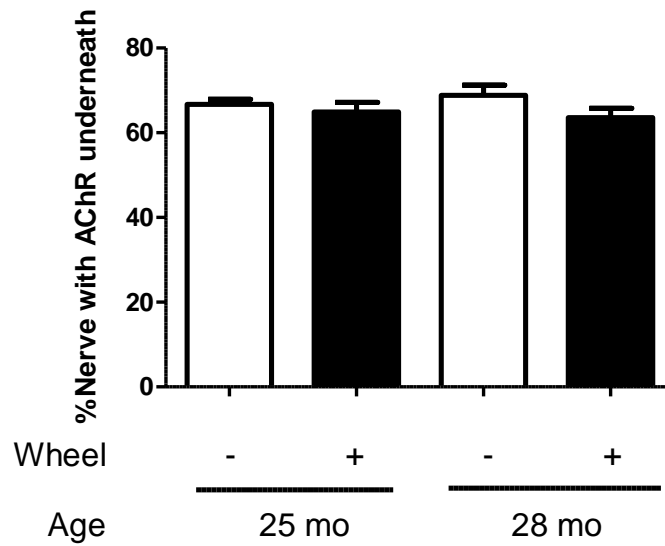


Figure 4-10 Effect of wheel access on percentage of nerve with AChR underneath. Column graph showing the average percentage of nerve that had AChRs present underneath with no significance in this case. There was no practical difference in the 25 month old animals ($P=0.54$). There was a small decrease with exercise seen in animals aged 28 months ($P=0.15$).

4.4 Chapter Summary and Discussion

Mice that had been given access to a running wheel from 18- or 21 months of age did a reasonable amount of exercise each day. The larger amount of running that was done at the beginning period is possibly due to the mice still having the fitness and ability to do a substantial amount of running. When they got older the ability of the mice to sustain the running effort for a long distance may be reduced or with their reduced ability they may also feel unmotivated to do exercise, this was seen as the reduced amount of running that the mice did each day as they got older. In previous studies it was seen that mice given in a running wheel at a young age and ones that were given a running wheel at a much older age the amount of running that was done by the young animals was substantially more. It was shown that young mice were running at an average of 12 km/day and old mice were running at average of 1km/day, which is much higher than what we found, but this is perhaps due to the high N number that the study included (Durrant, Seals et al. 2009).

Exercise appeared to have a large impact on presynaptic components of the NMJ, where there were significant larger nerve terminal area and area of overlap between the synaptophysin stained nerve terminal and the AChR clusters. On the postsynaptic side the AChR cluster area also increased significantly with exercise for those mice examined at 25 months after 4 months of running wheel access compared to aged matched mice without a wheel. The number of AChR clusters, the proportion of AChR covered by nerve staining and the proportion of nerve with AChR clusters underneath did not differ significantly between the exercise and control groups. AChR area did not change significantly with aging, but increased modestly in 25-month mice that were allowed to exercise. This shows that

while the AChRs cluster area was unaffected by the effects of aging, exercise may have the capacity to increase postsynaptic AChR cluster area. As shown in the previous chapter, nerve terminal area and the area of overlap between nerve terminal and AChRs decreased significantly with age, but exercise appeared to have a positive effect in reducing this decline.

While the proportion of AChRs covered by nerve declined significantly with age, exercise did not significantly affect this parameter. Interestingly the proportion of nerve with AChR underneath was very consistent throughout life even when young adult (8 month) and old (28 month) mice were compared, implying that this is a stable variable with little influential factors. Exercise had no significant effect upon the proportion of nerve with AChR underneath.

Overall the effects of aging can occur on some parameters, but not all and exercise can slow or prevent the age related decline in nerve terminal area and synaptic overlap. Exercise even showed a potential to increase AChR cluster area: a parameter that did not change with sedentary age. While exercise was able to produce some positive effects on certain parameters, but it did not affect all age related changes.

CHAPTER 5

EFFECTS OF AGE AND EXERCISE ON

MUSCLE FIBRES

5 Effects of age and exercise upon muscle fibre girth

5.1 Introduction

The third part of my investigation addressed the question of whether or not ageing and voluntary exercise had any effect on the cross sectional areas (CSA) of muscle fibres. This analysis was undertaken to see whether any changes in the girth of the muscle fibres might help explain the changes I found in the size of NMJs with age and exercise. For this analysis I used cross sections through tibialis anterior muscles from the same mice as I studied in the previous chapters.

5.2 Effects of ageing on muscle fibre CSA

Fig 5.1A portrays an example of a microscope field from a 2 months old mouse stained for basement membrane laminin. Such images were used to measure muscle fibre cross-sectional areas. It can be seen that the muscle fibres appear to be intact and are well orientated. Fig 5.1B is a cross-sectional image take from a 25-month old animal (no running wheel). It can be seen that in the aged animal the muscle fibres appear to be less-well orientated compared with the young animal. The laminin in the old animal also appears to be sparser compared to the young animal, which may lead to lower signal of laminin (Fig 5.1B).

A

B

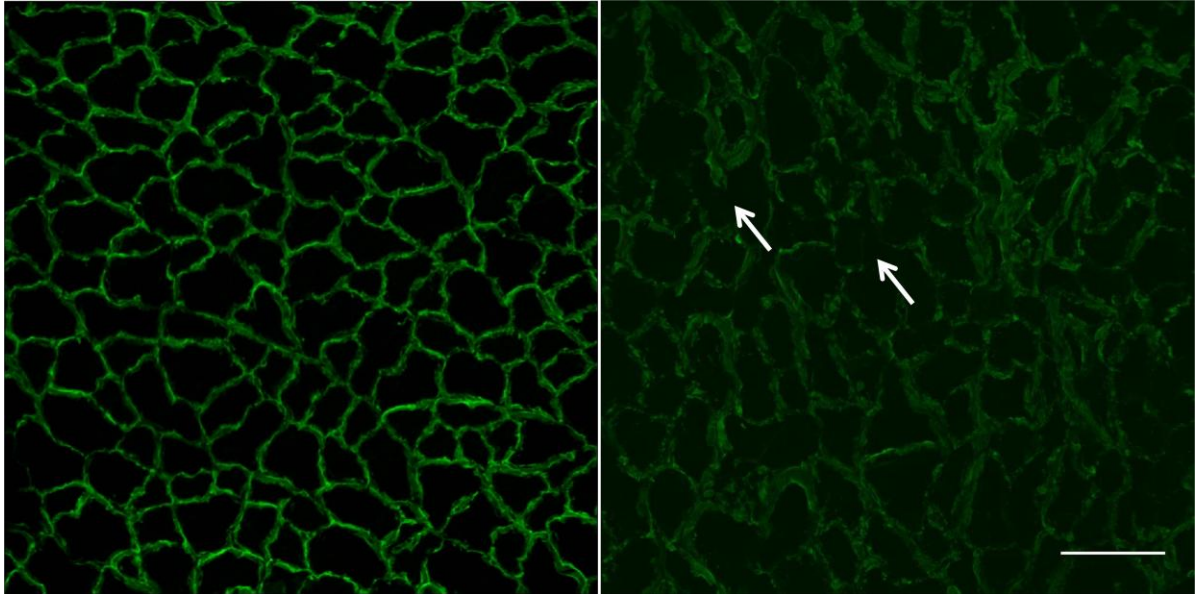


Figure 5-1 Muscle fibres of young and old animals from TA muscle. (A) Anti laminin stained muscle fibre in cross sectional projection in a young 2 month old animal. **(B)** Muscle fibres of a 25 month old animal, arrows indicate regions where anti laminin staining appeared incomplete and therefore leading to gaps in the membranes of fibres. Scale bar=100µm

The parameter I measured was the cross sectional areas of the muscle fibres in the TA muscle. In the aging timeline there was a trend towards increasing muscle fibre cross sectional area with age between 2 months and 14 months of age. Beyond 14-months there followed a decreasing trend. However 1-way ANOVA (with a Bonferonni post-test) did not reveal any significant changes in mean CSA with age. It seemed that at the younger ages there was less variation between mice in the average muscle fibre CSA. Older mice (25 and 28 months) showed much more individual-to-individual variation in mean cross-sectional area (Fig 5.2).

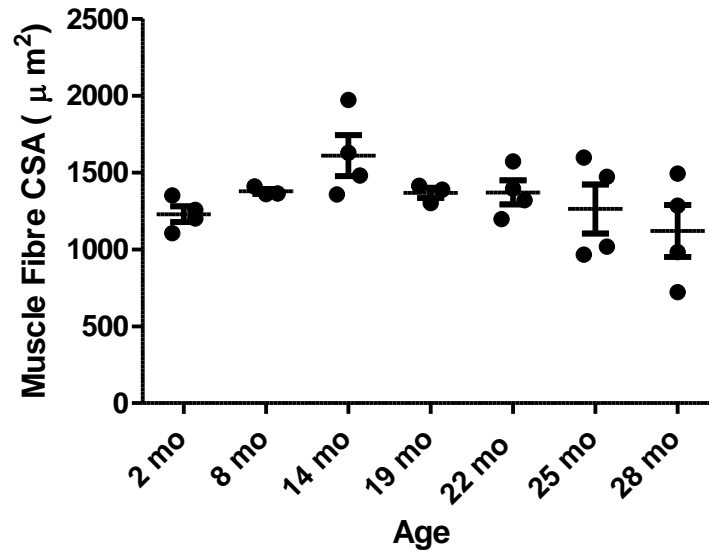


Figure 5-2 Alterations in muscle fibre size with age. Muscle fibre cross sectional areas with age. Each symbol represents the mean value for each individual mouse at the indicated age. Bars represent the mean \pm SEM for the animals studied.

Data from individual mice were pooled to construct frequency distribution graphs of muscle fibre CSA. At 14 months of age (middle age) fibre CSAs were normally distributed.

However, by 25 months of age fibre CSA had become skewed to the left, with an increased subset of muscle fibres being smaller than $1000 \mu\text{m}^2$ (Fig 5.3). A non-parametric Kruskal Wallis test showed that the distributions were significantly different from each other at 14- and 25-months of age ($P < 0.01$)

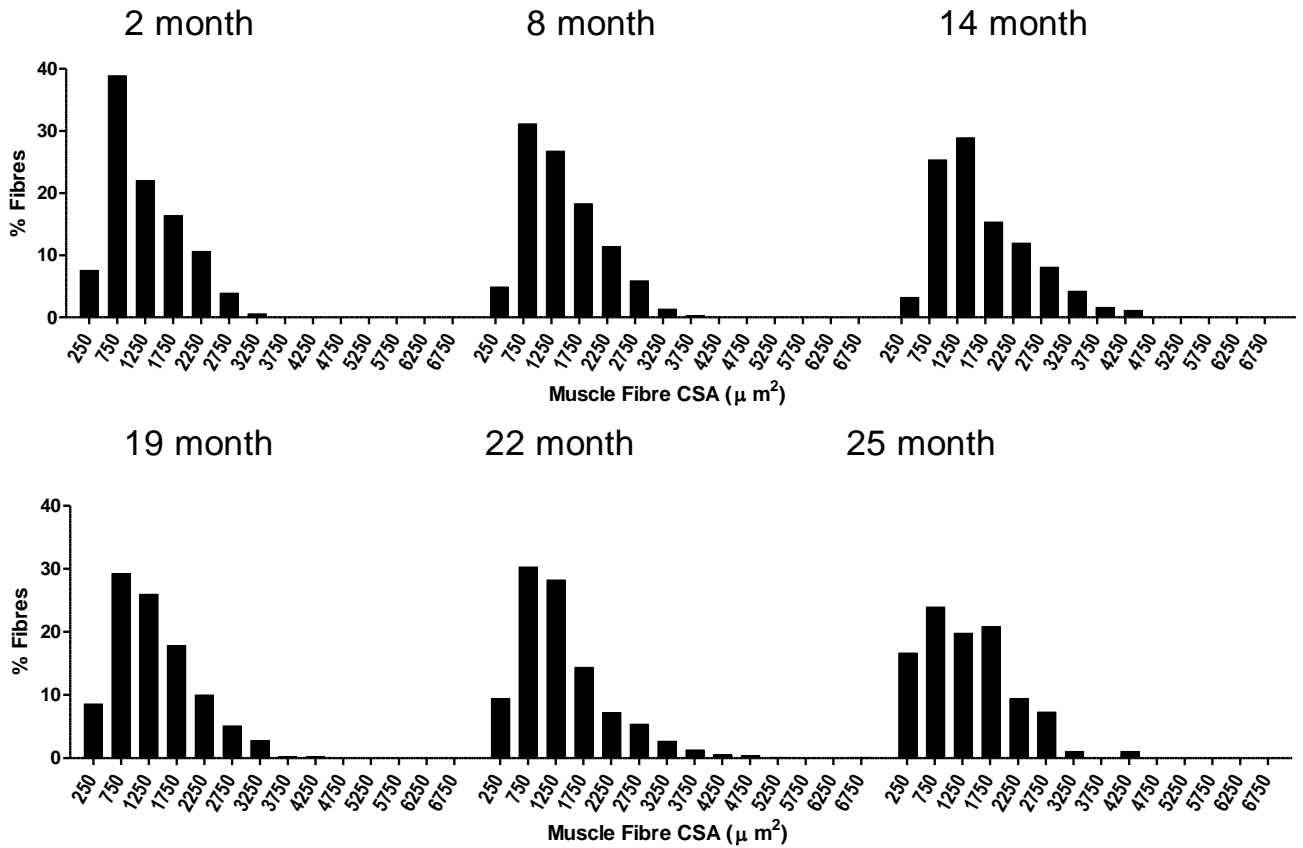


Figure 5-3 Range of muscle fibre CSA. Frequency distribution of muscle fibre cross sectional areas of mice at the indicated age groups. Each column represents the percentage of muscle fibres at the indicated BIN area.

5.3 Effects of exercise on Muscle CSA

I next compared the muscle fibre CSA between aged mice with and without a running wheel. The animals were given a running wheel as described previously where they received a running wheel from 18 to 28 months of age or 21 to 25 months of age and these animals were then compared to their age matched controls. Voluntary exercise had no significant effect upon muscle fibre CSA of old animals. In the 25 month age group the average muscle fibre CSA was roughly at $1300 \mu\text{m}^2$ in both the no wheel and wheel groups. At 28 months of age we a similar low average muscle CSA of roughly $1100 \mu\text{m}^2$ in both the no wheel and wheel groups. Exercise did not have any significant effect on muscle fibre CSA with either length of intervention (Fig 5.4).

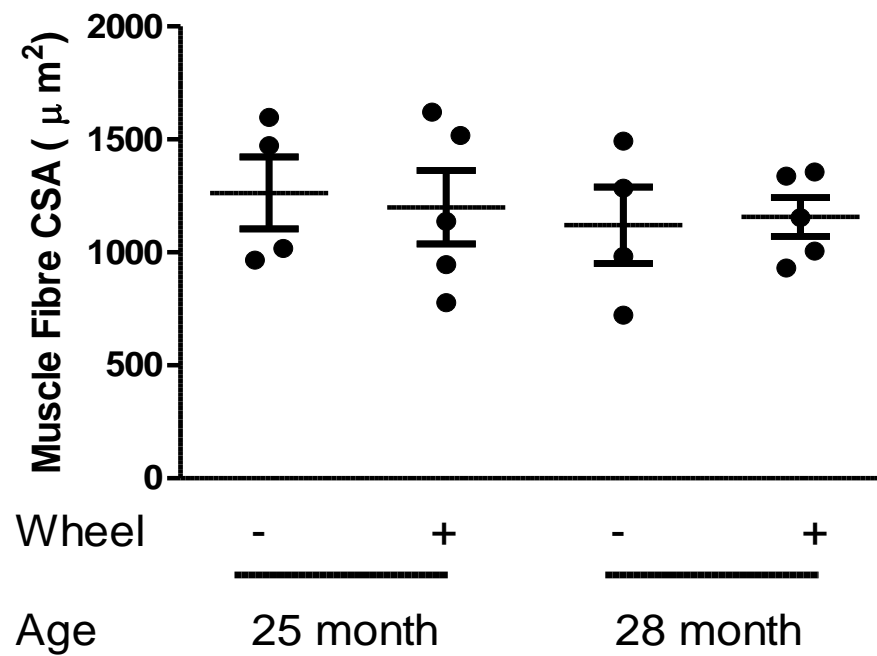


Figure 5-4 Average muscle fibre cross sectional areas of animals, which did or did not receive a running wheel. The + symbol represents the group where the animals received a running wheel and – indicates where animals did not receive a running wheel. Each symbol on the graph shows the average muscle fibre cross sectional area of each individual animal of the represented treatment and age group, error bars indicate the SEM of the animals in the age and treatment group.

5.4 Conclusion

The changes that were observed with ageing in muscle fibre size appeared to be quite subtle. We had expected some growth in muscle fibre girth but the growth up until middle age was modest. Between 2- and 14-months of age the mean CSA increased 31%. This difference was not found to be statistically significant by One-way ANOVA when all ages were included. After 14-months there was a trend towards decreasing muscle fibre CSA. For example between 14- and 19-months the average CSA declined 13%. Again this small difference was not statistically significant. Previous studies differed in their findings concerning the effects of age on muscle fibres CSA. In C57Bl/6J mice Chai et al reported that CSAs in the EDL muscle increased by 27 % between the ages of 3 and 29 months (Chai, Vukovic et al. 2011). Rowan and colleagues reported that in the soleus muscle of Male F344BN rats, between the ages of 8 months and 36 months, the CSA fell by 47%. In the gastrocnemius muscle CSA fell by just 22% in the same long interval (Rowan, Purves-Smith et al. 2011). While these previous studies reported either a significant increase or decrease with age this may have been facilitated by larger sample sizes and the animals that were used in these studies were older than the animals in my study by fair amount. In any event the mean CSA remained rather steady with only fairly modest changes with age in the present study.

In this investigation I used anti-laminin as a marker for laminin in the basement membrane of muscle fibres (Lesot, Kuhl et al. 1983). Although the animals in my study did a good amount of daily exercise it would appear that it had no effect on the muscle fibre CSA. This was perhaps due to the type of exercise. Voluntary running may be considered endurance

exercise, which is known to improve oxidative capacity and VO_2 max. In contrast resistance exercise is the type of exercise reported to improve muscle mass and fibre CSA (Rogers and Evans 1993). However some studies did not find that resistance exercise improved muscle fibre CSA (Deschenes, Judelson et al. 2000).

In summary, I observed only a non-significant trend toward increased muscle fibre CSA between youth and middle age followed by a non-significant trend to smaller CSA in old age. Increased animal-to-animal variability in older mice confounded any attempt to achieve statistical significance for changes in CSA with sedentary aging. Exercise had no effect on muscle fibre CSA in elderly mice. These results show that the decline in NMJ size after 14-months of age cannot be attributed to any significant age-associated atrophy of muscle fibres in the TA muscle.

CHAPTER 6

DISCUSSION

6 DISCUSSION

In the TA muscle on the postsynaptic side of the NMJ, the total perimeter area (which measures the total area occupied by AChRs and all the vacant spaces between AChRs) did not change significantly over the life span, although there was a gradual downward trend from 14 months of age to 28 months, this was not statistically significant. In previous studies a significant decrease was observed between rats aged 10 and 21 months of age in the total perimeter area, but this change was found in the plantaris (Deschenes, Roby et al. 2010). The transition from youth into middle age was marked by fragmentation of the large endplate AChR plaque into several smaller AChR clusters (Fig 3.9).

Total AChR-positive area of the endplate did not change significantly between 2- and 14-months of age, but then fell significantly between 14 and 19-months of age (Fig 3.7). Thus the main change to the endplate AChR clusters was the reduction in size by 31 % between 14 and 19- months of age, with no further changes in total AChR area after 19-months of age.

I found major changes in the size of the presynaptic nerve terminal in old age. Between 2 and 14 months of age there was not much change in the presynaptic nerve terminal area. However from the end of middle age (19months) the presynaptic nerve terminal became progressively smaller. By old age (28 months) the average area of the presynaptic nerve terminal at the endplate had decreased to less than 50% of the area observed at 14 months of age (Fig 3.10).

The interaction between the presynaptic nerve terminal and postsynaptic AChRs was also affected. The area of synaptic overlap, between the AChR-rich postsynaptic parts of the

endplate and the synaptophysin-positive presynaptic nerve terminal did not vary much between youth and middle age. From middle age the overlap area then experienced a decline similar to that of the presynaptic nerve terminal area. The gradual decline meant that by the age of 28 months the overlap area had decreased to less than 50% of its value at middle age (Fig 3.11). The change in the area of the presynaptic nerve terminal relative to the AChR-rich area of the endplate is important as it provides an idea of what portion of the endplate might remain a functional part of the synapse. The percentage of AChR-rich area of the endplate that was covered by nerve remained stable from 2- until 19- months of age. After 19-months the nerve terminal area declined while the AChR area stayed fairly steady. As a consequence the percentage of AChRs covered by nerve to decreased from 55% to 35% between 19 months and 28 months of age (Fig 3.13).

The second part of this project was to investigate whether exercise has any beneficial effects upon the age-related changes to the NMJ that were observed in the time course study. Mice were provided with a running wheel, which they were able to use at their leisure, starting from middle-age through to old age. The key findings were that in the exercise treatment group resulted in larger presynaptic nerve terminals when compared to age-matched control mice (no running wheel). Exercise also resulted in increases in the area of overlap between the nerve terminal and endplate AChR area, when compared to age-matched control mice. Thus voluntary exercise was able to dampen the age-related decline in nerve terminal area. In mice provided with a wheel from 21 through 25 months there was also a small but significant increase in AChR area. Mice provided with a wheel from 18 through 28months did not show a significantly enhanced retention of AChR area. The failure to reach

significance in the 28 month old mice might have been due to greater animal-to-animal variation in the AChR area at 28 months.

6.1 Is denervation of the NMJ simply an age related change?

The most substantial age-related change discovered in this project was the reduction in synaptophysin-positive nerve terminal area per endplate in the TA muscle. This is consistent with other studies where the nerve terminal appeared partially or completely withdrawn from some aged motor endplate (Valdez, Tapia et al. 2010; Punga and Ruegg 2012). In particular Chai et al reported that 20% of motor endplates in the EDL muscle of 29 month old mice appeared to be completely denervated (Chai, Vukovic et al. 2011). Valdez et al found that in the mouse gracilis muscle by 23 months of age 20-30% of endplates had become denervated (Valdez, Tapia et al. 2012).

Unlike Chai et al (2011) and Valdez et al. (2010) I found few endplates that appeared to be completely denervated. At most of the endplates I imaged small amount of dim anti-synaptophysin staining was still concentrated over the endplate AChR clusters, suggesting a small residual foothold by the motor axon at the endplate. This difference may be due to the differing imaging and measurement techniques that I used to detect and quantitate synaptophysin staining. Each endplate was individually imaged so as to optimize the tonal scale and resolution. This may have more effectively detected dim residual staining for presynaptic nerve terminal.

The prevalence of age-related changes can vary from muscle to muscle. Valdez and colleagues made a survey of NMJs in multiple muscles from different parts of the body of elderly mice. Some of the muscles they examined showed percentages of innervated

endplates similar to the high percentages found in young animals. Some other muscles showed a higher proportion of endplates with little or no innervation. In the case of the TA, by 24 months of age Valdez et al reported that 50% of endplates had become partially denervated and 15% were completely denervated. In the absence of quantitative criteria it is difficult to compare their reports of 50% "partially innervated" endplates to the current findings. Valdez et al. also observed that in elderly mice there was a relatively high prevalence of denervation in several other muscles located in the lower leg that are used for locomotory exercise and general mobility.

6.2 Fragmentation of the aged endplate

A commonly reported feature of the ageing NMJ is fragmentation of the endplate, where staining for synaptic specialisations such as AChR clusters become less contiguous: broken up into greater number of smaller patches (Valdez, Tapia et al. 2010; Chai, Vukovic et al. 2011; Li, Lee et al. 2011; Valdez, Tapia et al. 2012). In the present study I found a trend towards increasing number of AChR cluster fragments at endplates with age up until middle age, but this trend was not statistically significant. Moreover there was a downward trend in the number of endplate AChR cluster fragments at later ages (non significant). It is possible that the decrease in fragments with old age is associated with the decline in total AChR area such that remaining fragments of the endplate also decreased in number. In contrast to my findings some studies have reported fragmentation simply as a feature that became prominent once the animals progressed further with age (Valdez, Tapia et al. 2010; Chai, Vukovic et al. 2011; Jang and Van Remmen 2011; Valdez, Tapia et al. 2012).

The cause of endplate AChR cluster fragmentation in old animals may be related to degeneration and regeneration of the synapse. A recent study in which the same endplate on the surface of the mouse sternomastoid muscle was imaged over a period of several months has provided a longitudinal view of how endplate fragmentation comes about in old age. Fragmented endplates occurred after the underlying muscle fibre had (spontaneously) degenerated and regenerated. Degeneration of a portion of the muscle fibre happened sporadically but at an increasing frequency as the animal aged. When fibre degeneration occurred at the endplate, the endplate itself underwent reorganization. This was seen as a sudden change in the endplate rather than a gradual change where, after regeneration of the muscle fibre, the normal healthy endplate was rapidly replaced by fragmented islands of synaptic specialisations. Interestingly, in this study in the sternomastoid muscle, the sudden fragmentation of the endplate was not reported to be accompanied by loss of nerve terminal from the reorganised endplate (Li, Lee et al. 2011). In my study there was no significant change in endplate fragmentation or muscle fibre CSA with age. However I did not observe the same endplate and muscle fibres throughout the entire course of study. In future it may be interesting to approach this method of observation to obtain a more direct relationship between the two factors.

6.3 Benefits of Exercise for the NMJ

The second part of my study was to investigate whether voluntary exercise had any beneficial effects in preventing the age-associated changes in the NMJ. My results are broadly consistent with another recent study investigating the effects of voluntary exercise. Valdez et al (2010) reported that one month of voluntary wheel running produced a

reduction in the percentage of endplates classified as denervated in the gracilis and medial gastrocnemius muscles. In the TA the increase Valdez found was not statistically significant. Valdez et al (2010) also identified a subset of aged endplates having areas of dimmer AChR staining and a subset of endplates at which the AChRs were broken up into fragments. Both these classifications were reportedly reduced in aged mice by one-month of voluntary exercise (Valdez, Tapia et al. 2010). Once again the lack of a precise definition of 'dim areas' or of how AChR cluster fragments were quantitated limits the ability to compare results like these to findings in other studies, including my own. The most prominent impact of exercise I have found in this thesis was the preservation of presynaptic nerve terminal area with old age (fewer small nerve terminals). Perhaps the reason Valdez et al. did not find a significant reduction in the percentage of "denervated" endplates in the TA muscle of mice with a running wheel was that the elderly animals were only provided with the running wheel for 1 month prior to sacrifice. In my study the mice had access to the running wheel for at least 4 months.

As noted above I found that there was decrease in presynaptic nerve terminal area and AChR area with sedentary ageing, suggesting age-related shrinkage of the endplate. Some earlier studies in mice reported that endplates in the gluteus maximus muscle were larger in old animals when compared with young mice (Fahim 1997). When aged mice were forced to undergo endurance treadmill running there was a reduction in the endplate perimeter length nerve terminal area. This result appears to be the reverse of the effects of voluntary exercise that I observed.

Some points in this study that may be improved are how we monitor the activity pattern of the animals. Although the mice were allowed to exercise voluntarily, which is similar to how humans plan their own exercise schedules this may give rise to the question do all the mice exercise roughly the same amount. In this study the activity of each individual mouse was not monitored, but only the activity of each cage is monitored. In the case of controls, although they were not provided with a running wheel it cannot be assumed that they are completely inactive and do not exercise. It has been observed that some mice in the control groups at times will engage in cage top climbing and remain quite active by constantly moving around the cage. In future studies perhaps a better monitoring system will allow us to distinguish the animals that truly did do exercise when provided with a running wheel and also whether or not animals in the control group remain less active than the exercised animals.

6.4 Potential Implications of reduction in motor capacity

This projects investigation has mainly focused on the age related changes that occur in the NMJ. Do these structural changes in the NMJ have any influence on the neuromuscular function and the motor abilities of the elderly animals? To begin to address this question I examined the muscle fibre CSAs for the contralateral tibialis anterior muscle from the same aging mice. The reduction in the presynaptic nerve terminal area in sedentary aged mice suggested partial denervation whereas mice that exercised retained larger nerve terminals. In the sedentary aging mice there was a trend towards increasing muscle fibre CSA between youth and middle age. This was followed by a trend toward decreased CSA in old age. However there was no statistically significant change in fibre CSA across the lifespan of the

mice. A recent study reported a significant reduction in muscle fibre CSA associated with muscle fibres in elderly rats that displayed a molecular marker of denervation (Rowan, Rygiel et al. 2012). One difference was that Rowan et al studied rats rather than mice. Moreover the "old" rats in their study were 36 months, substantially older than our oldest mice. Perhaps if the mice in my study were allowed to age further into the advanced stages then I too might have observed a larger reduction in the muscle fibre CSA.

Mice that were provided with running wheels did not show a significantly greater mean muscle fibre CSA compared to my age-matched sedentary mice. In previous studies middle aged (12 month) rats were made to engage in resistance climbing exercise 7 weeks the CSA of fibres in the soleus muscle also showed no change post exercise (Deschenes, Judelson et al. 2000). These results are not directly comparable to my own because the rats were middle aged rather than aged and a different form of exercise was used. In contrast other studies with aged animals found that treadmill exercise consisting of 30 min sessions for 5 consecutive days was able to preserve the muscle fibre CSA of the gastrocnemius muscle in mice (Rosa, Silva et al. 2005). It remains uncertain whether this difference is due to the muscle studied, the age, intensity of running exercise or the type of exercise. Overall it appears that the changes in the muscle fibre innervation and the degree to which muscle fibres are affected may be largely dependent on the species of animals in the study and muscle that is focused upon. Thus if reductions in nerve terminal area cause muscle atrophy it may be too gradual to observe at 25-28months of age and may require studies be extended to much older mice.

Further studies should investigate the advanced stages of aging by allowing the mice to age to 36 months and this may also allow the extended periods of exercise to also be

investigated. Further in depths study of the effects of exercise on the aging NMJ could involve encouraging animals to exercise only from young up till middle age and observe whether or not the NMJ is preserved once the animals progresses onto old age. Overall further studies have to be carried out in order to gain more in depth knowledge about the aging NMJ and influences of exercise.

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