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The Role of Activity in Neuromuscular Synaptic Degeneration: Insights from *Wld*^{S} mice

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Declaration

I hereby declare that the work contained within, and the composition of, this thesis is my own and has not been submitted for any other degree

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Abstract

The nervous system is a dynamic structure. Both during development and in the adult, synapses display activity-dependent plasticity which can modify their structure and function. In the neonate, activity influences the stability of functional connections between the muscle and nerve. In adults, the process of neurotransmitter release and the structure of the postsynaptic muscle can also be altered by external stimuli such as exercise. It is important to understand this plasticity of the neuromuscular system, the ways in which it can be modified, and its relationship to the maintenance or degeneration of synapses.

After injury, peripheral nerve undergoes Wallerian Degeneration, during which the connections between axons and muscle fibres are lost, followed by the fragmentation of the nerve itself. The primary goal of this thesis was to determine whether activity modulates this process; that is, whether enhancing or reducing neuromuscular activity creates a susceptibility to degeneration or alternatively provides any protection against it. Developing greater understanding of this process is essential in relation to neurodegenerative disorders in which the benefits of activity, in the form of exercise, are controversial.

Using *Wld*^{s} mice, in which synaptic degeneration occurs approximately ten times more slowly than normal after nerve injury, I investigated the influence of both decreased (tetrodotoxin induced paralysis) and increased (voluntary wheel running) activity in vivo on this process. Paralysis prior to axotomy resulted in a significant increase in the rate of synapse degeneration. Using a novel method of repeatedly visualising degenerating synapses and axons *in vivo* I also established that this effect was specific to the synapse, as it did not affect the degeneration of axons. In contrast, voluntary wheel running had no effect on the rate of either axonal or neuromuscular synapse degeneration, but induced a slight modification of neuromuscular transmission.

To provide a more stringent test I developed a novel assay based on overnight, *ex vivo* incubation of nerve-muscle preparations at 32°C. I first demonstrated that this procedure separates the different degeneration time courses for neuromuscular synapse degeneration in wild-type and *Wld*^{s} preparations. I then extended the study

to investigate further ways of modulating synaptic degeneration. First, I tested the effects of electrical stimulation. Intermittent high frequency (100Hz) stimulation reduced the level of protection. Finally, I tested the effects of NAMPT enzymatic inhibitor FK866 on synaptic degeneration. Interestingly, the synaptic protection observed in *Wld*^{S} muscles was enhanced in the presence of FK866.

The results of my findings are relevant to understanding the plasticity of synapses and its relationship to degeneration. Together, these studies highlight the potential of genetic and epigenetic factors, including activity, to regulate neuromuscular synapse degeneration. My study also provides proof of concept for a novel organotypic culture system in which to identify pharmacological modulators of synaptic degeneration that could form part of a second-line screen for neuroprotective compounds or phenotypes.

My findings may be viewed in the wide context of neurodegenerative disease, since synaptic use or disuse is widely thought to influence susceptibility, onset and progression in such disorders.

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Abbreviations

ACh	Acetylcholine
AChR	Acetylcholine receptor
ANOVA	Analysis of variance
Bax	BCl-2 associated x protein
BCl-2	B-cell lymphoma 2
CD	Cervical dislocation
CNS	Central nervous system
CTX	Conotoxin
DRG	Dorsal root ganglion
EDL	Extensor digitorum longus
EPP	Endplate potential
FDB	Flexor digitorum brevis
GFP	Green fluorescent protein
LHS	Left hand side
LPN	Lateral plantar nerve
MEPP	Miniature endplate potential
MEPSP	Miniature excitatory postsynaptic potential
MiPTP	Mitochondrial permeability transition pore
MND	Motor neuron disease
MPS	Mammalian physiological saline
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Multiple sclerosis
NAD	Nicotinamide adenine dinucleotide
NaMN	Nicotinic acid mononucleotide
NAMPT	Nicotinamide phosphoribosyltransferase
NLS	Nuclear localisation sequence
NMDA	N-Methyl-D-Aspartic acid
NMJ	Neuromuscular junction
NMN	Nicotinamide mononucleotide

NMNAT	Nicotinamide mononucleotide adenylyltransferase
Npt	Nicotinic acid phosphoribosyl transferase
PNS	Peripheral nervous system
PUMA	P53 upregulated modulator of apoptosis
QC	Quantal content
RHS	Right hand side
RMP	Resting membrane potential
Sir2	Silent information regulator 2
SN	Sural nerve
SNARE	Soluble NSF attachment protein receptor
SNAP-25	Synaptosomal associated protein 25
SOD	Superoxide Dismutase
TA	Tibialis anterior
TRITC-α-BTX	Tetramethylrhodamine-isothiocyanate (TRITC) α-bungarotoxin
TTX	Tetrodotoxin
T50	Half decay time
Ube4b	Ubiquitination factor E4B
VCP	Valosin containing protein
WD	Wallerian degeneration
Wld^s	Wallerian degeneration slow
WT	Wildtype
YFP	Yellow fluorescent protein
4DL	Fourth deep lumbrical

1. General Introduction

1. General Introduction

Understanding synaptic degeneration is of utmost importance in neuroscience. The nervous system depends on normal synaptic functioning, and when this fails, the consequences can be fatal. For instance, many neurodegenerative disorders are characterised by loss of synapses. These diseases include Alzheimer's disease, Parkinson's disease, Huntington's disease and Motor Neuron Disease (MND). They have been widely researched for decades. However a cure or even an effective treatment has yet to be established for any of them. Thus, a clear understanding of the function and modulation of the nervous system is required if we are to achieve the goals of effective treatment or cure for neurodegenerative disorders.

1.1 Historical overview of the nervous system

The nervous system consists of the peripheral and the central nervous system (PNS and CNS, respectively). It controls multiple aspects of bodily function, including perception, emotion and behaviour. The survival of organisms depends on intact brain function.

Two of the most prominent figures in the history of our understanding of the nervous system were Santiago Ramón y Cajal and Camillo Golgi, who won a joint Nobel Prize for their studies in 1906. Ramón y Cajal's application of Golgi's staining technique lead to many advances in the field, in terms of investigating the connections and structure of the nervous system (Jones, 2007, Peters, 2007). Most importantly, it led to the development of the neuron doctrine; the idea that the nervous system consists of a variety of individual cells (reviewed by Jones, 1999, see also De Carlos and Borrell, 2007, Jones, 2007, Peters, 2007). A range of studies have since extended our knowledge and understanding of the structure of the nervous system, how it functions, and how communication within this entity and the surrounding body is maintained.

The nervous system consists of a number of different cell types. Neurons are responsible for the transmission of signals throughout the body. The term 'synapse' was first used by C.S. Sherrington to describe the connections neurons make with one another. Later it

emerged that these synapses display a high level of plasticity. The idea of plasticity of the nervous system was in fact addressed by Ramón Cajal in his 1894 lecture (Jones, 1994), and since then there has been a plethora of studies into synaptic plasticity, for instance in relation to learning and memory in the CNS (Kandel and Spencer, 1968, Albright et al., 2000, Martin et al., 2000). Peripheral synapses also show plasticity; showing alterations in their structure and function in response to use or disuse (Tsujimoto et al., 1990, Barry and Ribchester, 1995, Fahim, 1997).

1.2 The Neuromuscular Junction

During the development of the PNS, motor axons reach their target muscle and form synapses, or neuromuscular junctions (Grinnell, 1995, Pun et al., 2002). These junctions form the highly specialized connection between the motor nerve and muscle fibre (Luo, 2010). They comprise highly differentiated pre- and post-synaptic zones, separated by synaptic clefts containing the basal lamina (Birks et al., 1960a, Hughes et al., 2006).
The NMJ has been studied extensively, via multiple techniques ranging from electron microscopy (Desaki and Uehara, 1981), electrophysiology (Del Castillo and Katz, 1954c, Liley, 1956b) and immunofluorescent labelling (Marques and Santo Neto, 1998, Feng et al., 2000, Tabares et al., 2007). In comparison to the synapses of the CNS, NMJs are useful tools for investigating synaptic plasticity as their structure and function has been well documented, and they are easily accessible for experimental study (Hall and Sanes, 1993, Ribchester, 2009).

1.2.1 The Pre-synaptic Structure of the NMJ

The pre-synaptic terminal is capped by Schwann cells (Hughes et al., 2006) and fibroblast-like cells, recently named kranocytes (Robertson, 1956, Weis et al., 1991, Court et al., 2008). The nerve terminal contains clathrin-coated synaptic vesicles (De Robertis and Bennett, 1955, McMahon and Boucrot, 2011), each holding several thousand molecules of the neurotransmitter acetylcholine (Whittaker and Dowe, 1964, Kuffler and Yoshikami, 1975), which gather at active zones (Dreyer et al., 1973, Heuser and Salpeter, 1979, Nagwaney et al., 2009). The NMJ releases these packets, or quanta of ACh via the exocytosis of the vesicles. This release occurs spontaneously (Dale et al., 1936, Del Castillo and Katz, 1954b) and each individual quantum causes a small sub-threshold depolarization of approximately 1mV at the postsynaptic membrane. These responses are known as miniature endplate-potentials (Fatt and Katz, 1952, Boyd and Martin, 1956, Liley, 1956a, Elmqvist and Quastel, 1965).

1.2.2 The Post-synaptic Structure of the NMJ

In the post-synaptic region, the membrane folds around the area of the endplate in which acetylcholine receptors (AChRs) and voltage gated sodium (Na^+) channels are accumulated (Shotton et al., 1979, Salpeter and Loring, 1985, Flucher and Daniels, 1989). These postsynaptic folds appear to amplify the synaptic current, further contributing to the concept of the 'safety factor' of neuromuscular transmission (Slater, 2008), which will be discussed below.

1.2.3 Transmitter release at the NMJ

When an action potential arrives at the nerve terminal, the probability of transmitter release by exocytosis is increased in a calcium-dependent manner (Del Castillo and Stark, 1952, Dodge and Rahamimoff, 1967a, b, Katz and Miledi, 1967, Uchitel et al., 1992, Protti and Uchitel, 1993, Parnas and Parnas, 2010). Voltage-gated calcium channels (primarily P/Q type), present on the pre-synaptic membrane, are activated causing an increase in calcium influx which results in a significant increase in the rate of vesicle fusion and exocytosis (Robitaille et al., 1990, Wood and Slater, 2001, Hughes et al., 2006). In brief, vesicles dock at the active zone, a fusion pore opens and neurotransmitter is released. This process is mediated by so-called SNARE proteins, including synaptobrevin, syntaxin and SNAP-25, which form the fusion complex (Zucker, 1996, Sudhof, 2004). The number of vesicles released in response to the stimulus is known as the quantal content (QC), which will be discussed below. The subsequent diffusion of ACh to the postsynaptic membrane causes a depolarisation, or endplate potential (Hughes et al., 2006). This arises because ACh binds to nicotinic AChRs, inducing a conformational change that allows the channels to become permeable to Na^+ and K^+ ions (Unwin, 2005, Zouridakis et al., 2009).

1.2.4 The Safety Factor of Neuromuscular Transmission

At the NMJ a greater amount of neurotransmitter is released than that required to generate an action potential in the muscle fibre. This means that in normal NMJs, failure of transmission is rare. This excess of release is referred to as the 'safety factor', which can be calculated by measuring the number of quanta released at the NMJ to a stimulus, compared to the threshold amount required to trigger an action potential (Wood and Slater, 1997, 2001). Analysis of synaptic currents under voltage clamp established that the safety factor for the rat soleus muscle is approximately 3.5 whereas for the extensor digitorum longus muscle (EDL) it is estimated as 5.0.

1.2.5 Estimating Quantal Content at the NMJ

The quantal content (QC) of the EPP refers to the amount of transmitter released to a stimulus. The QC (m) is equal to the number of vesicles (n) multiplied by the probability of their release (p):

$$m=n.p$$

Reducing calcium (Ca^{2+}) or increasing magnesium (Mg^{2+}) reveals the quantal nature of neurotransmitter release, as it reduces the EPP until the smallest EPP is of the same amplitude as a MEPP. The distribution of MEPP amplitudes (and the amplitudes of uniquantal EPPs) is Gaussian. This lead to the suggestion that the EPP arises as a summation of the effects of the release of individual packets of ACh, and this observation allows for the calculation of QC (Del Castillo and Katz, 1954c, Boyd and Martin, 1956, Liley, 1956b).

The QC can be calculated several ways, the simplest being the 'Direct Method':

$$m = \text{mean EPP amplitude} / \text{mean MEPP amplitude}$$

However, the use of the direct method should be applied with caution, due to the potential occurence of small MEPPs that are indistinguishable from noise, or by the presence of giant MEPPs (Liley, 1956a), and by non-linear summation of EPPs when quantal content exceeds about 5 quanta (which will be discussed below).

An alternative method is to calculate the quantal content using the variance in the amplitude of the EPP, the variance method:

$$m=1/(CV)^2$$

where CV is the coefficient of variation, estimated as CV=standard deviation/mean (σ/μ)

When the mean quantal content is reduced to about 3 or less, then 'failures' of transmission are observed, that is, no response is observed when the nerve is stimulated. This occurs, for example, when the probability of release is reduced significantly in the

presence of increased Mg^{2+}. Under these conditions the quantal content can also be calculated using the 'failures' method. Following Poisson distribution:

$$P(0)= \exp(-m)$$

Therefore, if we apply logarithms define P(0) as equal to the number of failures divided by the number of stimuli, we obtain:

$$m=\ln (\text{number stimuli/number of failures})$$

The three methods of calculating QC produce remarkably similar values when QC is reduced in this fashion (Del Castillo and Katz, 1954c, Liley, 1956b).

When calculating the QC, it is essential to consider non-linear summation of EPPs. When the EPP is large, non-linear summation affects the calculated QC value. This is because each quantum produces a certain amount of leakage conductance. The additive effects of the amount of depolarisation contributed by each quantum decreases as the total number of quanta increases. Therefore estimation of 'm' by dividing the EPP size by the MEPP size produces underestimates of the QC and the use of the variance method overestimates QC (Del Castillo and Katz, 1954c, Martin, 1955, Wilson, 1977). However, non-linear summation can be corrected mathematically (Martin, 1955, McLachlan and Martin, 1981).

$$V'=V/(1-f.V/E)$$

where V'= corrected EPP amplitude, V=observed EPP amplitude, E= the voltage 'driving force' or synaptic current, in other words the difference between the resting membrane potential (RMP) and the reversal potential for the action of ACh at its receptor (about -10mV) and f = an arbitrary correction factor specific to the type of muscle under investigation. This correction factor varies depending on the type of muscle fibre used during the recording (McLachlan and Martin, 1981).

In addition quantal analysis using Poisson rules must be used carefully, as at some synapses Poisson analysis is not applicable (Johnson and Wernig, 1971, Wilson, 1977).

Non-linear summation of EPPs can be avoided using voltage clamping techniques to record synaptic currents. The relationship between the number of open ion channels included in generating this response allows a more accurate estimate of QC without the calculation errors imposed by non-linear summation in EPP recordings (Wood and Slater, 2001). However, this method is more technically challenging, and as such the yield of data is much smaller, which opposes its use for analysis of a large number of muscle fibres in order to screen for the effects of different treatments. Therefore, in this thesis, EPP analysis with correction for non-linear summation was used in all assays.

Aside from the problems posed by nonlinear summation, to record an EPP and therefore measure QC accurately from a preparation the electrode must be near the endplate to avoid the effect of attenuation of the EPP amplitude and time course resulting from the 'cable' properties of muscle fibres. This can be avoided using preparations in which the muscle fibres are very short, rendering them relatively isopotential such as the flexor digitorum brevis (Bekoff and Betz 1977).

The methods for calculating QC are employed to monitor the function of the NMJ. Both the structure of the NMJ and neuromuscular transmission can be altered by external stimuli, demonstrating the plasticity of the system.

1.3 The Plasticity of the Neuromuscular Junction

Both the structure of the NMJ and neuromuscular transmission can be altered by intrinsic environmental and epigenetic stimuli. This is true both in neonates and adults, and has been the subject of a substantial amount of research. The evidence discussed below highlights this plasticity of NMJs in response to both increased and decreasing neuromuscular activity. I focus mainly on the effects of enhanced activity by exercise or stimulation, and by pharmacological blockade of activity, as these are the methods I employed in my results experiments.

1.4 Activity and Synapse Elimination

When the axon first reaches the postsynaptic site from the spinal cord during development and the synaptic structure begins to assemble, other axons begin to make connections at the same site. This means that the motor-endplates of most muscle fibres are innervated by two or more motor inputs, a phenomenon referred to as polyneuronal innervation. This convergence is then removed postnatally by a process called 'synapse elimination' (Brown et al., 1976). During maturation, the supernumerary inputs retract until only one functional connection remains with each muscle fibre (mononeuronal innervation). It is not known exactly how the surviving input is determined, but there is compelling evidence that competition and activity are at least influential in this process. In fact, activity influences both the rate of axon withdrawal and maintenance of polyneuronally innervated synapses (Bennett and Pettigrew, 1974, Brown et al., 1976, Thompson et al., 1979, Thompson, 1983, Ridge and Betz, 1984, Hall and Sanes, 1993). Below I give a brief account of the effects of both increasing and decreasing neuromuscular activity on this process.

1.4.1 Inactivity and synapse elimination

Several studies have used toxins to inhibit or block activity at the developing synapse. While under normal conditions synapses are pruned to have only one remaining input, chronic local application of tetrodotoxin (TTX) or botulinum toxin preserves multiple innervation (Brown et al., 1981, Brown et al., 1982, Taxt, 1983, Barry and Ribchester, 1995, Costanzo et al., 2000). Paralysis caused by spinal cord transection also resulted in maintenance of polyneuronal innervation (Miyata and Yoshioka, 1980).

Multiple reports indicate the ability of paralysis to stabilise polyneuronal innervation. Using neonatal models with natural polyneuronal innervation, Brown *et al.* (1981) showed that application of botulinum toxin, induces maintenance of connections, and in 1982, Brown *et al.* also showed the ability of botulinum toxin to delay synapse elimination, although the effects were dependent upon the type of muscle (Brown et al., 1981, Brown et al., 1982). Thompson *et al.* (1979) also measured the level of polyneuronal innervation, using TTX to block nerve conduction and found that although short-term block had no effect, longer application preserved a large number of multiply innervated synapses.

In adult animals, nerve damage can be followed by reinnervation. The newly reformed NMJs become polyneuronally innervated, providing a model of synapse elimination in adults, that is easier to manipulate than neonates (Ribchester and Taxt, 1983, Rich and Lichtman, 1989, Barry and Ribchester, 1995, Costanzo et al., 2000). For instance, crushing the sural nerve (SN), one of the nerves innervating the fourth deep lumbrical (4DL) muscle, leads to collateral innervation of the denervated endplates by axons in the unharmed lateral plantar nerve (LPN). When the SN axon regenerates some muscle fibres become polyneuronally innervated. Ribchester and Taxt (1984) utilised this to show that if the LPN was blocked with TTX as the SN was reinnervating the muscle, the active SN displaced the inactive LPN inputs. In 1993, Ribchester utilised this same model but blocked both nerves during reinnervation by the SN. In this case, SN inputs were able to compete and displace the LPN inputs, indicating that perhaps activity is not actually required for the process of synapse elimination (Ribchester, 1993). Further

evidence that activity is not strictly necessary for synaptic elimination was published by Costanzo *et al.* (2000), who showed that synapse elimination still occurs at electrically silenced synapses in which nerve conduction and post-synaptic activity had been blocked by tetrodotoxin and α-bungarotoxin respectively.

However, another paper reported that TTX-paralysed NMJs had an advantage over active competitors during synapse elimination (Callaway et al., 1987), although the reliability of the methodology used and the interpretation of their data was questioned (Ribchester, 1988).

Another method of inducing reduced activity is by tenotomy. This procedure delayed elimination of polyneuronal innervation in young rats (Benoit and Changeux, 1975). In fact even when electrical activity is present, preventing or reducing mechanical activity delays synapse elimination (Greensmith et al., 1998), so muscle contractile activity appears to be vital in this process as well.

1.4.2 The influence of increased activity on synapse elimination

Increasing activity has been demonstrated to alter the time course of synapse elimination. Electrical stimulation was used by O'Brien *et al.* (1978) to increase the activity of polyneuronally innervated muscles. This procedure resulted in an increased rate of loss of polyneuronal innervation, demonstrated electrophysiologically, though these results were not replicated in their morphological data, (O'Brien et al., 1978). However, Thompson (1983) also showed that hastening of synapse elimination by electrical stimulation is dependent upon the rate of stimulation. A pattern of 100Hz accelerated removal of multiple inputs, but this was not the case with a repetitive impulse of 1Hz frequency (Thompson, 1983).

Activity also influences the outcome of synapse elimination as well as the rate. Ridge and Betz (1984) stimulated one of two motor units innervating a muscle with a dual nerve supply and found that the stimulated motor units were favoured over the unstimulated units.

In summary, it seems that removing activity stabilizes polyneuronally innervated synapses, whilst increasing the level of activity enhances the rate and influences the outcome of synapse elimination. However, when active units are present adjacent to those with activity block, they have a competitive advantage. The way in which activity exerts its effects is currently unknown.

1.5 Modification of the electrophysiological properties of the NMJ by activity

In the adult animal, neuromuscular synapses are normally innervated by a single input. However, like the neonate, this system remains responsive to activity levels and can be modified in various ways such as exercise, electrical stimulation and pharmacological block of activity. As the synapse conveys the flow of an electrochemical signal (action potential) from the axon to the muscle, any remodelling at the synapse would be expected to alter various electrophysiological properties and this has been demonstrated in multiple studies (Tsujimoto et al., 1990, Dorlochter et al., 1991, Deschenes et al., 1993, Barry and Ribchester, 1995), some of which will be discussed below.

1.5.1 Inactivity and electrophysiological properties of the NMJ

Chronic reduction of activity at the NMJ induces alterations in synaptic transmission and the electrical properties of the synapse. Activity block has been shown to result in an increase in QC (Snider and Harris, 1979, Plomp et al., 1992). In fact Snider and Harris (1979) found that 8-9 days of TTX block produced a ~40% increase in quantal content along with an increase in MEPP frequency in the soleus muscle. Paralysis has also been reported to reduce the resting membrane potential (Betz et al., 1980). Furthermore, Tsujimoto *et al.* (1990) confirmed the finding that TTX induces sprouting (Brown and Ironton, 1977, Tsujimoto and Kuno, 1988, Tsujimoto et al., 1990), yet confirmed that the increase in QC was not a result of sprouting itself. They demonstrated that when sprouting is blocked with calcitonin gene-related peptide, QC is still increased in blocked preparations.

1.5.2 Increasing activity and the electrophysiological properties of the NMJ

Exercise has been reported to cause an increase in MEPP amplitude coupled to a reduction in MEPP frequency compared to controls (Fahim, 1997). A 25% increase in QC was also reported in these animals along with an increase in nerve terminal area. In fact a similar increase (31%) in QC following prolonged exercise has been shown elsewhere (Desaulniers et al., 2001), along with an increased resistance to Mg^{2+} block (Dorlochter et al., 1991). Desaulniers *et al.* (2001) also reported an increase in MEPP frequency, but unlike Fahim and colleagues (1997) found no change in MEPP amplitude or rise time.

The response to a train of stimuli can also be used as readout of synaptic function; demonstrating the fatigability of muscles when repeatedly stimulated, and the level of depression or potentiation at the synapse. Dorlöchter *et al.* (1991) used a train of stimuli at 100Hz on the extensor digitorum longus (EDL) muscles of mice that had run for approximately 10km per day. The peak amplitudes within the train were reportedly larger in the runners.

1.6 Activity-dependent structural remodelling at the NMJ

The structure of the adult NMJ can also be remodelled by activity. Changes in the electrophysiological properties of exercised muscles, such as an increased transmitter release, may indicate alterations in neuromuscular junction morphology as the amount of transmitter released is correlated to the size of the nerve terminal (Kuno et al., 1971, Harris and Ribchester, 1979). Katz and Thesleff (1957) first demonstrated the relationship between MEPP amplitude and muscle fibre input resistance (R_{in}). This was also shown by Harris and Ribchester (1979), who demonstrated a positive correlation between MEPP amplitude and muscle fibre R_{in}, and also quantal size and R_{in}. As the R_{in} is inversely proportional to the fibre diameter, and there is a positive correlation between endplate area and fibre diameter (Kuno et al., 1971), it is clear that the properties of electrical transmission are related to the morphological characteristics of the endplate

and muscle fibre (Katz and Thesleff, 1957, Kuno et al., 1971, Harris and Ribchester, 1979).

Below I briefly outline a few of the reported changes in NMJ structure to both increased and decreased activity.

1.6.1 Inactivity and synaptic morphology

Similar to increased activity, removal or reduction of activity is also documented to cause morphological changes at the NMJ. Several reports show inactivity induced sprouting at the NMJ (Brown and Ironton, 1977, Fahim and Robbins, 1986, Fahim, 1989, Tsujimoto et al., 1990). A minimum of 3.5 days of TTX block was reported to be required for sprouting to be induced (Brown and Ironton, 1977). Furthermore, paralysed muscle fibres cause active nerve terminals to sprout (Betz et al., 1980).

1.6.2 The effects of increasing activity

Nerve terminal length is reduced in exercised rat EDL muscles compared to controls, along with a reduced number of branch points (Tomas et al., 1989). Waerhaug *et al.* (1992) also used a treadmill running protocol and showed adaptations in the NMJ in rats, but found that the responses were muscle specific. Exercise had limited effects on nerve terminal morphology in the EDL muscle, but induced an increase in both length and area in the soleus muscle. However, when Andonian and Fahim (1987) subjected mice to 8 weeks of treadmill training, they found that exercise induced an increase in size of nerve terminal in young animals in both the EDL and soleus, resulting in an increase in perimeter along with more branching. In fact they stated that the changes were more noticeable in the EDL than in the soleus, contradicting the work by Waerhaug and colleagues (Andonian and Fahim, 1987, Waerhaug et al., 1992). Thus it seems that the changes in morphology, similar to those found in electrophysiological

properties, are not only muscle specific but also dependent upon the species and type of exercise training, and remain controversial.

Exercise intensity may also be a contributing factor. One study showed that only high intensity treadmill training was capable of producing an alteration in branching pattern, but that both high and low intensity regimes increased terminal area (Deschenes et al., 1993). The same group later extended this study to the effects of both increasing and decreasing activity for 10 weeks, using hind limb elevation to reduce the exercise ability of adult rats, and treadmill training again as the exercise protocol. Increased activity increased the complexity and length of terminal branching, whereas hind limb elevation reduced post-synaptic endplate dimensions. Thus, at least in this study, increasing activity affects pre-synaptic sites, whereas decreasing activity affects post-synaptic morphology (Deschenes et al., 2006).

Muscle fibres themselves are also remodelled by activity. However, this too seems to be conflicting, depending on the species, experimental protocol etc. The differential effects of exercise can be easily observed, for example by comparing the outcome of the different training regimes used for example by endurance athletes and body builders. Some forms of exercise lead to enhanced muscle mass and size, whereas others enhance the endurance capabilities of muscles, and have different effects on blood supply and muscle force. In fact, different types of exercise recruit different fibre types (Knuttgen, 2007, Laughlin and Roseguini, 2008). Likewise, in some cases, exercise training in experimental animals has been insufficient to cause alterations in fibre diameter (Fahim, 1997). However, more intensive training, for example by increasing the load on the training wheels has proven effective (Ishihara et al., 2002) highlighting again the effect of training intensity.

In summary, altering activity pattern, and completely removing activity, has been shown to modify the process of synaptic elimination during development. In the adult, the structure and function of the synapse are both influenced by activity. The idea that the function of the neuromuscular synapse can be modified by environmental factors is of

great interest, particularly with regard to the pathogenesis and treatment of neurodegenerative disorders.

1.7 Activity and Neurodegeneration

Whether increased neuromuscular activity by exercise is beneficial or harmful is controversial in neurodegenerative disorders such as motor neuron disease (MND). Thus it seems vital to uncover whether physical activity can indeed elicit any control over the rate of synaptic degeneration.

There are several potential arguments for why exercise may be beneficial or detrimental in neurodegeneration. Exercise may induce excitotoxicity and increase intracellular Ca^{2+}, triggering degeneration (Liebetanz et al., 2004), or may increase free radical production (Itoh et al., 1998). However, adaptations to exercise may increase the function of antioxidant enzymes (Powers and Lennon, 1999). Exercise also upregulates growth hormones (Neeper et al., 1996, Carro et al., 2001). Since synaptic terminals appear to degenerate first in MND (Fischer et al., 2004), it is important to determine what effect, if any, exercise has on synaptic degeneration, and in turn what mechanism it acts by.

1.7.1 Exercise and MND – evidence from the clinic.

Axonal degeneration is a feature of MND, a fatal neurodegenerative disorder of unknown pathogenesis that causes paralysis. Degeneration begins at distal axons and synapses (Fischer and Glass, 2007). There is currently no adequate treatment for this disease, and as such investigating ways to protect the synapses from degeneration is critical.

In terms of MND, the role of activity forms the topic of an ongoing debate. Increasing activity in the form of physical exercise has been stated to enhance risk for developing

the disease. On the other hand some MND patients report anecdotally that exercising relieves their symptoms. One of the most referenced papers to refute a beneficial role for exercise argued that Italian football players were more at risk of developing the disease (Chio et al., 2005). However, other environmental factors were not considered. In contrast, a small study, incorporating low intensity exercise in a cohort of MND patients found that there were significant improvements in their walking distances. There was also an indication that patients felt less fatigued after the exercise, though this was not a longitudinal study – the exercise paradigm lasted for only 8 weeks. This study was also very small and a so larger study should perhaps be undertaken (Sanjak et al., 2010).

These conflicting views were not resolved by recent reviews (Kiernan, 2009), which therefore surmise that more research had to be done in the field. Drory *et al*. (2001) concluded that exercise in their study of MND patients produced short-lived beneficial effects, whereas another study (Dal Bello-Haas *et al.* 2007) showed functional resistance training had some capacity to improve patients function and well-being, although again both studies were small.

Other neurodegenerative disorders have also been featured in research aimed at understanding the effects of exercise. Some studies found no effect, either beneficial or detrimental, of strength training in myotonic dystrophy patients, whereas Charcot-Marie Tooth disease patients and also hereditary motor and sensory neuropathy patients showed an improvement in muscle strength, though again long term benefits were not studied (Lindeman et al., 1995, Lindeman et al., 1999). This seems to show a disease specific reaction to this type of exercise training.

An important consideration when evaluating clinical studies is environmental factors such as profession, smoking, drinking and other lifestyle differences. This makes the translation of this research to animal models attractive – there is less variation within studies in terms of genetics and habitat.

1.7.2 Evidence from animal models.

However, in animal models of MND, increasing activity in the form of exercise has also produced conflicting results. Using the superoxide dismutase (SOD) mouse model of MND, which replicates several features of MND including distal onset and axonal degeneration, various studies have shown that providing mice with free access to running wheels, or giving them an exercise regime consisting of treadmills for examples has no effect on lifespan (Liebetanz et al., 2004, Bruestle et al., 2009), whereas in others it has slowed onset (Carreras et al., 2010). However, Deforges *et al*. (2009) subjected SOD1 mice to either treadmill training or swimming. Running had no effect on lifespan, but swimming significantly increased survival. Swimming also reduced motor neuron loss and decreased apoptosis in the spinal cord, whereas running had no evident protective effects in these regions. Both training regimes however protected against atrophy in the soleus muscle (Deforges et al., 2009). Thus it seems that different types of exercise may be more effective than others. The variety of exercise regimes and measures of end stage, amongst other things, has made it virtually impossible to provide a definitive conclusion.

Of interest, other neurodegenerative models show positive effects of exercise. Some of the pathological features of Parkinson's disease can be replicated *in vivo* by application of the drug MPTP. Exercise has been shown to protect significantly against this toxicity, potentially by protection against the build up of free radicals (Faherty et al., 2005, Gerecke et al., 2010). These studies showed that animals that exercised the least lost more neurons than those that had longer exercise schedules. An increase in motor activity and improved lifespan as a result of exercise has also been shown in a rat model of ataxia (Uhlendorf et al., 2011), and exercise is beneficial in some Alzheimers and Huntingtons disease models (van Dellen et al., 2008, Yuede et al., 2009, Belarbi et al., 2011). However, the beneficial effects in the Huntingtons disease model have been contested (Potter et al., 2010).

The variation and inconsistency of protocols militates for the further study of the role of activity in modulating synaptic degeneration. To determine whether degeneration in the PNS can be modulated by activity, we must first address the complex mechanisms

involved in peripheral degeneration, then apply methods of reducing and increasing activity to a model in which the variability in onset of degeneration, and time course, is less problematic than in the SOD mouse, for example. This will be discussed further below.

1.8 Wallerian degeneration: overview

Maintenance of the functional connection between the nerve and muscle in the PNS, is essential for the correct functioning of muscle contraction and therefore movement. Occasionally, the contact between the NMJ and the axon body is interrupted. When this happens, both the distal axon and the nerve terminals undergo catastrophic breakdown, referred to as Wallerian degeneration (WD). This process was first characterized by Augustus Waller in 1850. Waller showed that when frog glossopharyngeal and hypoglossal nerves are cut, their distal entities undergo degeneration. This insight instigated a significant advance in the study of nerve degeneration (Pearce, 2000, Stoll et al., 2002).

In rodents, WD is normally complete after a period of approximately 48 hours. Loss of motor nerve terminals, and therefore of neuromuscular transmission, occurs within 12-24 hours (Vial, 1958, Slater, 1966. By contrast, peripheral nerve axons that have been lesioned appear normal for the first 36h after the section then display rapid fragmentation and loss of structure up until the 48h time point, at which the nerve appears completely fragmented (Miledi and Slater, 1970, Manolov, 1974, Gillingwater et al., 2003). The loss of axonal integrity is coupled to a failure of neuromuscular transmission (Birks et al., 1960b). Degeneration therefore appears to be a 'compartmentalised' process, with nerve terminals degenerating prior to the axon (Miledi and Slater, 1970, Levenson and Rosenbluth, 1990, Gillingwater and Ribchester, 2001).

The nerve terminal normally contains vesicles and mitochondria. One of the first steps of degeneration is mitochondrial swelling and a reduction in the number of vesicles. The nerve terminal fragments and Schwann cell processes enter the terminals. Degeneration advances rapidly and eventually Schwann cells envelop the nerve terminal (Birks et al.,

1960b, Miledi and Slater, 1970, Manolov, 1974, Winlow and Usherwood, 1975). Schwann cells and macrophages remove the myelin debris (Stoll et al., 1989). The rate of degeneration depends upon various factors, including temperature (Birks et al., 1960b, Wang, 1985, Sea et al., 1995, Tsao et al., 1999) and the length of the nerve stump (Miledi and Slater, 1970, Ribchester et al., 1995).

The exact mechanisms of WD are unknown. However, a recent paper demonstrated that axonal degeneration is dependent upon the function of the mitochondrial permeability transition pore (MiPTP). Barrientos *et al.* (2011) demonstrated that inhibiting the activation of MiPTP using cyclosporin A (CSA) delayed axonal degeneration in cultured WT nerves. CSA also delays the mitochondrial swelling noted to occur in advance of axonal degeneration (Barrientos et al., 2011).

1.8.1 Wallerian degeneration and neurodegenerative disorders

Wallerian degeneration occurs in the human population generally as a result of axon injury due to surgery, or some form of accident (Maxwell et al., 1997). However, it has been speculated that WD is similar to the process of degeneration seen in several different neurodegenerative disorders; these disorders being termed 'wallerian-like' due to similarities such as the compartmentalised loss of synapses prior to axons, in a distal to proximal manner (Dadon-Nachum et al., 2011). These include Charcot-Marie Tooth disease, motor neuron disease and multiple sclerosis (Coleman et al., 2005, Marcuzzo et al., 2011). Therefore the study of WD and how it can be modified is of interest regarding these disorders. The ultimate goal is to be able to develop methods of strengthening the NMJ against its susceptibility in dying-back disorders. A recently discovered mutation known as *Wld*^{s} (wallerian-degeneration slow) is aiding the study of WD and how to modify it, and will be discussed below.

1.8.2 Synaptic degeneration in the Wld^s mouse

Study of the mechanisms involved in WD has been aided by the discovery of the mutant mouse *Wld*^{S} (WD slow), in which the degenerative process is slowed significantly (Lunn et al., 1989, Lyon et al., 1993). This delay is approximately ten-fold in young adult homozygous *Wld*^{s} mice (Gillingwater and Ribchester, 2003, Beirowski et al., 2005). The phenotype was first characterized by Lunn *et al.* (1989) following axotomy of the sciatic nerve in *Wld*^{s} mice (initially called C57Bl/Ola mice).

Various studies utilizing techniques such as electrophysiology and immunocytochemistry have further highlighted the differences in the time course of decay at the axon compared to the synaptic terminals in these mice. Axonal degeneration can take several weeks after axotomy whereas synapses degenerate more rapidly, within 4-10 days (Gillingwater et al., 2002, Gillingwater and Ribchester, 2003, Beirowski et al., 2004, Beirowski et al., 2009) demonstrating a compartmentalised process similar to that seen in WT. In other respects, *Wld*^{s} mice show no obvious variation from C57 Bl6 (Lunn et al., 1989, Coleman et al., 1998). WD is still calcium dependent in Wld^s animals (Glass et al., 1994) and axonal transport is normal (Glass and Griffin, 1991, Smith and Bisby, 1993).

The phenotypic effects of this mutation appear to demonstrate age-dependency, as homozygous *Wld*^{S} mice >2 months old show a reduced synaptic protection, although *Wld*^{S} expression, either in brain or neurons, appears to be unaffected by age and axon preservation remains greater than that seen in wild-type animals (Mack et al., 2001, Gillingwater et al., 2002). Perry, Brown and Tsao (1992) reported the age dependency of *Wld*^{s}, stating that C57 nerves degenerate more slowly with age, yet *Wld*^{s} nerves degenerate more rapidly (Perry et al., 1992). This was contested by another paper, which reported preservation of axons in old *Wld*^{s} mice not dissimilar to that seen in younger animals (Crawford et al., 1995). However, Gillingwater *et al.* (2002) proved that the weakening level of protection in *Wld*^{s} mice with age is in fact apparent at the synapse, but not axons, and moreover is dependent upon the relative age of the synapse itself. It is not yet clear why this age-dependency occurs, yet it may be related to falling levels of nicotinamide adenine dinucleotide (NAD) in the ageing animal (Wang and He, 2009).

1.8.2.1 Mechanisms of action

The *Wld*^{S} mutation was discovered accidentally as a random mutation within a colony of mice (Lunn et al., 1989) and has since been identified as an autosomal dominant mutation (Perry et al., 1992) on chromosome 4 (Lyon et al., 1993), comprising tandem-triplication of an 85kb sequence. The active protein (figure 1.1) results from in-frame fusion of the N70 region of the ubiquitination factor E4B (Ube4b) and nicotinamide mononucleotide adenylyl transferase-1 (Nmnat-1) which synthesizes NAD (Coleman et al., 1998, Conforti et al., 2000, Emanuelli et al., 2001, Coleman and Freeman, 2010).
When the Ube4b/Nmnat chimeric gene is expressed in transgenic mice, the phenotype of the *Wld*^{S} mouse is reproduced in a gene-dose dependent manner (Mack et al., 2001).

Work to interpret which regions of Wld^s are essential for its function has generated controversy. In some reports, depletion of Nmnat activity has shown no detrimental effect on *Wld*^{s} activity, but has in others. The level of Nmnat activity is increased in the brains of *Wld*^{s} mice although interestingly NAD levels are not altered (Mack et al., 2001). In cultured DRG neurons, over-expression of Nmnat1 was reported to delay axonal degeneration in a manner similar to that of *Wld*^{s}, although expression of the Ube4b region was not, indicating that the Nmnat1 region is thus the region required for the *Wld*^{s} phenotype. Expression of the inactive forms of Nmnat1 and *Wld*^{s} resulted in a lack of phenotype, leading to the suggestion that it is the ability of Nmnat1 to produce NAD which is required for *Wld*^{s} mediated axonal protection (Araki et al., 2004). NAD added to the cultures also delayed degeneration but less so than over expression of Nmnat1 (Araki et al., 2004, Wang et al., 2005). It has also been shown that NAD levels fall rapidly after axotomy in axons, and expression of *Wld*^{s} or Nmnat1 delays this process (Wang et al., 2005). Furthermore, transgenic mice that over express Nmnat1 in their axons and cytoplasm are strongly protected from degeneration, and also do not seem to display the age-dependent phenotype (Sasaki et al., 2009).
These results have, however, been disputed by another study (Conforti et al., 2007). Overexpression of Nmnat1 to the level seen in *Wld*^{s} mice resulted in no protective phenotype. Thus although Nmnat1 overexpression protects at high levels *in vitro*,

protection *in vivo* in *Wld*^{s} mice cannot be solely due to Nmnat – there must be some other component of *Wld*^{s} involved. Further evidence for this comes from Watanabe and colleagues who showed that Nmnat is not as effective as Wld^{s} in protecting against vincristine toxicity (Watanabe et al., 2007).

One known interaction of the N70 region of Ube4b is with valosin-containing protein (VCP), which is involved in the ubiquitin proteasome system. In fact the N70 region controls the nuclear targetting of VCP (Laser et al., 2006, Wilbrey et al., 2008). Removing the N70 region abolishes the protective phenotype of *Wld*^{s}. It is the N16 region of N70 that is thought to be important in maintaining its function, as this is the region known to bind to VCP. Removal of this region alone abolishes axonal protection (Conforti et al., 2009), which was originally thought to indicate the importance of the VCP interaction. However, weakening the VCP association of N70 leaves the protective phenotype intact (Beirowski et al., 2009). Nonetheless it is clear that both the Nmnat and N70 regions are important in Wld^{s} function.

One recent theory regarding how *Wld*^{S} exerts its protective effect has been proposed by Michael Coleman and his colleagues. The idea is that *Wld*^{s} substitutes for a survival factor that is normally present within the axonal environment. This factor sustains healthy axons, but when the axon is injured and therefore axonal transport is interrupted the endogenous levels of this substance fall, leading to degeneration. *Wld*^{s} substitutes for this factor and thus slows degeneration. The evidence for this hypothesis was presented in a recent review of *Wld*^{s} and Wallerian degeneration (Coleman and Freeman, 2010). The survival factor is proposed by this group to be Nmnat2, an endogenous Nmnat isoform (Gilley and Coleman, 2010). SiRNA removal of Nmnat2 causes degeneration of uninjured axons, and the half-life of Nmnat2 following axotomy is short, corresponding to the time course of degeneration. Wld^{s} is more stable than Nmnat2, and may substitute for Nmnat2 long enough to produce the effects on synapse degeneration seen in the *Wld*^{s} mouse.

In their recent study, Barrientos *et al.* (2011) demonstrated that inhibiting the mitochondrial permeability transition pore (MiPTP) with cyclosporin A delays degeneration in WT nerves. However, when applied to *Wld*^{s} nerves, cyclosporin A had

no effect, yet activating MiPTP abolished the *Wld*^{s} phenotype. They suggested that part of the mechanism of Wld^{s} involves delayed activation of MiPTP, possibly via the action of Nmnat.

1.8.2.2 Location of action

Due to strong localisation of Wld^{s} to the nucleus, it was initially presumed that Wld^{s} must act via the nucleus (Mack et al., 2001, Gillingwater et al., 2006). However, low level staining has since been noted in the cytoplasm, and redistributing Wld^{s} from the nucleus to the cytoplasm enhances its protective efficacy (figure 1.1, Beirowski et al., 2009). By generating mice in which the Nmnat1 region was subjected to two point mutations in the nuclear localization sequence (NLS) to reduce the ability of Wld^{s} to locate to the nucleus (ΔNLS mice), it was shown that cytoplasmic Wld^{s} is more effective as in these transgenic mice there was enhanced synaptic and axonal protection. Interestingly, this enhanced phenotype is also preserved in 7.5 month old ΔNLS mice (Beirowski et al., 2009). Furthermore, targetting Nmnat1 to axons also improves the protective phenotype (Babetto et al., 2010) providing further evidence that it is the axonal localisation of Wld^{s} that is responsible for its function. Thus the location of Wld^{s} outwith the nucleus determines its efficacy and the cytoplasmic localisation may also have something to do with its age-dependent phenotype. The upshot however, is that both the Nmnat and N70 regions are necessary for Wld^{s} to inhibit axonal and synaptic degeneration.

1.8.3 Wld^s and Wallerian-like disorders

Thus, the *Wld*^{s} model is of interest for studying the mechanisms of WD, as it allows investigation of morphological and electrophysiological alterations over a longer time course. It also may provide the basis for the development of methods for intervening with synaptic degeneration, which is of particular interest in the field of neurodegenerative disorders and developing therapeutic interventions.

The *Wld*^{s} mutation has in fact proven to be effective at protecting against degeneration in several models, including Parkinsons disease (Sajadi et al., 2004, Hasbani and O'Malley, 2006) and global cerebral ischaemia (Gillingwater and Ribchester, 2003). *Wld*^{s} also extends the lifespan of progressive motor neuropathy mice by approximately 20 days (Ferri et al., 2003) and protects against myelin mutations (Samsam et al., 2003) along with taxol and vincristine induced degeneration (Wang et al., 2001, Wang et al., 2002).

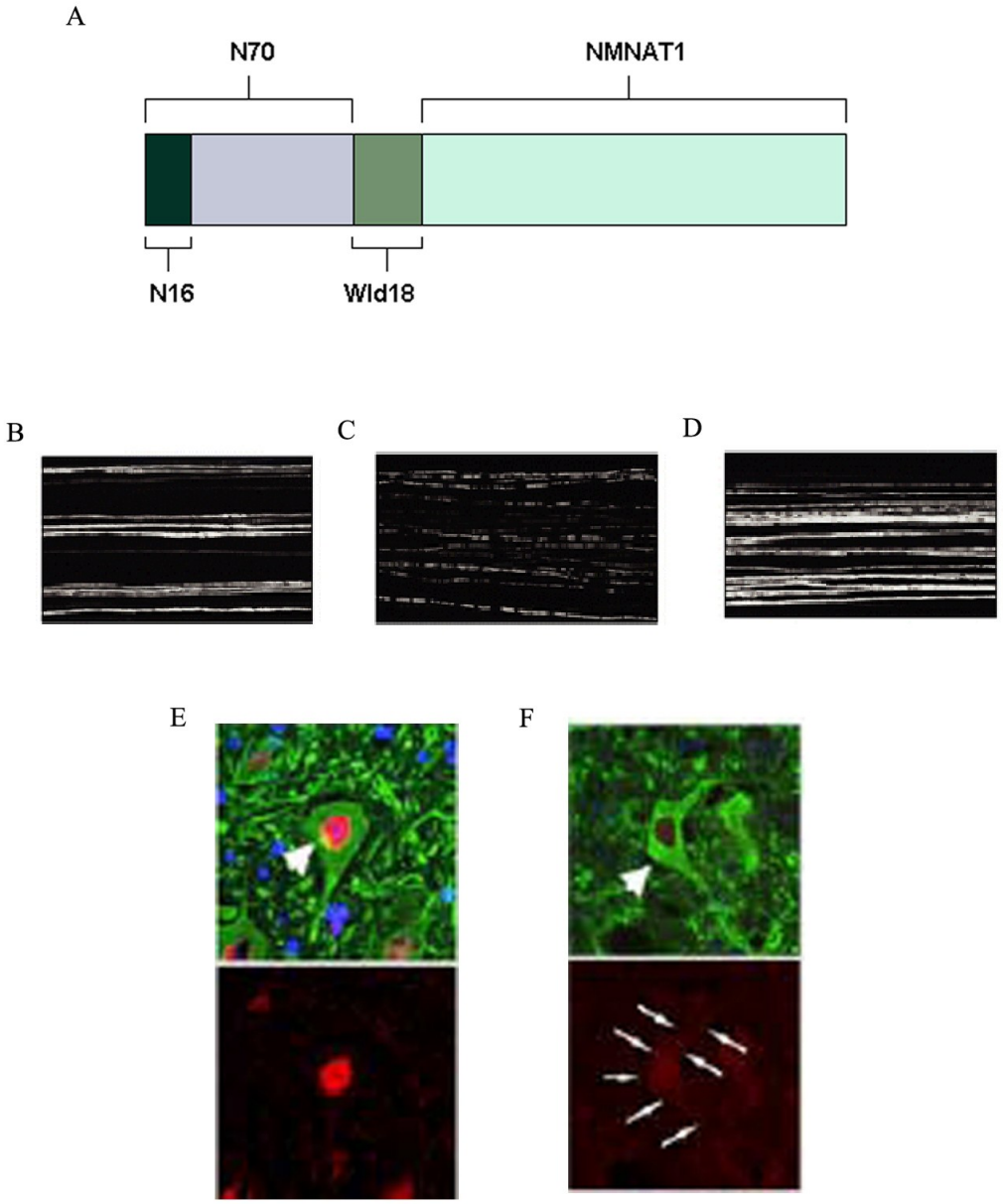
The protective effects of *Wld*^{s} are less evident in models in which the age-dependency of its synaptic efficacy is a confounding factor. For example, *Wld*^{s} has limited effects on gracile axonal dystrophy mouse model (Mi et al., 2005) and SOD mice (Fischer et al., 2005), although others have reported no effect at all (Vande Velde et al., 2004). However, in terms of studying ways of modifying synaptic degeneration, this is not of great concern, as the young animals can be used, and unlike models of neurodegenerative disorders, the timing of the onset of degeneration is controlled by the investigator, and is instigated by simply cutting the nerve. Furthermore, the time course of degeneration is well characterised in this mouse. It is therefore possible to predict at which time point approximately 50% or none of the synapses will remain innervated.

Figure 1.1 Composition of the Wld^s in-frame fusion protein, its protective effects and localisation

A. Wld^s comprises NMNAT1, the N70 region of Ube4b, and an 18 amino acid sequence 'Wld18', part of the normally untranslated region (5'UTR) region of NMNAT1. Based on Coleman and Freeman (2010).

B. Uncut tibial nerves show intact axons, whereas at 3 days post-axotomy WT axons are largely fragmented (C). However, heterozygous *Wld^s* axons remain largely intact 3 days post-axotomy (from Conforti et al. 2007).

E. Nuclear localisation of Wld^s, shown by an arrow, can be seen clearly in lumbar spinal cord sections from *Wld^s* mice (Wld18 antibody stain, red; ß-III tubulin, green; Hoechst 33258, blue). F. The ΔNLS mutation redistributes Wld^s outside of the nucleus. Arrows indicate the outline of individual cell bodies (from Beirowski et al. 2009).


A
N70
NMNAT1
N16
Wld18
B
C
D
E
F

1.9 Aims and Outline

In order to develop methods of intervening with neuromuscular synapse degeneration in neurodegenerative disorders, we must first address the basic principle of how to modify properties of the NMJ. As discussed above, both the structure and function of the NMJ are modified by activity, and furthermore the role of activity in the onset and progression of neuromuscular disorders is debated. Further evidence for the role of activity in the modulation of synaptic degeneration comes from the study of neonatal synapse elimination, which appears to be an activity-dependent process.

The potential of activity to modulate neuromuscular degeneration has been studied in animal models such as the SOD1 MND mouse. However, the results have been conflicting and no definitive conclusion has been reached. This is more than likely a result of the variation in protocols used, and in the age of symptom onset and lifespan between animals.

The aim of this thesis is therefore to test the hypothesis that synaptic degeneration is modulated by activity. For this, I utilised the *Wld*^{s} mouse.

The objectives in the three Results chapters are as follows:

1) I examined whether removing activity at the NMJ can influence the rate of synaptic degeneration in *Wld*^{s} mice. This was done using tetrodotoxin-induced paralysis of the hind limb prior to axotomy, and the effects were noted using a combination of intracellular electrophysiological recordings, morphological analysis of fluorescent NMJs, and a novel confocal microendoscope to monitor degeneration *in vivo* (Chapter 3).

2) Next, I attempted to measure the effects of increased activity on NMJ degeneration, and on the muscle itself, by giving *Wld*^{s} mice access to running wheels to stimulate aerobic activity then studied the rate of synaptic degeneration following axotomy (Chapter 4).

3) Finally, I developed an *ex vivo* model to establish the effects of electrical stimulation on the rate of synapse degeneration. This was then extended to investigation of pharmacological modulators of degeneration (Chapter 5).

Taking all the data from these studies together, I conclude that a period of inactivity in vivo leads to sensitization of NMJs to degeneration following nerve axotomy, whereas voluntary exercise has no effect on the rate of synapse degeneration. High frequency stimulation *in vitro* also lead to accelerated synapse degeneration in isolated FDB nerve muscle preparations. These findings may lead to the development of a mechanism which links the process of activity dependent plasticity and neurodegeneration.

2. Materials and Methods

2. Materials and Methods

The methods set out below are common to at least two of the following results chapters. Techniques specific to individual chapters will be described within that chapter.

2.1 Animal care and housing

Animals were maintained in the facilities at the University of Edinburgh in accordance with UK Home Office regulations (United Kingdom Animals (Scientific Procedures) Act, 1986). All mice used were housed in standard light (12h light 12h darkness) and temperature conditions and unless otherwise stated, in cages of 6 or fewer. Mice had access to food and water *ad libitum*.

Yellow fluorescent protein (YFP), a variant of the jellyfish green fluorescent protein (GFP) can be transgenically expressed in mice and labels both nerve terminals and axons, making it a valuable resource for the study of the degeneration process morphologically (Beirowski et al., 2004, Beirowski et al., 2005). These mice are originally generated using a modified thy1 gene to promote expression of YFP. Several strains of YFP mice have been generated, some of which are commercially available (Jackson Laboratories, USA). In these experiments I primarily used the Jackson Laboratories line thy1.2 YFP16 (Caroni, 1997, Feng et al., 2000, Lichtman and Sanes, 2003). In some experiments I used line thy1.2 YFPH mice, in which YFP expression is limited to around 3% of axons and thus allows clear visualization of individual axons (Lichtman and Sanes, 2003, Beirowski et al., 2004). Mice were genotyped for fluorescent protein expression by observing axons in ear punches. When weaned, ear punches were removed from the mice and mounted on slides, then the presence of fluorescence noted using a fluorescence microscope (see below).

Age matched (5-10 weeks old) male and female C57/Bl6, thy1.2-YFP16 Bl6, C57 Wld^s and thy1.2-YFP16 Wld^s mice are used throughout these chapters. The age was

monitored in order to take account of the age-dependence of the *Wld*^{s} phenotype (Gillingwater et al., 2002).

2.2 Animal sacrifice

At the end of the experiment mice were killed by cervical dislocation, in accordance with the UK Home Office regulations, schedule 1.

2.3 Sciatic and tibial nerve axotomy

Mice were anaesthetised in a lidded box using Isoflurane (Merial Animal Health Ltd., UK) and medical gas (95% oxygen, 5% CO_2) delivered by inhalation (4 litres min^{-1} containing 5% Isoflurane initially, which was reduced to 3% when the animal was fully anaesthetised). Once deeply anaesthetised (determined by absence of reflex reaction to foot pinch) the mouse was removed and placed on the operating table where the level of anaesthesia was maintained via a face mask. The flow of anaesthetic was reduced and surgical procedure begun under aseptic conditions. The hip or ankle region was shaved and wiped with an antiseptic solution (70% ethanol, 0.5% Hibitane), then the sciatic or tibial nerve was exposed by blunt dissection and a small section removed to prevent regeneration. The wound was sutured using 7-0 Mersilk suture (Ethicon Products, UK) and the animals' recovery monitored**.**

2.4 Flexor digitorum brevis muscle preparation

Directly after sacrifice the hind limbs of the animal were removed and the skin stripped. The limbs were then placed in fresh oxygenated mammalian physiological saline (MPS; 120mM NaCl, 5mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 0.4mM NaH_2PO_4, 23.8mM $NaHCO_3$, 5.6mM Glucose) until dissection. Each leg was then removed and pinned in a Sylgard-lined Petri dish. The FDB nerve-muscle preparation was dissected and returned to bubbling physiological solution ready for

electrophysiological analysis. The rest of the leg was either kept for lumbrical muscle dissection or discarded.

The *Wld*^{s} protective phenotype has already been quantified in the FDB (Ribchester et al., 1995, Gillingwater et al., 2002) and it is a very practical muscle for electrophysiology: FDB muscle fibres are short and therefore isopotential along their lengths (Bekoff and Betz, 1977). This means that EPPs can be faithfully recorded wherever the electrode is placed in the muscle fibre. It has also been shown that co-expression of YFP does not impair the protective phenotype in *Wld*^{s} mice (Gillingwater et al., 2002, Bridge et al., 2009).

2.5 EPP recording in μ-conotoxin

Prior to recording, isolated nerve-muscle preparations were bathed in physiological saline containing 2.5μM μ-conotoxin (GIIIB μ-CTX; Scientific Marketing Associates, UK) for 20 minutes, or until the muscle twitch disappeared. This toxin originated from the venom of the marine snail *Conus geographicus*. It blocks voltage dependent sodium channels at the muscle ($Na_v1.4$) whilst having no discernible effect on the sodium channels of the nerve**,** allowing recording of a stable endplate potential without disruption from muscle twitch (Cruz et al., 1985, Hong and Chang, 1989, Li and Tomaselli, 2004).

Following incubation in μ-CTX, the muscle was pinned in a Sylgard-lined recording chamber. The nerve was held inside a glass suction electrode. The internal and external silver wires were connected to a Digitimer D52 stimulator, controlled by a Digitimer D4030 programmer. Electrode glass (1.5mm o.d x 0.86mm i.d, Harvard Apparatus, UK) was pulled on a Flaming/Brown micropipette puller (Sutter Instrument Co., USA) to make microelectrodes with a final tip resistance of around 30-70 MΩ. Muscle fibres were impaled and the membrane potential was recorded using an Axoclamp 2B amplifier (Axon Instruments, USA). A CED 1401 interface transferred data to a personal computer (pc, Dell), which was then analysed using WinWCP software (Dr John Dempster, University of Strathclyde, UK). This

program was used to measure and calculate the various parameters of the EPP, including peak amplitude, rise and decay time and quantal content. Decay time was analysed as T50 and T90, i.e. the time for the EPP amplitude to decay to 50% or 90% of its original value, respectively (Figure 2.1). Interference was minimized using a Humbug filter (Digitimer Ltd., UK).

From a total of 30 randomly selected fibres, the percentage of innervated fibres was estimated by scoring a) the number of fibres to give an evoked response upon stimulation, b) MEPPs only or c) no response.

When scoring the number of innervated fibres, I counted the number giving an evoked response to stimulation with or without MEPPs, those giving only MEPPs and those giving no response at all. This is therefore a low stringency test of functional innervation, since those fibres showing only spontaneous MEPPs were scored as 'functional'. Synapses undergo stages of denervation, from fully occupied, partially occupied to denervated. If an axon breaks but synaptic boutons are still present, only spontaneous MEPPs can be recorded.

2.6 Recording and analysis of miniature endplate potentials

Spike-2 software (Cambridge Electronic Design, UK) was used to record MEPPs. The records were then analysed using Minianalysis (Synaptosoft, USA), which is designed to automatically detect and measure the parameters of MEPPs. Spontaneous MEPP activity was recorded for approximately 100s for each fibre. As resting membrane potential (RMP) can affect MEPP amplitude, recording were only taken from fibres with an RMP between –60 and –70mV.

2.7 Quantal analysis

A train of 30 pulses was recorded from each muscle fibre. Using WinWCP software quantal content was analysed in most cases using the Variance Method as discussed in Chapter One. Where applicable, the 'failures' method was used to calculate quantal content by counting the number of failures in responses to trains of stimuli. The correction factor 'f' for non linear summation was set to 0.3 and the reversal potential to -10mV. All methods of calculating QC have been shown to produce comparable values, and thus are interchangeable to a certain degree when m has a low value (Del Castillo and Katz, 1954c, Liley, 1956b). Without the appropriate correction for non-linear summation (McLachlan and Martin, 1981), estimates of quantal content are high (Harris and Ribchester, 1979, Wood and Slater, 1997)

2.8 Lumbrical muscle dissection and staining

As with the FDB dissection, the hind limbs were removed, skinned, and placed in oxygenated MPS. Either after or without previous FDB dissection, lumbrical muscles were dissected, pinned out in Sylgard lined Petri dishes and immersed in MPS containing 5µgml^{-1} Tetramethylrhodamine-isothiocyanate (TRITC) α-bungarotoxin (Molecular Probes, USA) which labelled postsynaptic acetylcholine receptors (AChRs). The preparations were then placed on a rocking platform (Stuart Scientific, UK) for 10 minutes, then washed twice with MPS for 10 minutes each time. To fix the muscle tissue it was bathed in 4% paraformaldehyde (Electron Microscopy Sciences, USA) for 15 minutes then washed twice with MPS for 10 minutes each time. Once fixed and stained, the muscles were mounted on a slide in Vectashield (Vector laboratories, UK) and covered with a coverslip ready for fluorescence microscopy.

Directly after sacrifice the hind limbs of the animal were removed and the skin stripped. The limbs were then placed in fresh oxygenated mammalian physiological saline (MPS; 120mM NaCl, 5mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 0.4mM NaH_2PO_4, 23.8mM $NaHCO_3$, 5.6mM Glucose) until dissection. Each leg was then removed and pinned in a Sylgard-lined Petri dish. The FDB nerve-muscle preparation was dissected and returned to bubbling physiological solution ready for electrophysiological analysis. The rest of the leg was either kept for lumbrical muscle dissection or discarded.

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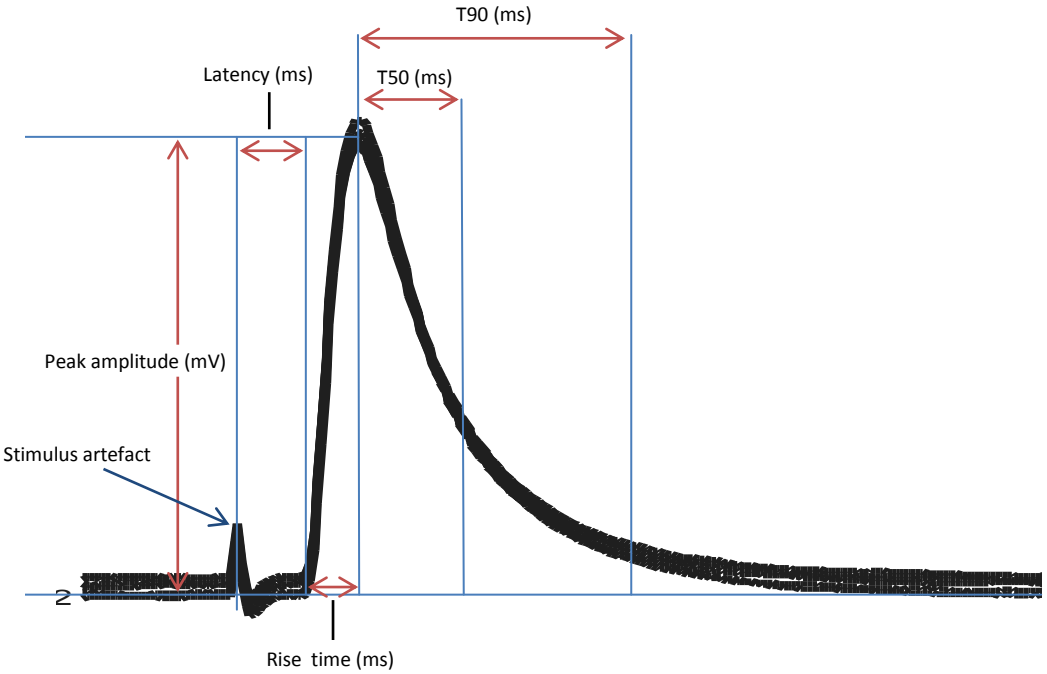
Figure 2.1 Analysis of endplate potential parameters

WinWCP software (Dr John Dempster, University of Strathclyde, UK) was used to analyse the various properties of evoked endplate potentials (EPPs) in FDB muscles. The stimulus artifact appears at the time of stimulation. As illustrated, measurements include the peak amplitude of the EPP, the time between the stimulus and the beginning of the response (latency) and the rise time of the peak. Decay time was analysed as T50 and T90, i.e. the time for the EPP amplitude to decay to 50% or 90% of its original value, respectively


T90 (ms)
Latency (ms)
T50 (ms)
Peak amplitude (mV)
Stimulus artefact
2
Rise time (ms)

2.9 Fluorescence microscopy

Nerve terminals and intramuscular axons were visualised using a fluorescence microscope (Olympus, UK). Both 10x and 20x Olympus objectives were used. Images were obtained using a Hamamatsu Orca-ER camera coupled to an Apple Mac computer, and processed using both OpenLab (Improvision, UK) and Adobe Photoshop.

Endplates were scored as innervated when the TRITC-α-bungarotoxin labelled endplate showed any coverage with a YFP labelled axon. Denervated endplates were scored as those with no YFP covering.

2.10 Statistical Analysis

All statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc, USA), using mainly analysis of variance (ANOVA) and unpaired t-tests. All data are represented as mean±SEM, unless otherwise stated. 'N' refers to the number of animals, whereas 'n' represents the number of muscles. Conventional star ratings are used to denote the level of significance ($p<0.05$ = *, $p<0.01$ = **, $p<0.001$ = ***).

3. Effect of paralysis on synaptic degeneration

3. Effect of paralysis on synaptic degeneration

3.1 Background

Neuromuscular junctions display activity-dependent plasticity, which modifies both their structure and function. Various models have been used to reduce or block neuromuscular activity. These include limb immobilisation, limb suspension and pharmacological block of synaptic transmission (Snider and Harris, 1979, Betz et al., 1980, Pachter and Eberstein, 1984, Templeton et al., 1984, Desplanches et al., 1987, Costanzo et al., 2000). These studies focus not only on the plasticity of the polyneuronally innervated synapse (Brown et al., 1982, Costanzo et al., 2000), but also on the ability of reduced activity to alter the adult synapse (Wilson and Deschenes, 2005). I therefore tested the effects of inactivity on the vulnerability of the NMJ to axotomy induced Wallerian degeneration.

One way of reducing neuromuscular activity is by pharmacologically blocking voltage-gated sodium channels using tetrodotoxin (TTX). TTX was originally extracted from the liver of a species of puffer fish, but has since been identified in many more species (reviewed by Kao, 1966). TTX blocks some, but not all, subtypes of voltage gated sodium channels, results in loss of nerve conduction and therefore paralysis. When systemic levels reach a critical value, death ensues from paralysis of respiratory muscles (Yoshida, 1994, Lee and Ruben, 2008).

Topical administration of TTX to peripheral nerves does not cause degeneration *in vivo* (Lavoie et al., 1976, Betz et al., 1980), and it also does not affect axonal transport (Lavoie et al., 1976, Pestronk et al., 1976a, Czeh et al., 1978). Chronic block has however been documented to affect both properties of synaptic transmission, and also the structure of the NMJ (Brown and Ironton, 1977, Snider and Harris, 1979) in adult animals in which it provokes nerve sprouting and increased quantal content (Tsujimoto et al., 1990). Chronic TTX block maintains polyneuronal innervation in adults (Barry and Ribchester, 1995, Costanzo et al., 2000) and neonates (Thompson et al., 1979, Brown et al., 1982). This demonstrates

the implications of removing activity for synaptic function and raises questions regarding its effects on synaptic degeneration.

Sustaining effective nerve block can be problematic (Betz et al., 1980, Ribchester and Taxt, 1983, Martinov and Nja, 2005). Techniques need to minimise systemic diffusion of TTX and avoid toxicity. Martinov and Njå (2005) modified previous nerve block techniques by using small glass microcapillaries capable of sustaining nerve block for weeks, whilst producing minimal adverse side effects and being relatively easy to implant. The tube is maintained in its position by a polythene cuff. This technique is preferable to other forms of removing activity, such as tenotomy, which is suspected to not always be completely effective (Bray et al., 1979). It is also economically preferable to using osmotic minipumps, which have been utilised by my thesis supervisor in many nerve conduction block studies using rats (Ribchester and Taxt, 1983, 1984, Ribchester, 1993, Barry and Ribchester, 1995).

As TTX prevents loss of axons during developmental pruning, we might hypothesise that synaptic degeneration induced by other stimuli would also be activity-dependent, or at least influenced by activity. One prediction from this hypothesis is that block of activity would protect degenerating synapses. In support of this, there is evidence that blocking sodium channels may be neuroprotective (Stys, 2005). On the other hand exercise increases the oxidative capacity of muscles (Kirkendall and Garrett, 1998) and physical activity has also been shown to improve motor function in ageing people (Lang et al., 2007).

In this chapter I first investigated whether TTX application alone is capable of inducing synaptic degeneration or damage. Then I studied the electrophysiological adaptations of the FDB muscle to one week of paralysis in Wld^s mice.

To apply TTX to the sciatic nerve I adapted the method of handmade microcapsule implantation from Martinov and Njå (2005), who used the tubes for studying chronic nerve block in rats and mice. I successfully modified the protocol to make similar tubes, inducing effective hindlimb paralysis for one week after implantation adjacent to the sciatic nerve in mice.

Second, I investigated the effect of TTX on synapse degeneration induced by axotomy. I focussed primarily on *Wld^s* mice in which WD is retarded by 4-10 days. I

also studied the effect of paralysis in axotomised WT NMJs. Following one week of TTX block, the tibial nerve was cut and the level of degeneration compared at various time points to that seen in mice with no previous surgery or those treated with saline instead of TTX. I used electrophysiological measurement of synaptic transmission to estimate functional survival in the FDB following axotomy. I also studied the morphology of NMJs in lumbrical muscles and also the axons in the distal tibial nerve to visualize the level of degeneration. For some of these studies I applied a method for monitoring degeneration *in vivo* using confocal microendoscopy, shown to be effective at monitoring both synaptic and axonal degeneration *in vivo* (Pelled et al., 2006, Vincent et al., 2006, Wong et al., 2009).

One week of TTX block prior to axotomy in *Wld*^{s} mice significantly reduced the amount of synaptic protection in response to the nerve lesion, although it had no effect on axonal degeneration. TTX block alone caused no detectable degeneration, but was sufficient to cause some subtle adaptations to neuromuscular transmission.

3.2 Methods

The following methods are specific to this chapter. For general methods see chapter 2.

3.2.1 Tetrodotoxin

1mg TTX powder in citrate buffer (Alomone Labs, Israel) was dissolved in sterile saline (Hameln Pharmaceuticals, UK) containing 200µg/ml Ampicillin (Sigma Aldrich, UK) to make a final concentration of 15mM. I reduced this from the 30mM concentration used by Martinov and Nja (2005) as I found this dose caused too many deaths, presumably due to systemic toxicity.

3.2.2 Microcapsule manufacture

Electrode glass (1.2mm x 0.94mm Harvard Apparatus, UK) was pulled on a Flaming/Brown micropipette puller. The tip was then broken and rounded using a micro forge (Narishige, Japan) to produce a tip with an external diameter of approximately 200µm. The other end of the electrode was then scored with a diamond pencil and broken to obtain an overall length of 5mm, and rounded using an ethanol lamp. The microcapsules were filled by capillary action with either 15mM TTX or sterile saline containing 200µg ml^{-1} Ampicillin (Hameln Pharmaceuticals, UK; Sigma Aldrich, UK) and the larger end sealed with a small amount of dental wax (Kemdent, UK; figure 3.1a). The tip was protected by inserting it into a small length of polythene tubing (SF Medical, USA).

3.2.3 Microcapsule implantation

thy1.2 YFP16 Wld^{s} (N=35) or C57 Bl6 mice (N=4), aged 5-8 weeks, were anaesthetised with Isoflurane (Merial Animal Health Ltd., UK) and an incision made

at the level of the sciatic notch. The muscle was parted by blunt dissection to reveal the sciatic nerve, which was then enclosed within a small cuff made from polythene tubing (SF Medical, USA; figure 3.1). The microcapsule was first dipped in sterile saline to remove any excess TTX from the exterior and then the tip inserted inside the cuff to hold it in place. The wound was sutured and the recovery of the animal monitored. Initially the tip was inserted into the epineurium but this was discontinued after it was found that the block was as effective at this concentration when just lodged inside the cuff, which also reduces the risk of nerve damage.

Block was monitored daily by observing the animals grip with its hind limb on the cage bars, walking gait, toe spreading reflex and response to pinching the foot pad. Absence of toe spreading, and toe-pinch, reflex has previously been used to determine the presence and effectiveness of nerve block (Lavoie et al., 1976, Brown and Ironton, 1977, Betz et al., 1980, Ribchester, 1993, Barry and Ribchester, 1995).

To further check the efficacy of the nerve block, in one anaesthetized *Wld*^{s} mouse the sciatic nerve was stimulated both above and below the level of the block. Stimulation of the sciatic nerve produced no twitch in the foot muscle when stimulated above the site of block, but a twitch was present in the leg and foot when the nerve was stimulated below the implant (data not shown).

3.2.4 Tibial nerve cuts

Tibial nerve cuts were performed after one week of block and in age-matched, litter-matched control animals, which had either no previous surgery or had a microcapsule containing saline implanted one week earlier. Mice were anaesthetised using isoflurane and an incision made on the medial side of the leg close to the ankle. The tibial nerve was exposed and a small section removed to prevent regeneration. The wound was sutured and the recovery of the animal monitored.

Mice were killed by cervical dislocation and electrophysiological and morphological studies performed by the methods described above. In Wld^{s} mice this was performed at 3 (N=3), 5 (N=22) and 7 (N=3) days post axotomy, whereas in WT mice (N=4) the

level of degeneration was assessed 12hr after nerve cut. In some cases the contralateral side was used as an uncut control.

3.2.5 In vivo monitoring of axonal degeneration

A handheld fibre-optic confocal microendoscope (Mauna Kea Technologies 'Cell Vizio', France) was used to monitor synaptic degeneration *in vivo*, allowing the degeneration of motor nerve terminals to be observed within the same animal at various time points. A 1.5mm handheld probe (S-1500) connected to its laser scanning unit with an excitation wavelength of 488nm allows the nerve and neuromuscular junctions to be imaged through a small incision. The tip of the probe was placed in direct contact with the tissue. The observation depth is approximately 15µm. Fluorescence signal was collected between 500-650nm, and image-capture was at 12 frames per second. Images were processed using Cell Vizio software on an Apple Mac computer.

The validity of the technique was proven in anaesthetised WT and Wld^s mice 3 days post axotomy (Wong et al., 2009). Furthermore, imaging of the flexor digitorum longus muscle 3, 4 and 5 days post axotomy in a YFP16 Wld^s mouse demonstrates the utility of this technique for repeatedly visualising degenerating endplates (Wong et al., 2009).

To determine the effects of one week of TTX block on axonal degeneration following tibial nerve section, thy1.2 YFP16Wld^s (N=2) mice were anaesthetised and a microcapsule containing 15mM TTX implanted in the RHS as before. 7 days later, a bilateral tibial nerve cut was performed, meaning that the unblocked LHS could be used as an internal control. As controls, the tibial nerves of one thy1.2 YFP16 Bl6 and one thy 1.2 YFP16Wld^s were cut bilaterally. The nerves were exposed and visualised at 0 and 3 days post-axotomy in all mice, extending to 5 and 7 days in the Wld^s mice.

3.3 Results

3.3.1 Efficacy of the TTX block

Unilateral hindlimb paralysis was successfully maintained for one week. However, three animals died before one week had elapsed, presumably from systemic toxicity. Two animals were sacrificed early as the block was not maintained for one week. This may have been due to variation in the flow rate from the individually made TTX-filled tubes. The block was completely effective in 30 out of 35 animals.

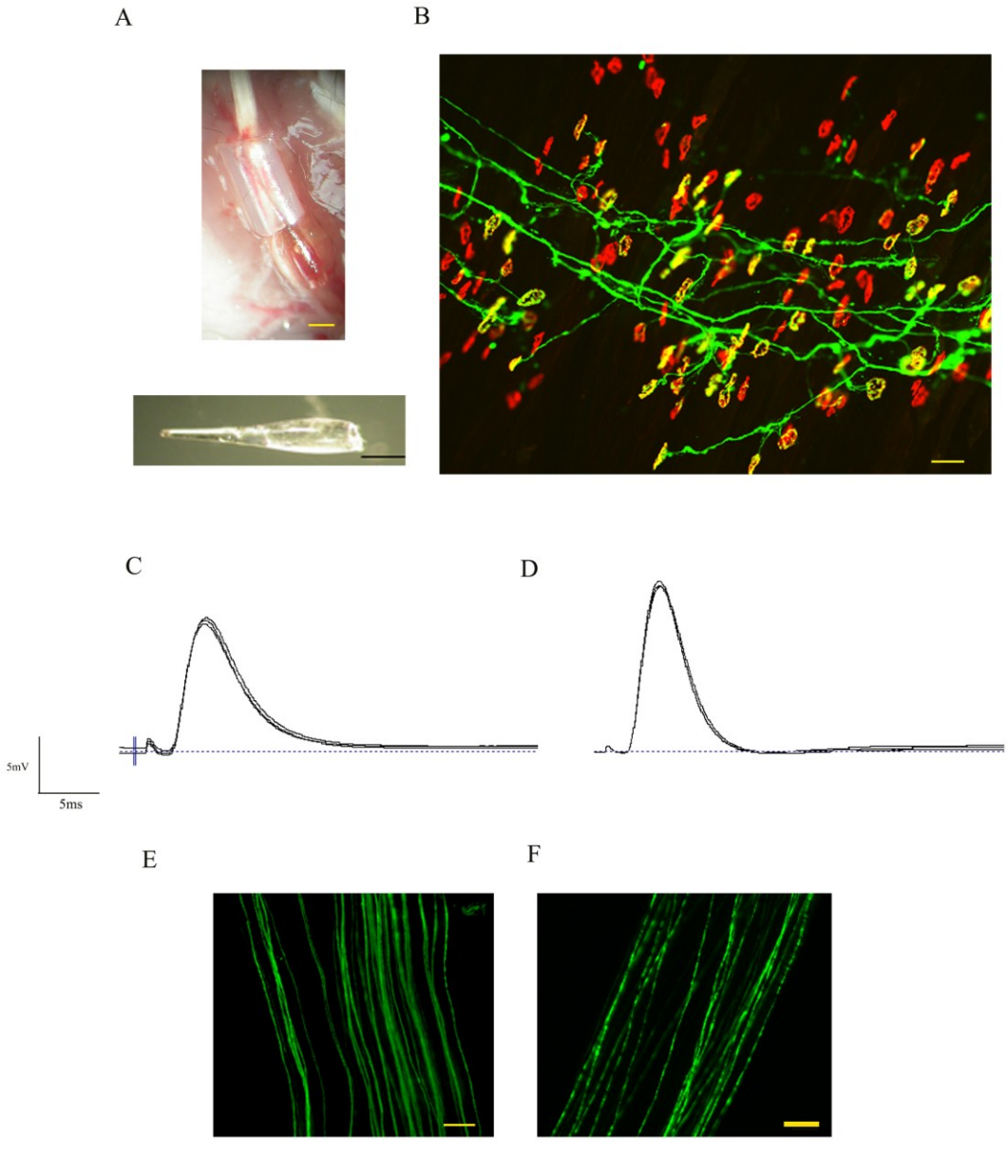
3.3.2 TTX does not damage axons or cause degeneration

First, it was important to establish that the nerve blocking technique did not directly damage axons. TTX application via the implant caused no discernible axon degeneration in either *Wld*^{s} or WT mice. Implanting a capsule containing either saline or 15mM TTX produced no significant synaptic degeneration when compared to the contralateral side 7 days after the implant ($p>0.05$). There was also no discernible axonal degeneration, as shown by fluorescence imaging of YFP expressing axons (figure 3.1e,f). Electrophysiological analysis also revealed no significant difference in the number of fibres showing an evoked response in FDB muscles in mice with sciatic nerve block with TTX for 1 week compared to contralateral controls or those treated with saline (87.78±7.78% n=3 muscles; 91.88±2.834% n=6; 85.00±2.152% n=4 respectively, figure 3.1c,d, 3.4).

Therefore, neither the implant nor its TTX content caused any axonal or synaptic degeneration, making this a suitable technique for studying the effects of chronic nerve block prior to axotomy.

Figure 3.1: Implantation of microcapsules containing TTX or saline does not damage axons or NMJs

A. Mice were implanted with microcapsules containing either saline or TTX at the mid-thigh region. The small implant (scale bar = 3mm) was held in place adjacent to the sciatic nerve by a short length of polythene tubing. B. Morphological analysis was completed using lumbrical preparations stained with TRITC-α-BTX (red) from mice expressing YFP in their axons (green). The number of endplates showing no YFP coverage was scored as a percentage of the total number of endplates. Scale bar = 30μm. One week of TTX application, or microcapsule implantation, did not cause any detectable axonal degeneration as FDBs displayed robust EPPs upon stimulation in both TTX-treated preparations (C) and saline treated controls (D). E. YFP labelled axons showed no signs of fragmentation, which is noticeable in Wld^s animals 7 days post axotomy (F, scale bars = 30 μm).


A
B
C
D
5mV
5ms
E
F

3.3.3 TTX block changes some electrophysiological properties of the FDB muscle

Next I ascertained whether one week of TTX block affected any of the physiological properties of the FDB muscle.

Previous studies have illustrated the effect of nerve conduction block using TTX on properties of transmitter release at the NMJ (Snider and Harris, 1979, Tsujimoto and Kuno, 1988, Tsujimoto et al., 1990, Wong et al., 2009). Therefore, I analysed the properties of EPPs in FDB muscles in which nerve conduction had been blocked *in vivo* for one week previously.

Application of TTX for 7 days produced a significant increase in quantal content at the NMJ in the FDB muscle compared to controls (40.67±5.32 n=6 muscles, 25.42±3.26 n=7, $p<0.05$ respectively, figure 3.2b), indicating an alteration in transmitter release from the silenced synapses. There was also a significant increase in peak amplitude (26.10±1.25 vs 16.51±0.14, $p<0.01$), rate of rise (25.04±1.28 vs 18.59±1.47, $p<0.05$), half decay time (T50, 4.47±0.36 vs 2.96±0.23, $p<0.05$) and 90% decay time (T90, 12.46±1.50 vs 7.30±0.79, $p<0.05$, figure 3.2, n=5 TTX treated muscles, n=3 control muscles for all). Rise time (1.47±0.09 vs 1.22±0.09), latency (2.57±0.06 vs 2.43±0.12) and RMP (-67.33±2.38 vs -71.59±2.38) were unchanged ($p>0.05$ for all three).

I also observed the properties of MEPPs. Surprisingly, TTX block produced no alteration in amplitude compared to controls (1.59±0.17, 1.17±0.10), rise (4.44±0.85, 5.21±1.15) or decay time (3.69±0.32, 3.10±0.36 respectively, $p>0.05$, figure 3.3). There was also no significant alteration in MEPP frequency (23.68±4.47 n=6, 30.18±4.97 MEPPs min^{-1} n=4, $p>0.05$).

Figure 3.2: Effect of TTX-induced paralysis on properties of neuromuscular transmission

A. Schematic of the experiment design. Following one week of TTX-block, animals were killed by cervical dislocation (CD) and electrophysiological analysis of the FDB muscles was carried out. Results were compared to untreated controls.
In comparison to untreated controls, one week of sciatic nerve paralysis via TTX application caused a significant increase in QC (B, n=7, 6 respectively, $p<0.05$). Peak amplitude (C, $p<0.01$), rate of rise (E, $p<0.05$), T50 (F, $p<0.05$) and T90 (G, $p<0.05$, n=3, 5 respectively, all unpaired t-tests). No change in the rise time was induced by TTX however (D, $p>0.05$).

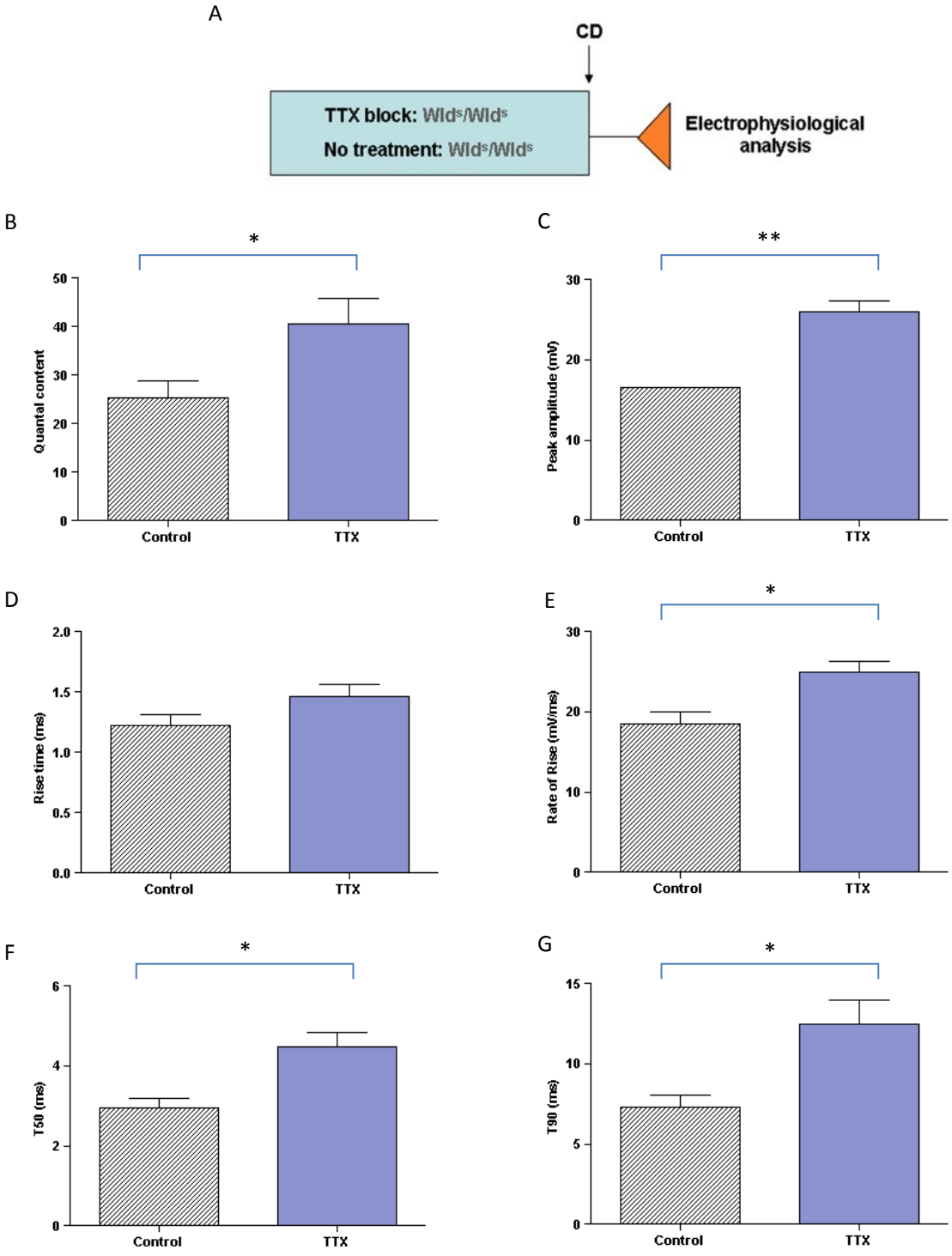
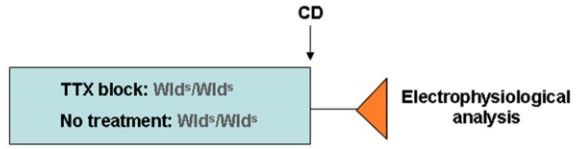

A
CD
TTX block: Wld^s/Wld^s
No treatment: Wld^s/Wld^s
Electrophysiological analysis
B
Quantal content
Control
TTX
C
Peak amplitude (mV)
Control
TTX
D
Rise time (ms)
Control
TTX
E
Rate of Rise (mV/ms)
Control
TTX
F
T50 (ms)
Control
TTX
G
T90 (ms)
Control
TTX

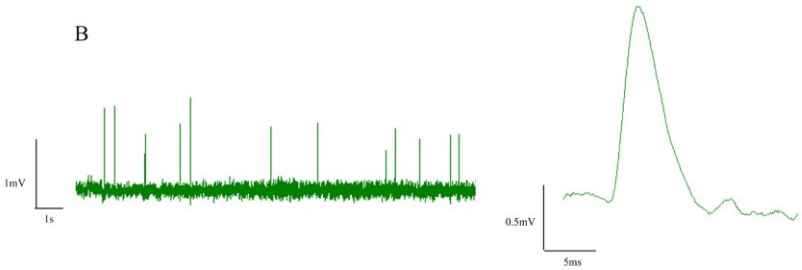
Figure 3.3: TTX paralysis does not significantly alter properties of spontaneous transmitter release

A. Schematic of the experiment design. Following one week of TTX-block, animals were killed by cervical dislocation (CD) and electrophysiological analysis of the FDB muscles was carried out. Results were compared to untreated controls.
B. Miniature endplate potentials were recorded in both TTX treated (n=6) and control (n=5) FDB muscles. Trains of EPPs were recorded using Spike-2 Software (Cambridge Electronic Design, UK), the individual MEPPs analysed. One week of TTX block did not alter the properties of spontaneous transmitter release. C. There was a slight increase in MEPP amplitude, but this was not statistically significant ($p=0.094$) and T50 (D, $p=0.275$) or rise time (E, $p=0.596$ unpaired t-test) were also not significantly altered.

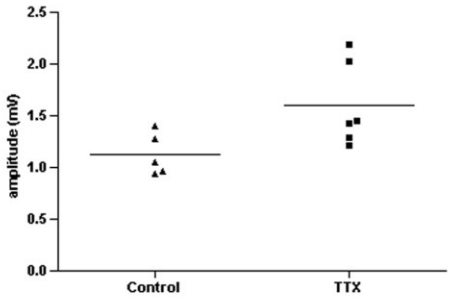
A


CD
TTX block: Wld^s/Wld^s
No treatment: Wld^s/Wld^s
Electrophysiological analysis

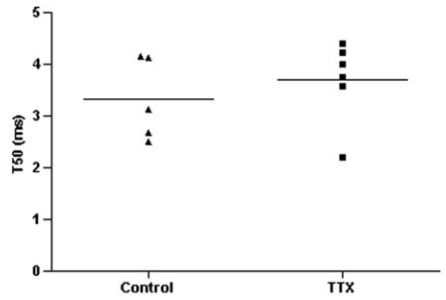
B


1mV
1s
0.5mV
5ms

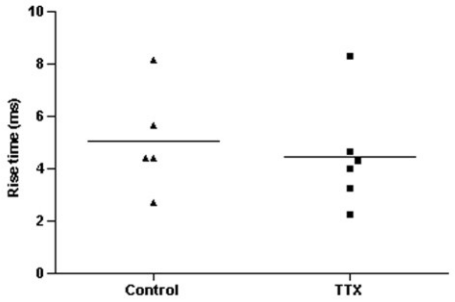
C


amplitude (mV)
2.5
2.0
1.5
1.0
0.5
0.0
Control
TTX

D


T50 (ms)
5
4
3
2
1
0
Control
TTX

E


Rise time (ms)
10
8
6
4
2
0
Control
TTX

3.3.4 TTX pre-treatment hastened synaptic degeneration in Wld^s mice

Finally, I measured the effects of 7 days of paralysis on the degeneration of NMJ's induced by axotomy. TTX pre-treatment for 7 days resulted in an increase in the number of degenerated synapses 5 days after axotomy in *Wld*^{s} mice, revealed by both electrophysiological and morphological assessments. Analysis of the number of remaining fibres capable of producing an evoked response to stimulation at 5 days post axotomy revealed about a five-fold reduction in the number of responsive fibres compared to those treated with saline prior to axotomy, or no previous surgery (TTX 8.57±2.51%; saline 55.56±5.88%; no previous surgery 43.34±10.11%, n=7, 3, 6 respectively $p<0.05$, figure 3.4). Including fibres with spontaneous transmitter release only into this analysis further indicated the substantial reduction in the number of functional fibres in TTX treated preparations compared to controls (TTX 13.81±4.27%, saline 77.78±4.44%, no previous surgery 78.89±4.61% respectively, one way ANOVA $p<0.01$, figure 3.4). The shift in the rate of degeneration was consistent over time, with a reduction in the number of responsive fibres, including those with spontaneous activity only, in paralysed muscles compared to controls at 3 (38.34±18.13% vs 88.75±3.15% n=4 per group, $p<0.01$), 5 (13.81±4.27% vs 81.90±4.93% n=7 per group, $p<0.01$) and 7 (0 vs 34.58±14.74% n=4 per group, $p<0.01$) days post-axotomy (figure 3.5). The same pattern was noted when considering only fibres giving an evoked response to stimulation.

YFP labelled axons, coupled with TRITC-α-bungarotoxin staining of endplates revealed substantially more vacant endplates compared to controls 5 days post axotomy (figure 3.6). In preparations treated with TTX prior to axotomy, an average of 75.75±3.65% of endplates were unoccupied (n=4), compared to 23.58±7.578% in axotomised Wld^s muscles (n=6). The number of denervated endplates in innervated contralateral muscles was negligible (0.07±0.05, n=7).

Figure 3.4: TTX block enhances susceptibility to synaptic degeneration following axotomy in *Wld*^{S} mice

The number of fibres in FDB muscles responding to stimulation with EPPs (A), MEPPs (B) or no response (C) was recorded in experimental and control muscles.
D. One week of TTX paralysis (n=7) significantly reduced the number of fibres producing EPPs compared to saline application (n=3) or those with no previous surgery (n=6) 5 days post axotomy, and also all uncut controls (n=3 TTX treated, n=4 saline treated, n=6 contralateral control, $p<0.01$). Neither TTX block alone nor an implant containing saline caused any notable degeneration compared to contralateral controls ($p>0.05$). E. Analysis of the number of fibres responding with EPPs and/or MEPPs strengthened the significance of this observation ($p<0.001$, one way ANOVA)

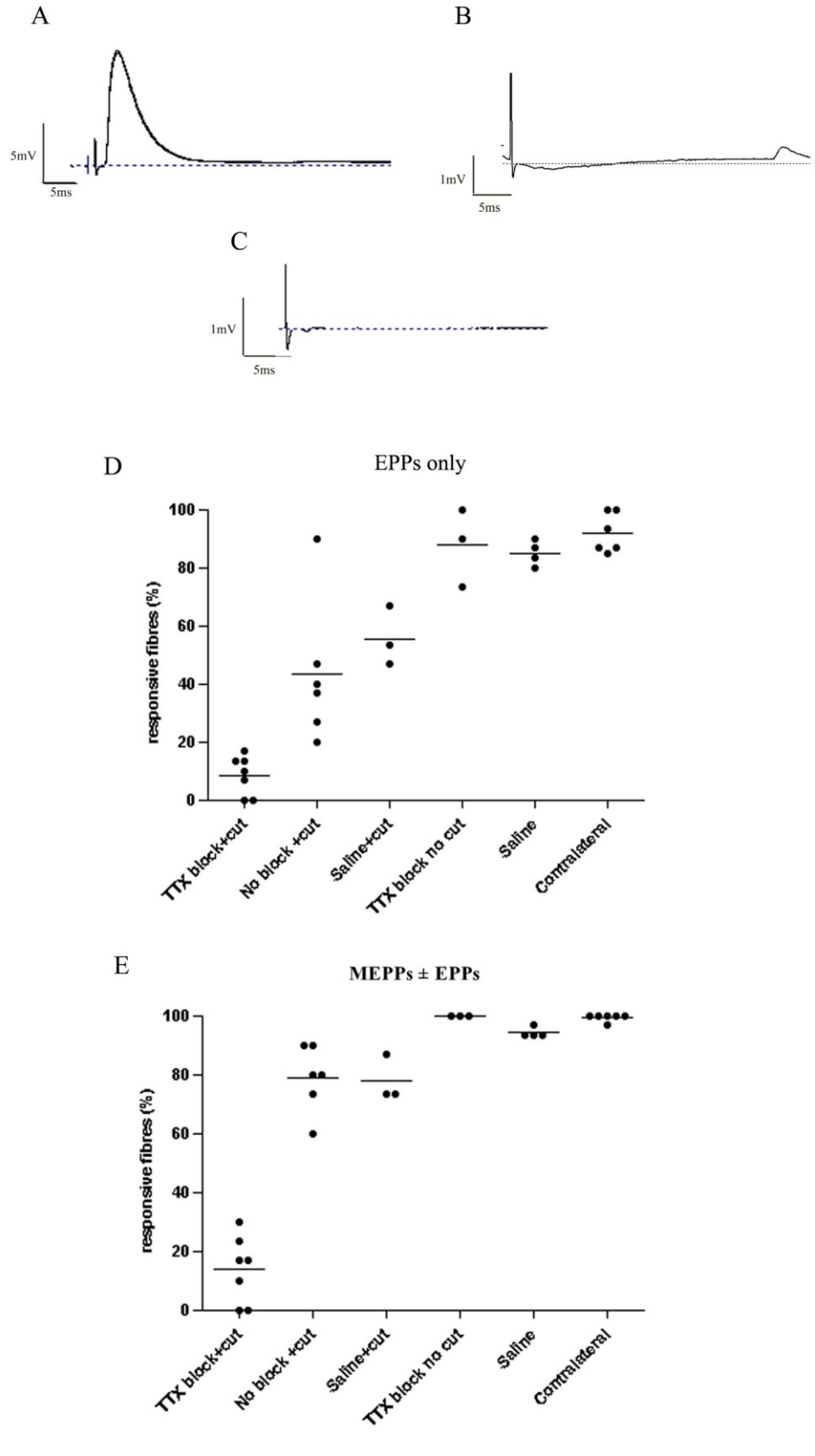
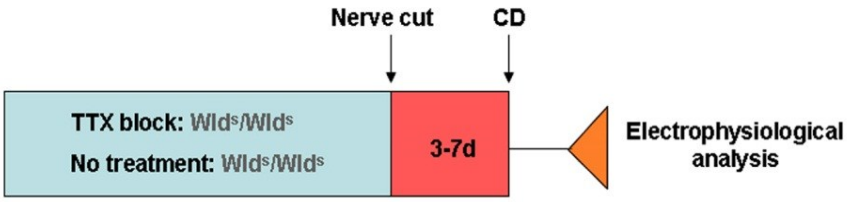

A
5mV
5ms
B
1mV
5ms
C
1mV
5ms
D
EPPs only
responsive fibres (%)
100
80
60
40
20
0
TTX block+cut
No block +cut
Saline+cut
TTX block no cut
Saline
Contralateral
E
MEPPs ± EPPs
responsive fibres (%)
100
80
60
40
20
0
TTX block+cut
No block +cut
Saline+cut
TTX block no cut
Saline
Contralateral

Figure 3.5: Time course of synaptic degeneration in TTX blocked muscle

A. Schematic of the experimental design. Following one week of nerve block, the tibial nerve was cut and electrophysiological analysis carried out 3-7 days later. Results were compared to axotomised controls. B. The reduction in the number of responsive fibres found after pre-treatment with TTX was maintained at earlier and later time points post-axotomy. The number of responsive fibres was significantly reduced in TTX-blocked muscles 3 (n=4 per group), 5 (n=7 per group) and 7 (n=4 per group) days post-axotomy compared to untreated axotomised controls ($p<0.01$, two way ANOVA).

A


Nerve cut
CD
TTX block: Wlds/Wlds
No treatment: Wlds/Wlds
3-7d
Electrophysiological analysis

B

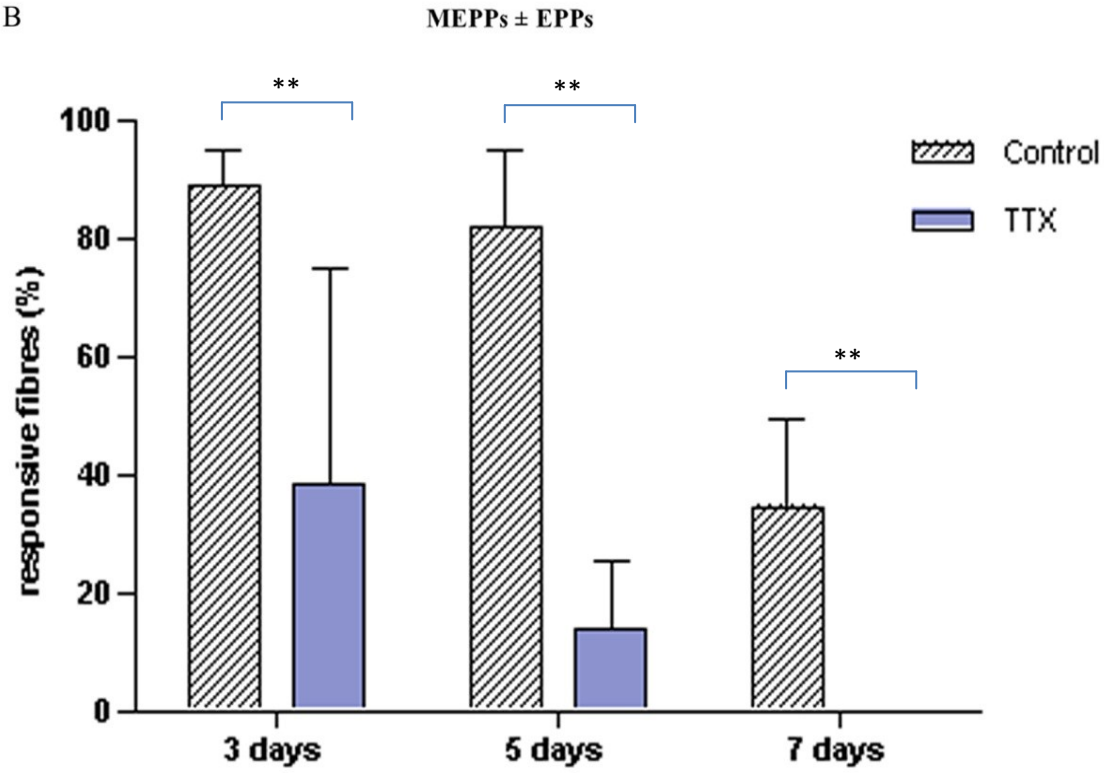
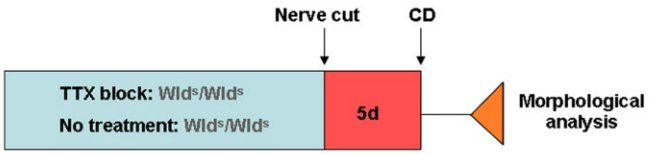

MEPPs ± EPPs
responsive fibres (%)
100
80
60
40
20
0
**
**
**
Control
TTX
3 days
5 days
7 days

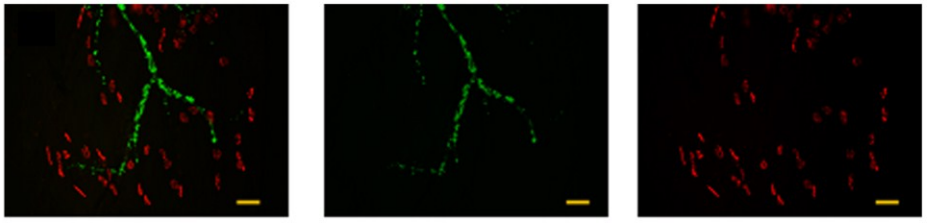
Figure 3.6: Morphological analysis reveals heightened sensitivity of NMJs to degeneration initiated by axotomy, following one week of nerve conduction block

A. Schematic of the experimental design. Following one week of nerve block the tibial nerve was cut and the animal killed by cervical dislocation 5 days later. Morphological analysis of the number of unoccupied endplates was then carried out.
B. 5 days post-axotomy in lumbrical muscles paralysed previously for one week with TTX, denervated endplates are common, along with fragmented intramuscular axons.
C. In untreated control *Wld*^{S} muscles 5 days post-axotomy, innervated endplates occurred more frequently, as reflected by analysis of the average percentage of unoccupied endplates in TTX-blocked (n=4) compared to untreated axotomised (n=6) and contralateral controls (n=7, D, $p<0.001$, one way ANOVA). Scale bars = 30μm.

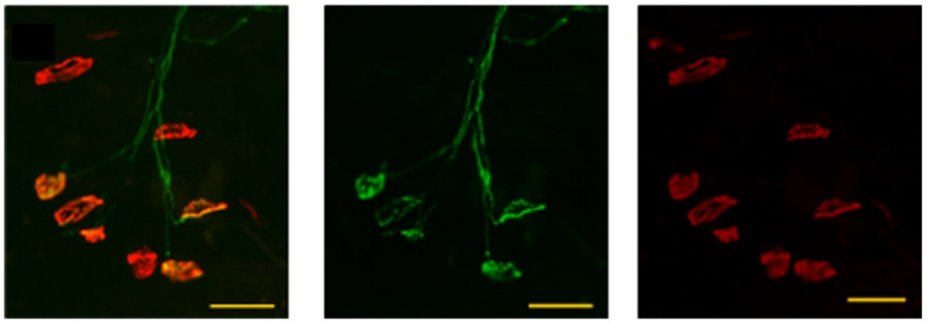
A


Nerve cut
CD
TTX block: Wlds/Wlds
No treatment: Wlds/Wlds
5d
Morphological analysis

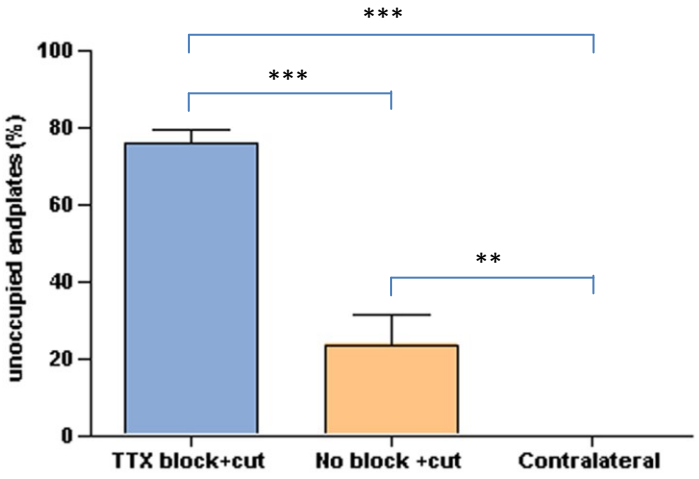
B



C



D


***
***
**
100
80
60
40
20
0
unoccupied endplates (%)
TTX block+cut
No block +cut
Contralateral

3.3.5 TTX block does not affect degeneration of the axon in *Wld*^{s} mice

The confocal microendoscope (Cell Vizio) allows repeated visualization of degenerating endplates and axons *in vivo*. We initially validated the technique using YFP expressing C57 and *Wld*^{s} mice, and confirmed that at 3 days post sciatic nerve axotomy, the difference in degeneration rates from WT mice could be clearly detected using this technique. It was also possible to repeatedly visualise a particular group of endplates and monitor their degeneration over a few days (figure 3.7). These results have been published (Wong et al., 2009).

Using *Wld*^{s} and C57 mice expressing YFP in their axons we were able to study the rate of axonal degeneration over time following nerve block and cut. Figure 3.8 shows the difference in degeneration rate between WT and *Wld*^{s} axons. However, there was no discernible difference between the level of axonal degeneration at any time point in *Wld*^{s} mice after tibial nerve axotomy and those preconditioned with paralysis, despite a clear difference in the level of synaptic degeneration at all time points after axotomy.

3.3.6 Paralysis hastens degeneration in WT mice

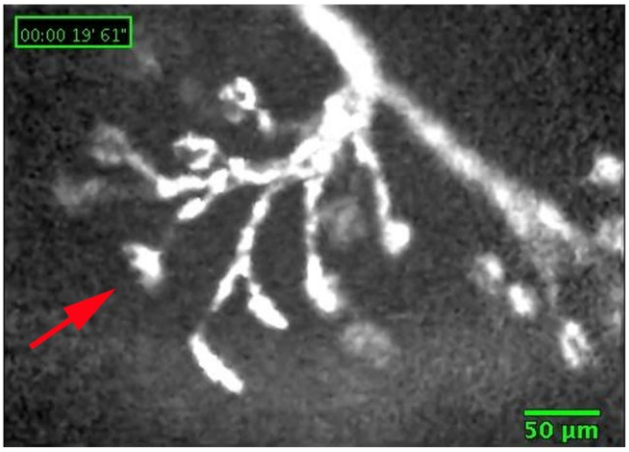
The effect of inactivity on synaptic degeneration is difficult to detect in WT mice, because the process is normally so rapid, and complete within 15-36h (Slater, 1966, Gillingwater et al., 2002). However, I found that one week of TTX paralysis also significantly increased the level of synaptic degeneration in WT animals, observed at 12hrs post-axotomy. A mean of 61.67±17.56% fibres produced an evoked response to stimulation in the FDB of WT mice at 12hrs, compared to 6.25±3.75% in those previously exposed to TTX ($p<0.05$, unpaired t-test n=4 in each group, figure 3.9). When fibres giving MEPPs only upon stimulation were included in the analysis, this strengthened the difference between the two, with 85.00±8.44% fibres responding to stimulation in controls, and 37.50±6.29% in TTX pretreated muscles 12hours post-axotomy ($p<0.01$, unpaired t-test, figure 3.9). These results suggest that inactivity

accelerates axotomy-induced degeneration, rather than affecting the mechanism activated by *Wld*^{s}.

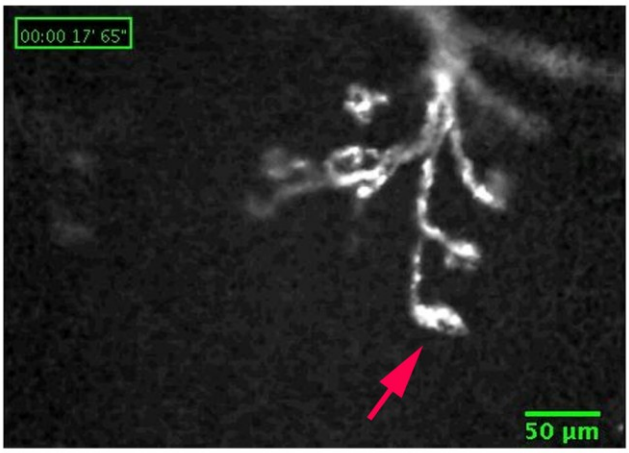
Figure 3.7: Confocal microendoscopy enabled longitudinal imaging of degenerating axons and synapses *in vivo*

A confocal microendoscope was used to monitor nerve degeneration *in vivo*. These images show degeneration of thy1.2-YFP16 Wld^s NMJs in the flexor digitorum longus muscle 3 (A), 4 (B) and 5 (C) days post sciatic nerve axotomy in the same mouse. The degeneration of individual nerve terminals can be clearly seen over the three days. Arrows indicate nerve terminals which degenerate over the 3 day imaging period (Wong *et al.* 2009).

A



B



C

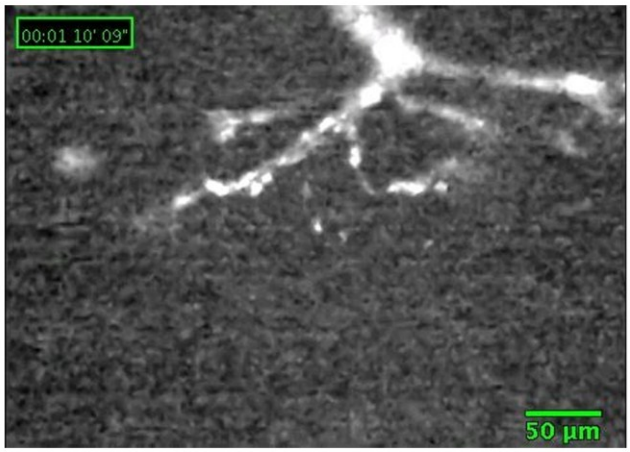


Figure 3.8: TTX block does not enhance nerve degeneration following axotomy

Repeated visualisation of the tibial nerve was conducted by confocal microendoscopy at the time of the surgery, then 3, 5 and 7 days post-axotomy. In an untreated YFP16 Bl6 mouse, progressive nerve degeneration can be clearly visualised over 7 days**,** with substantial fragmentation observable even by 5 days, yet there is no clearly discernible difference between degenerating axons in one YFP16 Wld^s and those that from a YFP16 Wld^s mouse that had only been subjected to TTX block for 7 days pre-axotomy. Scale bars = 50μm.

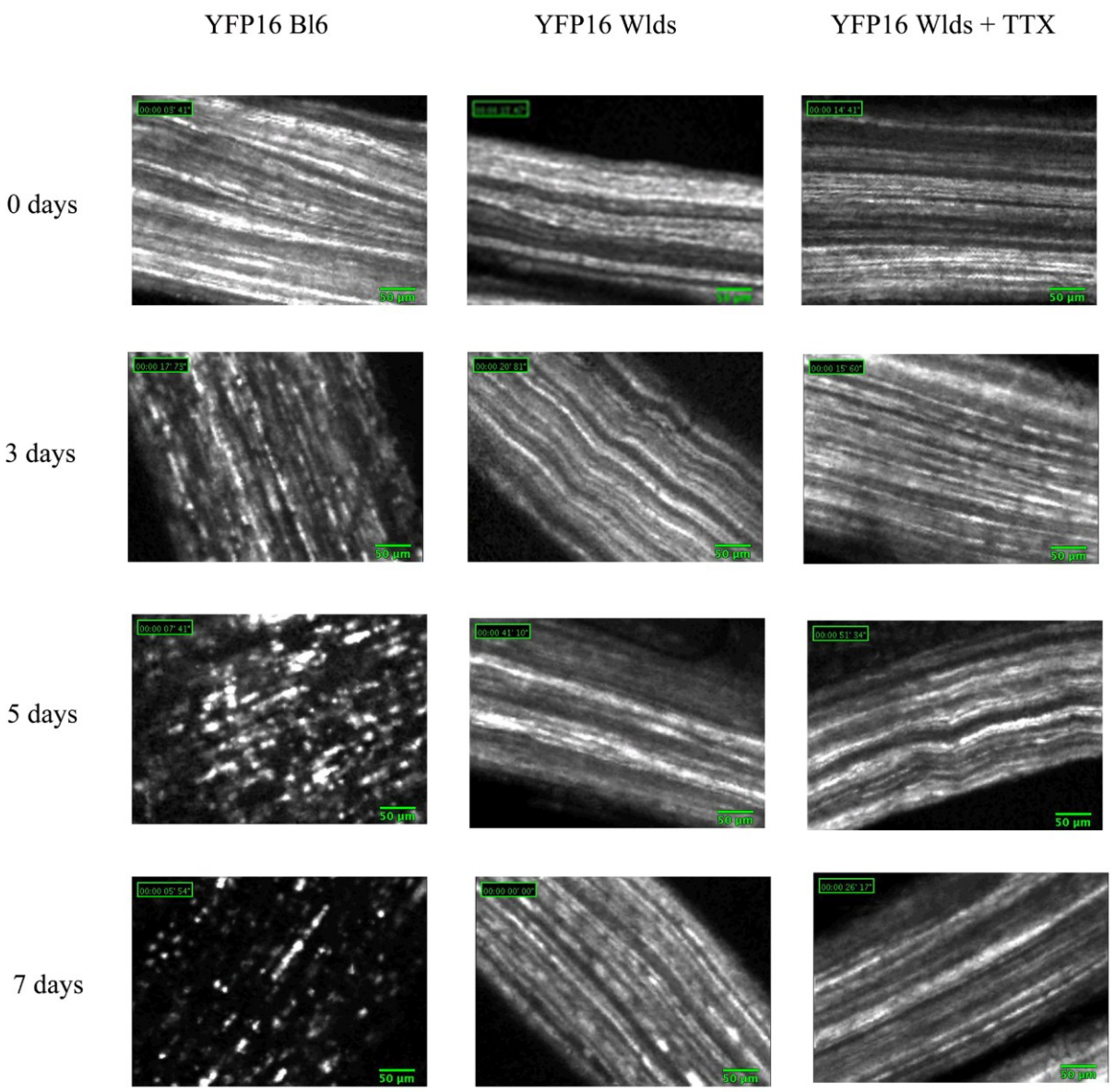
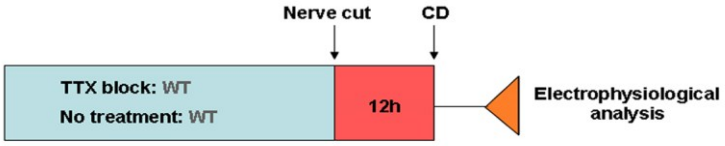

YFP16 Bl6
YFP16 Wlds
YFP16 Wlds + TTX
0 days
3 days
5 days
7 days

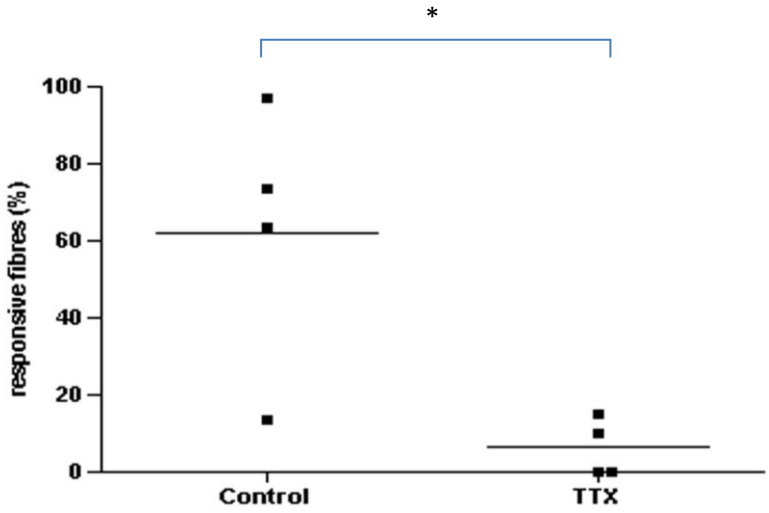
Figure 3.9: TTX block also enhanced the rate of synaptic degeneration in WT mice

A. After one week of TTX block, the tibial nerves of WT animals were cut. 12h later, animals were killed by cervical dislocation (CD) and electrophysiological analysis of the FDB muscles carried out. Results were compared to axotomised controls.
B. In WT animals, 12hr post-axotomy, a significant reduction could be seen in the number of responsive fibres giving EPPs in response to stimulation when axons were blocked with TTX for one week prior to nerve axotomy ($n=4$ per group, $p<0.05$, unpaired t-test). C. When fibres giving MEPPs only in response to stimulation were included in the analysis of responsive fibres, this strengthened the significance of this relationship ($p<0.01$, unpaired t-test).

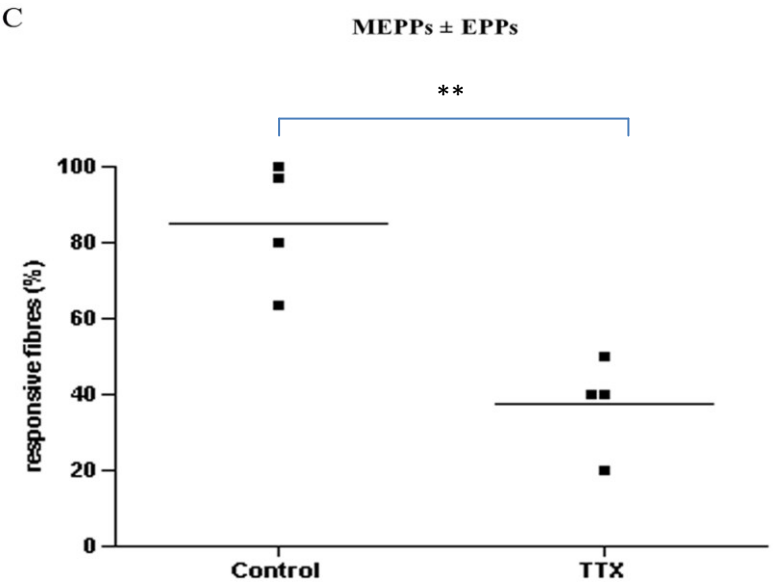
A


Nerve cut
CD
TTX block: WT
No treatment: WT
12h
Electrophysiological analysis

B


EPPs only
*
responsive fibres (%)
100
80
60
40
20
0
Control
TTX

C


MEPPs ± EPPs
**
responsive fibres (%)
100
80
60
40
20
0
Control
TTX

3.4 Discussion

The hypothesis that synaptic degeneration is modulated by activity was tested in this chapter by suppressing neuromuscular transmission with a TTX block of the nerve. It is clear from these data that TTX-induced paralysis accelerates neuromuscular synapse degeneration after axotomy in *Wld*^{s} mice and possibly in WT mice as well. However, TTX block itself did not cause any discernible damage to the axon or synapse prior to nerve axotomy.

3.4.1 Tetrodotoxin does not damage the NMJ but produces alterations in synaptic transmission

The efficacy of TTX-mediated block of neuromuscular transmission was demonstrated by observable paralysis in the hindlimb and the response to stimulation above and below the nerve in an anaesthetised animal. After 7 days neither the implant nor the TTX block caused any degeneration or damage, in agreement with previous studies (Lavoie et al., 1976, Martinov and Nja, 2005). However, an increased QC and adaptations to the amplitude, rise and decay time of the evoked response were noted.

An increase in QC has previously been reported following application of TTX (Snider and Harris, 1979, Tsujimoto and Kuno, 1988, Tsujimoto et al., 1990). It was first assumed that this adaptation of transmission was a result of disuse-induced sprouting or increase in terminal size, noted to be greater in paralysed muscles than controls. However, Tsujimoto and Kuno (1988) showed that blocking sprouting with CGRP does not affect the increase in quantal content. Further refuting the idea that the two are correlated, Tsujimoto *et al.* (1990) showed that the increase in transmitter release occurs at an earlier time point than enhanced sprouting or increased terminal size in disused muscles. In fact, an increased transmitter release was recorded from terminals that did not have sprouts or were not above average dimensions, and this increase in release was maintained even after removal of the TTX block. They

postulate that the change arises as a result of an increased number of active zones. In fact, it has been shown by others that a reduction in activity leads to an alteration in the number of vesicles in the ready releasable pool and in active zone size and protein expression in alternative models (DiAntonio et al., 1999; Murthy et al., 2001; Weyhersmuller et al., 2011).

A reduced RMP has also been reported in TTX blocked preparations (Mills et al., 1978, Bray et al., 1979, Betz et al., 1980, Gundersen, 1990), but the present data did not confirm this finding. Surprisingly, analysis of the properties of MEPPs revealed no change in amplitude, rise or decay time or in average frequency either. Snider and Harris (1979) reported an increase in MEPP frequency following paralysis, but no change in amplitude. However, giant MEPPs have been reported after a longer period of block but this was not coupled to an overall change in MEPP frequency (Gundersen, 1990). Gundersen also reports an increased input resistance. Other studies have also reported an increase in MEPP frequency (Tsujimoto et al., 1990). Similar to Snider and Harris (1979) I did not find an increase in MEPP amplitude, suggesting that the period of block was insufficient to induce such changes. Previous studies are also mainly conducted on rats, which may indicate some species differences in muscle and nerve terminal response to paralysis. Another difference between this and previous studies is the muscle used. Gundersen (1990), for example, used the extensor digitorum longus, a leg muscle, whereas I studied the FDB, a muscle involved in flexion of the foot. It is possible that these muscles would respond differently to different environmental modulators.

I therefore report an increase in quantal content without any accompanying change in MEPP amplitude. It would be interesting to know whether the fibre input resistance changes, and whether any the increased transmitter release is related to any presynaptic alterations, such as an increase in the number of active zones.

3.4.2 Tetrodotoxin enhances susceptibility to axotomy-induced synaptic degeneration

Although these results differ in some respects to previous studies, it is clear that one week of TTX block does induce functional adaptations in the FDB. The enhanced degeneration compared to WT observed in TTX-blocked preparations can be assumed to be a result of the TTX block itself, as mice treated with saline or with no previous surgery prior to axotomy showed less degeneration. In *Wld*^{s} mice the hastened synaptic degeneration was observable in both the functional response in the FDB and the number of innervated endplates in the lumbricals. It was also consistent over time, as a greater level of degeneration was noted at 3, 5 and 7 days post axotomy in comparison to controls. In WT mice, the degeneration response was studied at a much earlier time point as in WT synapses degenerate within 24h (Slater, 1966, Miledi and Slater, 1970). However, enhanced degeneration was noted in these animals 12h post axotomy. It therefore seems unlikely that TTX enhances the rate of synaptic degeneration by interfering with the protective mechanisms conferred by *Wld*^{s}.

I also applied a relatively novel technique for monitoring synaptic degeneration, the confocal microendoscope. I showed that it can be successfully used to monitor synaptic degeneration *in vivo*, and to repeatedly visualise individual groups of endplates and track their degeneration over time in *Wld*^{s} and WT mice. I also showed the degeneration of axons over time.

Interestingly, in the TTX blocked animals, the enhanced degeneration appears to be specific to the synapse as repeated visualisation of the axon 3, 5 and 7 days post axotomy using the confocal microendoscope revealed no signs of enhanced degeneration at the level of the axon. Thus one week of TTX application results in a synapse specific enhancement of degeneration.

It is known that synapses degenerate more rapidly than axons in WT mice, and in *Wld*^{S} mice the protective gene is less effective at protecting the synapse than the axon (Slater, 1966, Gillingwater and Ribchester, 2001). In heterozygous *Wld*^{S} mice the gene confers no synaptic protection whereas axonal degeneration remains delayed

(Wong et al., 2009), and as *Wld*^{S} mice age the synaptic, but not axonal, protection decays (Gillingwater et al., 2002). Thus it is clear that the mechanisms of synaptic degeneration differ from that of the axon and cell body, with degeneration occurring in a compartmentalized manner (Gillingwater and Ribchester, 2001). Therefore, in both animal models, and presumably by the same mechanism, TTX interferes with the timing of neuromuscular synapse degeneration by interfering with a process that specifically affects the synapse. The mechanism underlying the compartmentalisation of Wallerian degeneration and hence how the synapse degenerates first remains unknown. However, results from other studies may provide some insight. It has been suggested that axotomy interrupts the supply of trophic substances to the nerve terminal, which leads, or contributes, to their degeneration. TTX sequesters all but spontaneous transmitter release, which may interfere with the supply of such substances to the postsynaptic region and therefore cause an enhanced susceptibility to degeneration. It might be of interest to determine whether stimulation at the same time as TTX block prevents the enhanced degeneration rate.

Although several *in vivo* studies have shown that TTX treatment does not lead to degeneration without axotomy, some culture studies have suggested that TTX alone can be toxic. Although focused on the CNS, it is worth considering the findings of a recent paper (Schonfeld-Dado and Segal, 2009), which noted that culturing rat pup brains for 7 days in the presence of TTX resulted in degeneration that was apoptotic in nature. TTX was found to induce p53 and BAX, two genes involved in the process of apoptosis, prior to cell death. Thus it seems that TTX in culture activates an apoptotic cell death process. In fact the same group has shown that treatment with TTX, even after the toxin is removed from the culture media while the neurons still appear healthy, is enough to result in eventual neuronal death (Schonfeld-Dado et al., 2009). This seemed to be caused by an increase in intracellular calcium. They hypothesise that in blocked preparations the remaining spontaneous activity is enough to provoke apoptosis via a steady increase of intracellular Ca^{2+}. In fact, in an earlier paper (Fishbein and Segal, 2007) the same group found that TTX treated cortical neurons exhibit very large spontaneous MEPSPs and degenerate rapidly. However, when the MEPSPs are blocked the neurons are protected from

degeneration. They showed that TTX induced a slow elevation of Ca^{2+}, which Schonfeld-Dado *et al.* (2009b*)* then extended to show that in fact Ca^{2+} levels fail to return to normal regardless of TTX removal. The findings of Schonfeld-Dado *et al.* (2009b) also indicate the involvement of calpain, as TTX treated cultures showed higher levels of breakdown products of calpain, and blocking calpain activity enhanced protection in neurons from TTX-mediated toxicity. They also report mitochondrial damage and the activation of caspase-3 and PUMA, which are involved in apoptosis. Similar effects of TTX have been found in other culture studies (Heck et al., 2008).

A potential rise in calcium concentrations was not examined. It could be possible that application of TTX *in vivo* at concentrations low enough not to cause systemic toxicity do over time lead to the onset of apoptotic cell death, but this is certainly not noted in previous studies with up to 5 weeks of nerve block (Pasino et al., 1996).

It is unlikely that the enhanced degeneration rate is a result of a block of axonal transport, as TTX application has been proven to not affect this process (Lavoie et al., 1976, Pestronk et al., 1976a, Czeh et al., 1978). However, TTX has been reported to produce similar changes *in vivo* to those produced by axotomy (Cangiano et al., 1977, Czeh et al., 1978, Prakash et al., 1999). In fact, chronic TTX block has been shown to induce similar adaptations to those caused by denervation in enzymatic activity, and the structure and function of the NMJ. The effect of TTX paralysis compared to sciatic nerve axotomy on enzymatic activity in the spinal cord has been studied (Carr et al., 1998). Though the effects of axotomy were greater, 7 and 14 days of TTX paralysis resulted in similar changes. TTX paralysis of the sciatic nerve for 6 days also results in alterations in gene expression comparable to those observed after sciatic nerve cut, including calpain-3, which is involved in apoptotic pathways (Tang et al., 2000). Muscle protein content has also been shown to decline in TTX treated muscles to the same extent as in denervated muscle. This was shown after 21 days of TTX paralysis, a longer time point than this study was extended to. Of particular interest with regards to this study though, is that four days after axotomy in rats which had TTX applied to the sciatic nerve for 7 days previously, the level of

resistance to TTX typically noted in axotomised preparations was increased beyond the level seen in preparations only axotomised (Cangiano et al., 1977).

Muscle fibre atrophy has also been confirmed to occur in response to TTX paralysis to the same extent as that caused by denervation (Bray et al., 1979, Labovitz et al., 1984, Buffelli et al., 1997). TTX has also been shown to induce an increase in AChR expression, though this is less than that caused by denervation (Lavoie et al., 1976, Pestronk et al., 1976a). It may be possible that some factors aren't affected as much by paralysis as they are by denervation as the maintenance of spontaneous transmitter release during TTX application maintains some communication with the postsynaptic region.

Thus pharmacological removal of activity leads to similar changes to physical removal of activity, but it seems that without the interruption of axonal transport, degeneration does not occur. It may be that paralysis makes the system more susceptible to insult (Cangiano, 1985), and perhaps particularly the synapse which already exhibits a weaker level of protection. In terms of ACh sensitivity and resistance to TTX, coupling TTX paralysis to partial denervation is as effective as denervation itself and much more effective that partial denervation or paralysis alone (Cangiano et al., 1984b). Therefore if functional denervation results in similar adaptations to physical denervation, these may render the system incapable of coping with further insult. The subsequent removal of axon transport by tibial nerve cut in this study may therefore result in enhanced degeneration in a system that has already been sensitised to injury.

In consideration of this theory, it may be of interest to extend our findings to an alternative model, and determine whether inactivity enhances synaptic susceptibility to other forms of injury. One such example could be to study the response to hypoxia, which occurs due to a lack of oxygen supply and causes nerve terminal degeneration, but mechanistically distinct from WD (Baxter et al., 2008).

3.4.3 Summary

The present results were surprising, partly because previous studies show that motor nerve innervation is preserved by paralysis of polyneuronally innervated synapses (Thompson et al., 1979, Brown et al., 1982, Costanzo et al., 2000). By contrast, I have shown here that TTX induces a faster rate of axotomy-induced synaptic degeneration in both *Wld*^{s} and WT mice, not a slower one. The mechanism for this increase in degeneration or the loss of protection by Wld^{s} is unclear. It may be of interest firstly to test whether TTX does in fact lead to a greater susceptibility to other forms of insult, perhaps testing its effects on the response to hypoxia in comparison to nerve cut.

These results also indicate the importance of studying the opposite paradigm – the effects of enhanced activity on synaptic degeneration. Stimulation can reverse the adaptations to denervation and paralysis (Czeh et al., 1978, Akaaboune et al., 1999), showing the importance of neuronal and muscle activity in maintaining normal function, and indicating that neuronal activity can rescue the apparently detrimental effects of paralysis. I address this possibility in the next chapter

In order to elucidate these mechanisms, it may prove useful to extend this work to an ex vivo preparation. Ex vivo muscle can be easily accessed and manipulated by techniques such as drug application and electrical stimulation. In this case the work will be extended to remove the FDB nerve-muscle preparation after one week of nerve block *in vivo* and monitor the degeneration of the FDB *ex vivo*. Should the enhanced degeneration be replicated in this manner, it will be possible to test methods of interfering with this process, such as by interfering with the NAD cycle and thereby evaluate potential mechanisms. I address this possibility in chapter 5.

4. Does voluntary activity mitigate synaptic degeneration in *Wld*^{S} mice?

4. Does voluntary activity mitigate synaptic degeneration in *Wld*^{S} mice?

4.1 Background

NMJs are remodelled by external stimuli. Both neonatal and adult NMJs have been shown to display plasticity (structural and functional alterations) in response to altered activity levels. In the Introduction (Chapter 1) I referred to debate over the role of exercise in neurodegenerative disorders and discussed its role in modulating the properties of the muscle. In adult animals, exercise alters neuromuscular transmission (Dorlochter et al., 1991, Fahim, 1997) and also evokes plasticity in relation to the structure of the NMJ (Tomas et al., 1989, Fahim, 1997)– for more references see Chapter 1). In terms of neurodegenerative disorders, some studies report that exercise has no effect on disease progression in models such as the SOD1 mutant (Bruestle et al., 2009) but this conflicts with those that report enhanced survival or delayed onset (Veldink et al., 2003, Deforges et al., 2009, Carreras et al., 2010).

There are also reports that electrical stimulation can reverse the effects on neuromuscular morphology seen after denervation (Lomo and Rosenthal, 1972, Wilson and Deschenes, 2005, Gordon et al., 2008, Tomori et al., 2010), and the recent popular idea of 'use it or lose it' which implies that more active use of neurons and muscles enhances their strength and survival (Shors et al., 2011).

Although increased neuromuscular activity induces a wide range of changes, its potential to modulate synaptic degeneration has not been fully investigated. Given the evidence for modulation of intact neuromuscular synapse by activity, it seems reasonable to hypothesise that synapse degeneration is modulated by activity. However, one of the major problems confronting a test of this hypothesis is the variability in age of symptom onset and lifespan between disease models. This variation makes it difficult to draw definitive conclusions: any effects can easily be buried in pathological 'noise' (Scott et al., 2008). I therefore studied the effect of exercise on a more controllable and predictable model of synapse degeneration after axotomy in the *Wld*^{S} mouse.

In this first set of experiments my primary aim was to measure the effects of voluntary activity on the time course of axotomy-induced synaptic degeneration. By enriching the environment of the mice via the provision running wheels, I allowed both homozygous and heterozygous *Wld*^{s} mice two or four weeks of voluntary exercise prior to sciatic nerve axotomy. A few days after nerve cut, the level of synaptic degeneration was measured by electrophysiology of the flexor digitorum brevis (FDB) muscle. Where possible, mice expressing YFP in their axons were used to measure degeneration by studying NMJ morphology in the lumbrical muscles. Combining the YFP labelled nerve terminals with TRITC α-bungarotoxin stain of the endplate allows visualisation, and counting, of denervated endplates by fluorescence microscopy (see methods).

I employed a voluntary running paradigm as it has been reported to produce less indicators of stress in animals than paradigms involving forced exercise (Rosenheimer, 1985, Ke et al., 2011). It also requires little human intervention, and allows mice to follow their natural circadian pattern, with more activity at night (Rodnick et al., 1989). A voluntary running protocol also avoided stressors as stimuli for exercise, such as electrical shocking. It has previously been demonstrated that stress can also induce adaptations at the NMJ (Rosenheimer, 1985). Unforced running should avoid this. However, it is difficult to determine the exact level of activity sustained by the animal in a voluntary running situation. Therefore, it can be argued that this paradigm represents environmental enrichment, rather than a method of increasing exercise per se.

Briefly, the results suggest that voluntary wheel running has no mitigating effect on neuromuscular synaptic degeneration. Voluntary wheel running had no effect on degeneration in either heterozygous or homozygous *Wld*^{s} mice. It was therefore natural to enquire whether the exercise protocol had any other physiological effect on the properties of the muscle. I measured properties of EPPs and their response to repetitive stimulation, their sensitivity to $Ca^{2+/}Mg^{2+}$, and miniature endplate potential parameters within exercised muscles compared to controls. I also measured muscle

fibre diameter and endplate area and length, as these have previously been reported to change in exercised animals (Tomas et al., 1989, Waerhaug et al., 1992, Ishihara et al., 1998, Ishihara et al., 2002, Pellegrino et al., 2005). To my knowledge, no study has determined the effect of exercise on the characteristics of the FDB muscle. I therefore aimed not only to test the hypothesis that synaptic degeneration is activity dependent, but also to produce novel data on the effects of voluntary exercise on physiological and morphological properties of the FDB muscle.

In response to four weeks of voluntary wheel running, FDB muscles show an increase in MEPP amplitude, coupled with a decreased quantal content in response to a train of stimuli. No alterations were found in fibre diameter or endplate measurements.

4.2 Methods

4.2.1 Experimental design

Age matched, litter matched *Wld*^{s} homozygous or heterozygous mice of both sexes were individually housed. The experimental groups had free access to an inclined running wheel (figure 4.1) connected to a revolution counter calibrated to account for the circumference of the wheel. Distance run was recorded daily.
Control animals were individually housed with no environmental enrichment. Originally, control mice were provided with disabled wheels, but these were removed after noting that the mice were running around on the top of the immobilized wheel.

4.2.2 Monitoring circadian rhythm

The open field activity patterns of male Wld^{S} and C57 Bl6 mice (Harlan, UK; N=8 per group) were monitored using an in-house modified passive infrared (PIR) system build and used by Professor A.J. Harmar and colleagues. Animals were housed individually and exposed to a 12h light-dark cycle for 10 days. Activity readouts were measured in arbitrary units using Clocklab Software (Actimetrics). A further group of *Wld*^{s} mice were provided with voluntary access to a running wheel in order to establish whether running activity followed the normal circadian pattern of the mice (N=8). Activity was measured in 6 minute bins for each mouse. The recording and analysis of this section was performed by John Sheward (Queens Medical Research Institute, Edinburgh).

4.2.3 Homozygous Wld^{s} mice

Mice had access to a wheel for two weeks (N=10), or four weeks (N=7). Following the voluntary running period, mice were anaesthetised with isoflurane (Merial Animal Health limited, UK) and an incision made on the right-hindlimb (RHS) at the level of the sciatic notch. The sciatic nerve was exposed by blunt dissection,

transected and a small section removed. Five days later electrophysiological assessment of the FDB was performed (see main methods, Chapter 2, for EPP recording) and compared to controls for each group.

4.2.4 Heterozygous Wld^s mice

Heterozygotes were also provided with a running wheel for two (N=6) or four (N=4) weeks. In this case, the level of synapse degeneration was measured 3 days post-axotomy, since heterozygous mice show more rapid synaptic degeneration than homozygotes. Results were compared to controls with no wheel-running (N=8). In some animals the lefthand side (LHS) was used as a contralateral (uncut) control.
Thy1.2 YFP16 expressing mice were used to enable visualisation of axons and terminals. The lumbrical muscles were dissected and stained with TRITC-α -BTX (see main methods) to determine morphologically whether any innervated fibres could be detected in exercised mice 3 days post sciatic nerve axotomy.
In a subset of mice (N=4), ~18g total weight was added to the wheels to increase the load, and therefore the energy output required to turn the wheel.

4.2.5 Physiological Analysis of Synaptic Transmission

4.2.5.1 Train of four and MEPP analysis

Train-of-four is a technique used by clinicians to monitor neuromuscular blockade, allowing monitoring of facilitation or depression of the response to stimuli (Strange et al., 1997). In this instance, train-of-four stimulation was used to further investigate the effects of activity, with the aim of testing whether voluntary wheel running affects the facilitation or depression response seen in the FDB.
Wld^s mice were provided with running wheels (N=5) or housed in cages with no such environmental enrichment (N=4) for four weeks. Animals were then killed by cervical dislocation. Isolated FDB nerve muscle preparations were dissected and

subjected to electrophysiological analysis. Initially, four stimuli with a frequency of 10Hz were applied, and repeated for 30 cycles with a delay of 1s between each train of four. This was done in 30 fibres from each muscles, and also at 30 and 50Hz. For each EPP in the train, WinWCP software (Dr J Dempster, University of Strathclyde, UK) was used to calculate the quantal content and peak amplitude of the response. Miniature endplate potentials were also analysed (see Chapter 2 for methods).

4.2.5.2 Effect of $Ca^{2+/}Mg^{2+}$ ratio on synaptic transmission in 'exercised' mice

Homozygous *Wld*^{s} mice were provided with an inclined running wheel or given no environmental enrichment for four weeks (N= 5 mice, n=5 muscles for each group). To investigate the Mg^{2+} sensitivity of the exercised FDB in comparison to controls with no environmental enrichment, muscles were bathed in MPS in which the $CaCl_2$ concentration was reduced from 2mM to 1mM and $MgCl_2$ was increased to 2, 3, 4 and 5mM. Recordings were begun 15 minutes after changing the solution. The concentrations of other ions were unchanged. Quantal analysis, as discussed in chapter 1, was performed at each concentration for each muscle, using the failures method where necessary.

4.2.6 Analysis of morphological properties of the FDB muscle fibre and endplate

Endplate and muscle fibre sizes were measured in four exercised mice (n=7) and three control mice (n=6). The muscles were stained with TRITC α-bungarotoxin and then fixed in paraformaldehyde. They were then transferred to a 50% glycerol solution and groups of muscle fibres were teased apart from the tendons using mounted needles. The teased fibres were then transferred to 100% glycerol and individual muscle fibres teased and mounted on a slide. Measurements made using a fluorescence microscope (Olympus) connected to a camera (Hamamatsu Photonics)

and OpenLab software (Improvision) calibrated with both an eyepiece and a stage graticule.

4.3 Results

4.3.1 Circadian activity of Wld^s mice

Figure 4.1 shows individual open field activity traces for C57 Bl6 and *Wld*^{s} mice. Activity, measured in arbitrary units, was averaged and is shown in figure 4.2. *Wld*^{s} mice have a circadian rhythm that was not discernibly different from C57/Bl6.

Clocklab Software was used to analyse activity onset and duration, depicted by black spikes in the activity traces (figures 4.1, 4.2). Average activity was recorded in bins for each mouse, then the mean for each group taken to produce an average activity plot for both strains. It is clear that both strains of mice were at their most active during the dark cycle, between 7 am and 7 pm (figure 4.1). Activity peaks at the beginning of the dark cycle, falling off towards the end of the night. Mice provided with a running wheel were also active mainly in the dark hours, as shown by the representative individual activity trace in figure 4.2.

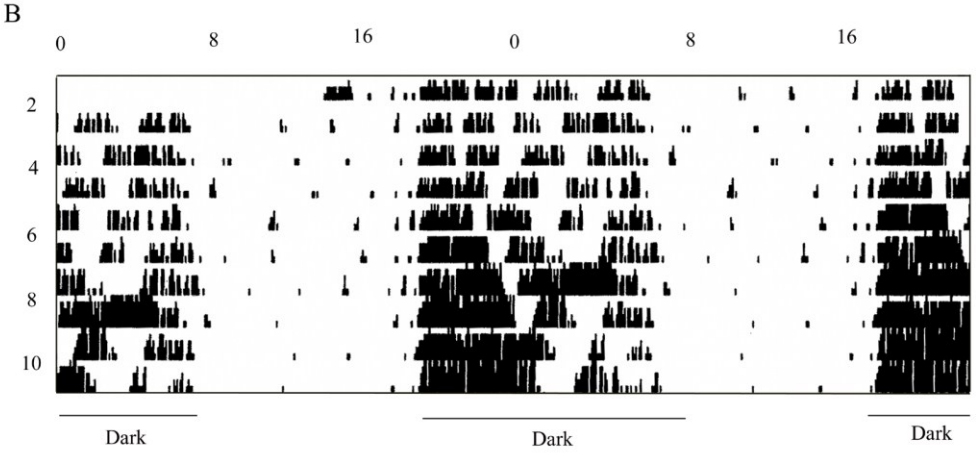
Animals ran an average of 6225±1759m/24hours, which is comparable with other voluntary wheel running studies (Allen et al., 2001, De Bono et al., 2006).

Figure 4.1: Circadian rhythms of WT and *Wld*^{s} mice can be monitored using a modified passive infrared system

A. A subset of mice were provided with running wheels (scale bar 5cm) to test whether increasing voluntary activity modulated synaptic degeneration.
Open field circadian activity was monitored in *Wld*^{s} (B) and WT (C) mice with no wheel access using a modified passive infrared system for 10 days, with 12 hour light-dark cycles.

A

B


0
8
16
0
8
16
2
4
6
8
10
Dark
Dark
Dark

C

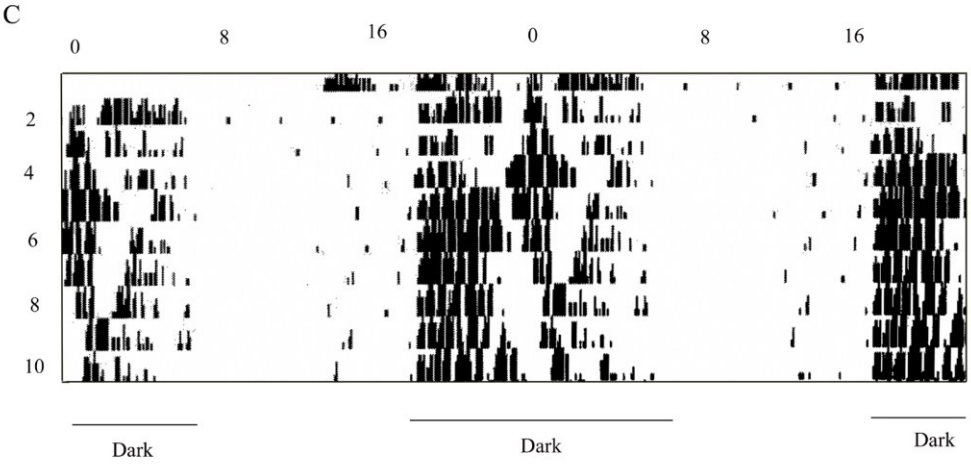
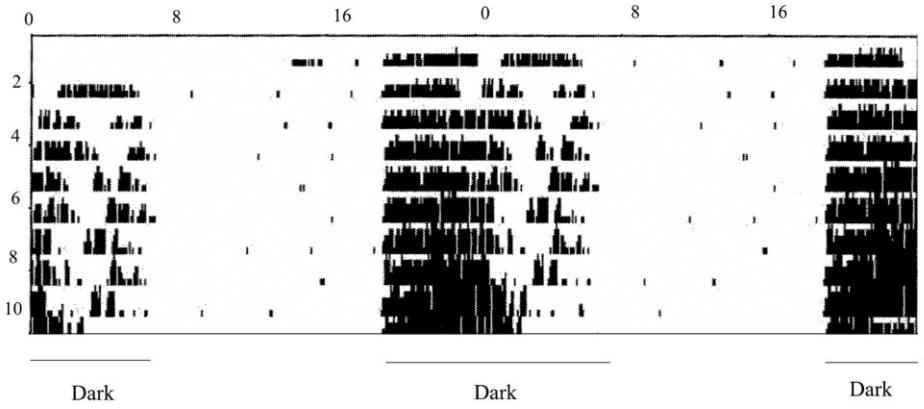

0
8
16
0
8
16
2
4
6
8
10
Dark
Dark
Dark

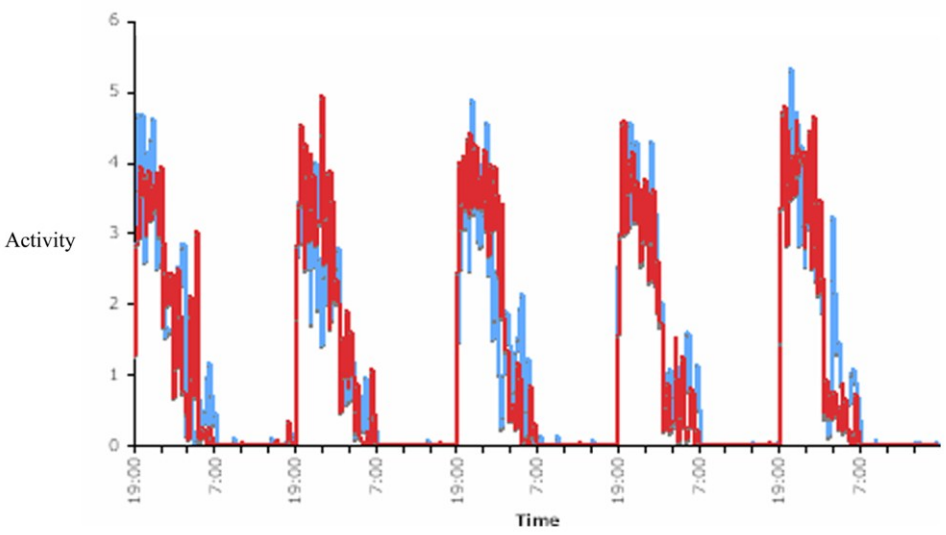
Figure 4.2: Running wheels do not alter the circadian rhythm of Wld^s mice

A. A representative circadian rhythm chart from a *Wld*^{s} mouse with voluntary access to a running wheel. During 12 hour light-dark periods over 10 days, it was observed that mice were mainly active during the hours of darkness, as with the open field recordings (figure 4.1). B. The average activity level was measured in arbitrary units, and averaged across time for each mouse (N=8 per group). When combined, there was no difference between *Wld*^{s} (blue) and C57/Bl6 (red) mice.

A


0
8
16
0
8
16
2
4
6
8
10
Dark
Dark
Dark

B


Activity
6
5
4
3
2
1
0
19:00
7:00
19:00
7:00
19:00
7:00
19:00
7:00
Time

4.3.2 Effect of exercise on synapse degeneration

4.3.2.1 Homozygous mice

Voluntary wheel running was used to investigate the effect of activity on synapse degeneration. The number of fibres 5 days post-axotomy (p-a) were scored as those capable of producing an evoked response, spontaneous activity only, and those with no response to stimulation. Comparison of only the number of remaining fibres 5 days p-a that gave an evoked response to stimulation showed that two weeks of voluntary wheel running did not affect the rate of degeneration in homozygous *Wld*^{s} muscles compared to axotomised *Wld*^{s} muscles with no wheel access (48.00±7.88% vs 54.45±11.44%, n=10, 6 respectively $p>0.05$, figure 4.3). However, both were significantly reduced compared to the number of responsive fibres in uncut control muscles (95.83±2.50%, n=4 $p<0.05$). Four weeks of voluntary exercise also did not alter the level of responsive fibres compared to controls 5 days p-a (19.43±5.57% n=7 vs. 32.67±5.10% n=5, $p>0.05$, figure 4.4), yet both groups were again significantly reduced compared to uncut muscles (90.69±3.79%, n=4 $p<0.01$). However, if fibres giving MEPPs only were also included in the analysis, four weeks of wheel access significantly reduced the number of innervated fibres 5 days p-a compared to controls 5 day p-a (27.24±6.73% n=7 vs. 52.00±5.54% n=5, $p<0.05$, figure 4.4). Both groups remained significantly reduced compared to uncut controls (96.61±1.96%, n=4, $p<0.01$). However, there remained no significant difference following 2 weeks access, with 59.67±8.81 responsive fibres in the exercised FDBs (n=10), and 76.67±6.89% fibres remaining in axotomised controls (n=6). Although the number of fibres remaining in exercised muscles remained significantly reduced compared to uncut controls (99.17±0.83% n=4, $p<0.05$), this analysis removed the significant reduction between axotomised and uncut controls ($p>0.05$, figure 4.3).
The number of active fibres remaining was not correlated to the distance run or the age of individual mice (Pearson r= -0.2313, p=0.29 figure 4.5).

Figure 4.3: Two weeks of voluntary running activity does not alter the level of synaptic degeneration 5 days post-axotomy in *Wld*^{S} FDB muscles

A. Following two weeks of voluntary running-wheel activity, mice underwent sciatic nerve axotomy. 5 days later, animals were killed by cervical dislocation and electrophysiological analysis of the axotomised FDB muscles carried out.
B. Measurement of the number of fibres giving an evoked response to stimulation 5 days post-axotomy revealed no significant difference between muscles from mice that had access to a running wheel for two weeks (n=10) and those that had not (n=6), though both were significantly reduced in comparison to controls (n=4, $p<0.05$). When including fibres giving EPPs and/or MEPPs in response to stimulation (C), there was no significant difference between exercised and control muscles 5 days post-axotomy, but whereas a significantly greater number of inputs remained degenerated in exercised muscles compared to uncut controls ($p<0.05$), the axotomised controls were no longer significantly different from the unoperated controls ($p>0.05$, one way ANOVA).

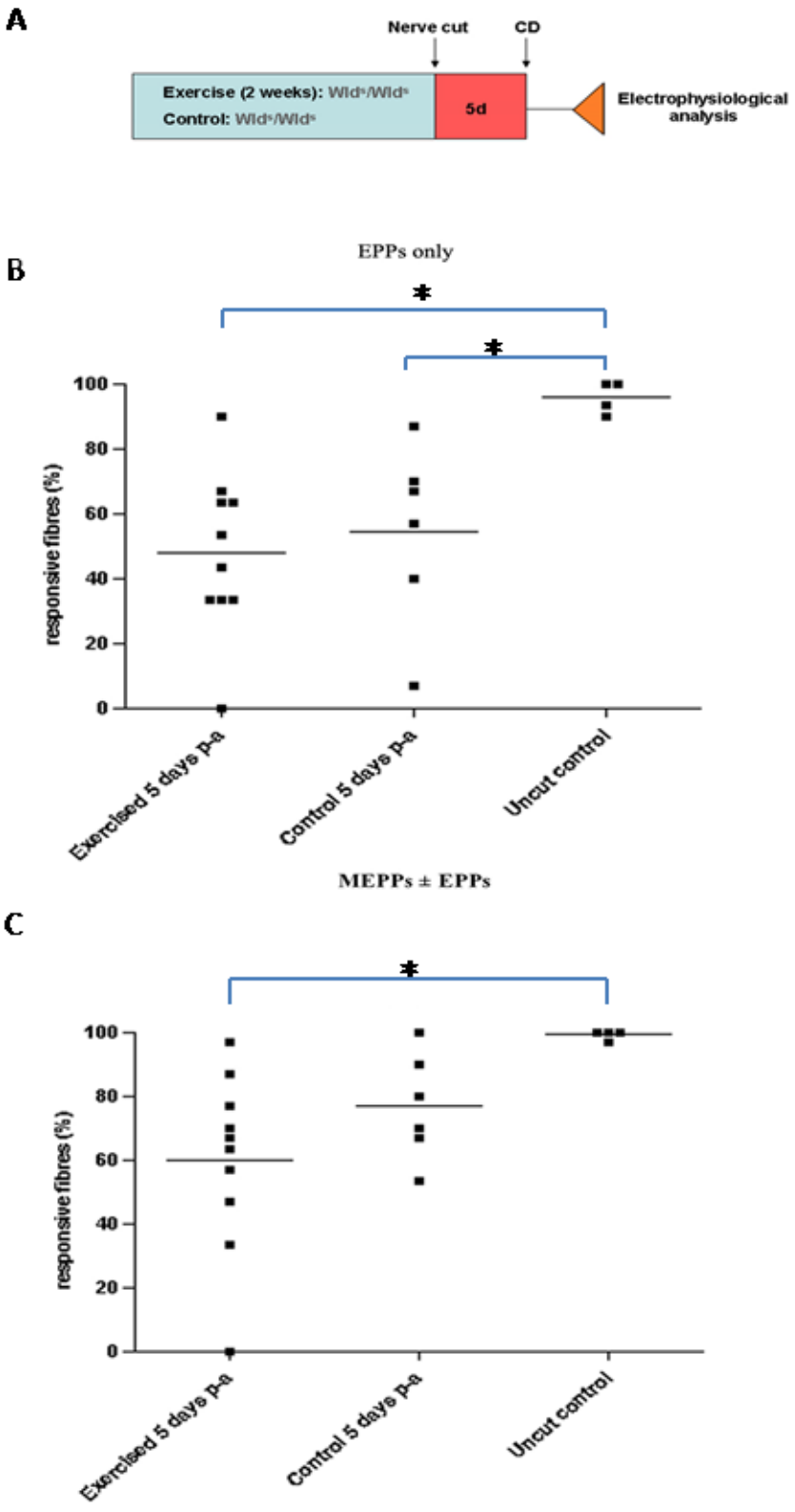
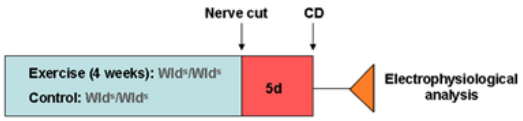

A
Nerve cut
CD
Exercise (2 weeks): Wlds/Wlds
Control: Wlds/Wlds
5d
Electrophysiological analysis
B
EPPs only
*
*
responsive fibres (%)
100
80
60
40
20
0
Exercised 5 days p-a
Control 5 days p-a
Uncut control
MEPPs ± EPPs
C
*
responsive fibres (%)
100
80
60
40
20
0
Exercised 5 days p-a
Control 5 days p-a
Uncut control

Figure 4.4: Four weeks of voluntary running activity produces only slight alterations in the level of neuromuscular degeneration observed 5 days post-axotomy in Wld^s mice

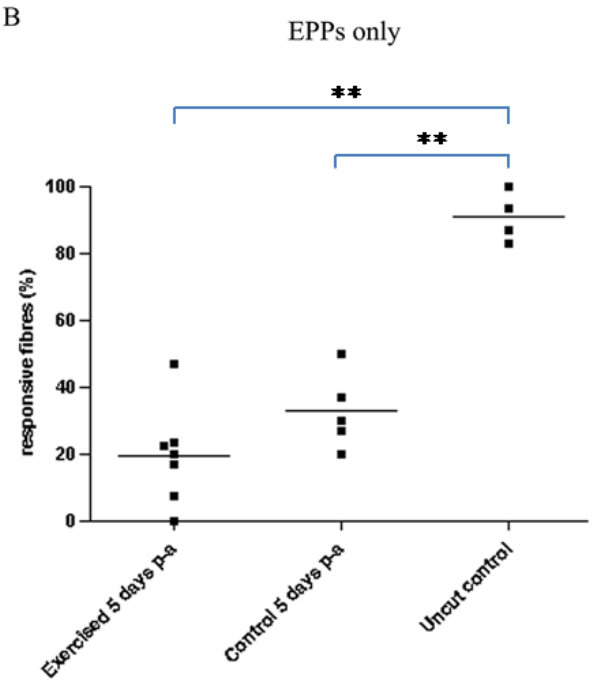
A. Following four weeks of voluntary running-wheel activity, mice underwent sciatic nerve axotomy. 5 days later, animals were killed by cervical dislocation and electrophysiological analysis of the axotomised FDB muscles carried out.

B. Four weeks of wheel access (n=7) did not significantly alter the number of fibres giving an evoked response 5 days post-axotomy compared to operated controls (n=7, 5 respectively, $p>0.05$), though both showed significantly less responsive fibres compared to unoperated controls (n=4, $p<0.01$). C. Analysis of fibres responding with EPPs and/or MEPPs, however, revealed a significant reduction in responsive fibres in exercised muscles compared to controls 5 days post-axotomy ($p<0.05$) and both groups has a significantly reduced number of responsive fibres compared to uncut controls ($p<0.05$, one way ANOVA).

A


Nerve cut
CD
Exercise (4 weeks): Wld^s/Wld^s
Control: Wld^s/Wld^s
5d
Electrophysiological analysis

B


EPPs only
**
**
responsive fibres (%)
100
80
60
40
20
0
Exercised 5 days p-a
Control 5 days p-a
Uncut control

C

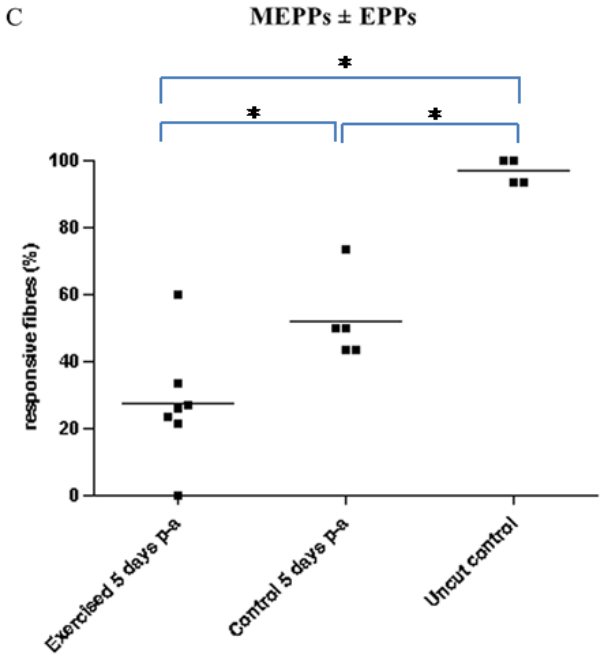
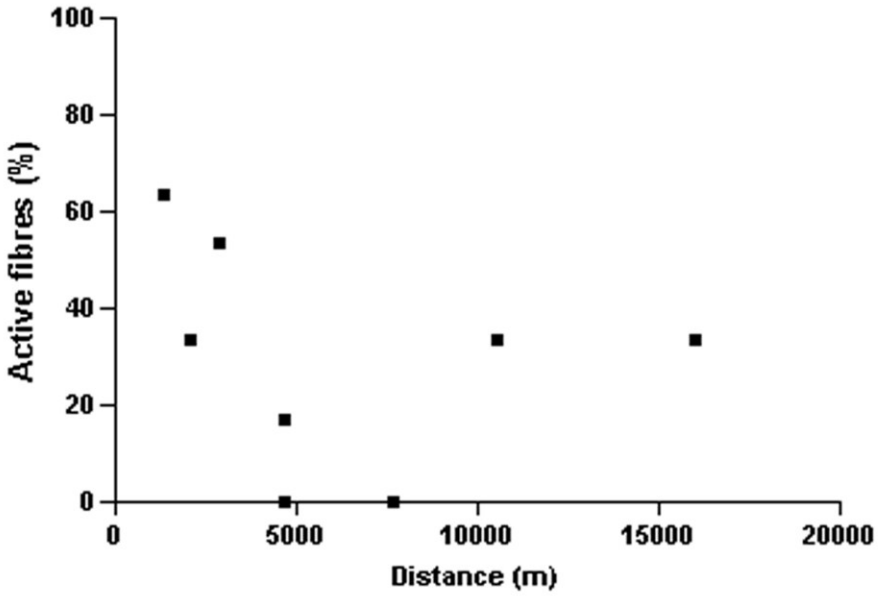

MEPPs ± EPPs
*
*
*
responsive fibres (%)
100
80
60
40
20
0
Exercised 5 days p-a
Control 5 days p-a
Uncut control

Figure 4.5: The number of fibres giving an evoked response to stimulation was not significantly correlated to the distance run over four weeks.

The total distance run was recorded for each mouse. Plotting this against the number of responsive fibres remaining 5 days post axotomy revealed no correlation (n=8, Pearson r = -0.2313, p=0.29).


100
80
60
40
20
0
Active fibres (%)
0
5000
10000
15000
20000
Distance (m)

4.3.2.2 Heterozygous mice

Four weeks of environmental enrichment through provision of running wheels also had no effect on the amount of synaptic degeneration in young heterozygous *Wld*^{s} muscles (n= 10 exercised, n=8 axotomised controls). By 3 days post-axotomy, all axotomised FDBs were unresponsive to stimulation compared to contralateral controls (98.15±0.807%, n=9, figure 4.6). This was reflected in the morphological studies of the lumbrical muscles from the same animals. Inspection of innervation in YFP16 mice established there were no remaining innervated endplates 3 days post axotomy (figure 4.6, 100% denervated in both groups).

It has been previously noted that increasing the load of running wheels has more significant effects in skeletal muscle mass and cross sectional area than unloaded wheel running (Ishihara et al., 1998, Ishihara et al., 2002, Konhilas et al., 2005). Thus, in n=4 heterozygous mice, extra load was added to the running wheel. However, this had no effect on synapse degeneration in heterozygous mice as no synapses remained active three days post-axotomy (n=4), whereas nearly 100% were active in the contralateral muscles (figure 4.6).

4.3.3 Train of four

Train-of-four stimulation was used to investigate the whether voluntary wheel running affects the facilitation or depression response seen in the FDB. At 10 and 30Hz, no significant effect was demonstrated in any of the parameters investigated (figure 4.7, 4.8). However, a stimulation frequency of 50Hz revealed a significantly reduced quantal content in mice with access to running wheels compared to controls (figure 4.9). Despite the reduced QC, peak amplitude was not significantly different from control values, although there was significant facilitation of peak amplitude in both groups (figure 4.10).

4.3.4 Response of exercised muscle to altering Ca^{2+}/Mg^{2+} concentration

Calcium and magnesium ions demonstrate a competitive action on transmitter release at the NMJ. Decreasing the available concentration of Ca^{2+} ions, and increasing Mg^{2+} ions reduces transmitter release, and as such QC is decreased (Del Castillo and Stark, 1952, Del Castillo and Engbaek, 1954, Del Castillo and Katz, 1954a, Dodge and Rahamimoff, 1967b). It is possible that the sensitivity to these changes in ion concentration is affected by external stimuli, such as exercise, that would alter transmitter release. Exercised mice showed no difference in sensitivity of neuromuscular transmission to Ca^{2+}/Mg^{2+}. Increasing Mg^{2+} produced a decrease in transmitter release based on measurements of quantal content of EPPs as transmitter release was decreased. In the exercised muscles, the decline in quantal content observed with increasing Mg^{2+} followed the same pattern as seen in the control mice suggest that the Mg^{2+} sensitivity of synaptic transmission remained unchanged after four weeks of voluntary wheel access (figure 4.11).

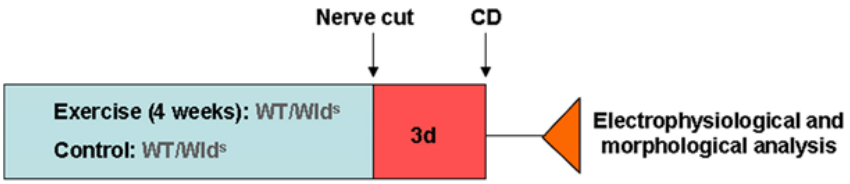
4.3.5 Miniature endplate potentials

Due to the relationship between MEPPs and EPPs, and the calculation of the QC, a decrease in quantal content without a change in EPP amplitude implies an alteration in MEPP amplitude (Del Castillo and Katz, 1954c, Liley, 1956b). Therefore in additional muscles exercised for four weeks (n= 6 runners, 5 controls), I analysed the properties of miniature endplate potentials. The mean MEPP amplitude was increased in mice with access to a running wheel compared to controls (1.445±0.185 vs 0.9309±0.050 mV; $p<0.05$, figure 4.12). The half-decay time was unaffected ($p>0.05$), along with MEPP frequency (37.26±14.23 n=6 vs 27.79±6.363 n=5, MEPPs min^{-1} figure 4.13).

Figure 4.6: Rapid synaptic degeneration in heterozygous *Wld*^{S} mice cannot be delayed by voluntary wheel running activity

A. Heterozygous *Wld*^{S} mice were provided with voluntary wheel access for four weeks. Following this, the sciatic nerve of each mouse was unilaterally denervated, and 3 days post axotomy the FDB and lumbrical muscles analysed electrophysiologically and morphologically, respectively. B. Electrophysiological analysis revealed no protective benefits of four weeks of voluntary running wheel activity, with or without added load, 3-days post axotomy in heterozygous *Wld*^{S} mice. This was confirmed by morphological analysis of the number of innervated endplates 3 days post axotomy in lumbrical muscles from these mice.

A



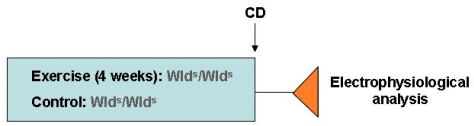
B

	Average evoked activity (%)	Average occupied endplates (%)
Operated leg		
control	0, 0, 0, 0	0, 0, 0, 0
2 weeks wheel access	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0
4 weeks wheel access	0, 0, 0, 0	0, 0, 0, 0
4 weeks wheel access + load	0, 0, 0, 0	0, 0, 0, 0
Unoperated leg		
control	96.67, 96.67, 100, 100	100, 100, 100, 100
2 weeks wheel access	100, 100, 100, 100, 100, 100	100, 100, 100, 100, 100, 100
4 weeks wheel access	93.33, 96.67, 100, 100	99, 99, 100, 100
4 weeks wheel access + load	96.67, 96.67, 100, 100	100, 100, 100, 100

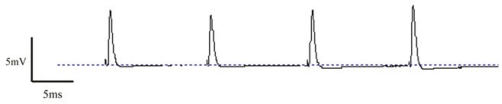
Figure 4.7: Trains of four at 10Hz do not reveal any adaptation as a result of voluntary wheel running

A. Following four weeks of voluntary running-wheel activity, animals were killed by cervical dislocation and electrophysiological analysis of FDB muscles carried out.
B. Thirty trains-of-four at 10Hz were applied to exercised (n=5) and control (n=4) FDB muscles. The properties of each EPP (peak 1, 2, 3 and 4 respectively) were averaged across the thirty trains. Results from mice with voluntary running wheel access for four weeks showed no difference in quantal content when compared to unexercised controls (C). Average EPP amplitude (D) and percentage change in EPP amplitude (E) was also unchanged in mice with access to running wheels compared to controls ($p>0.05$ for all, two-way ANOVA).

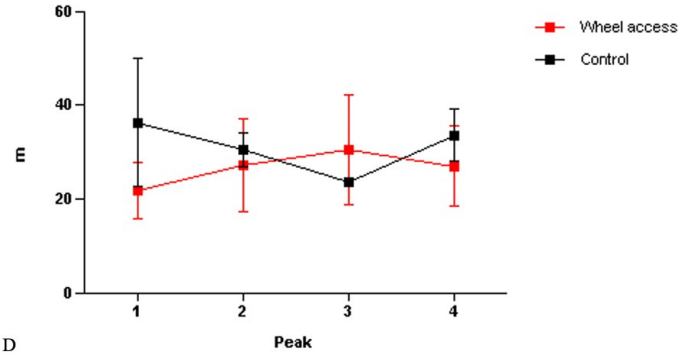
A


CD
Exercise (4 weeks): Wlds/Wlds
Control: Wlds/Wlds
Electrophysiological analysis

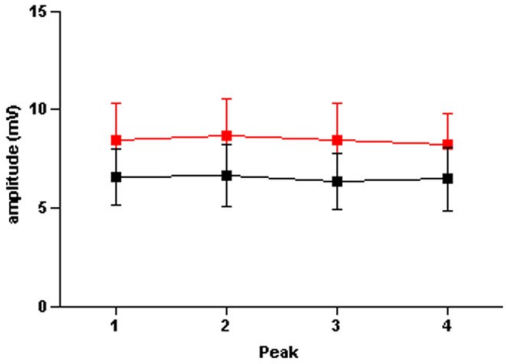
B


5mV
5ms

C


Wheel access
Control
m
Peak

D


amplitude (mV)
Peak

E

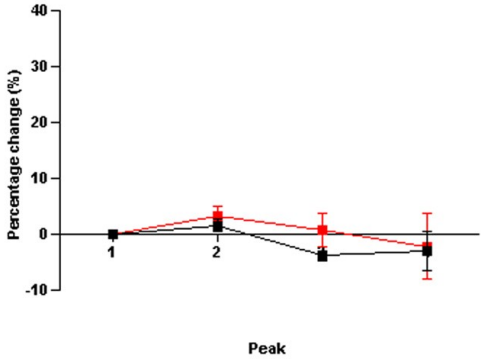

Percentage change (%)
Peak

Figure 4.8: Trains of four at 30Hz do not reveal any adaptation as a result of voluntary wheel running

A. Following four weeks of voluntary running-wheel activity, animals were killed by cervical dislocation and electrophysiological analysis of FDB muscles carried out.
B. Thirty trains-of-four at 30Hz were applied to exercised (n=5) and control (n=4) FDB muscles. The properties of each EPP (peak 1, 2, 3 and 4 respectively) were averaged across the thirty trains. Mean quantal content (C) EPP amplitude (D) and percentage change in EPP amplitude (E) were unchanged after four weeks of voluntary wheel running compared to controls ($p>0.05$ for all, two-way ANOVA).

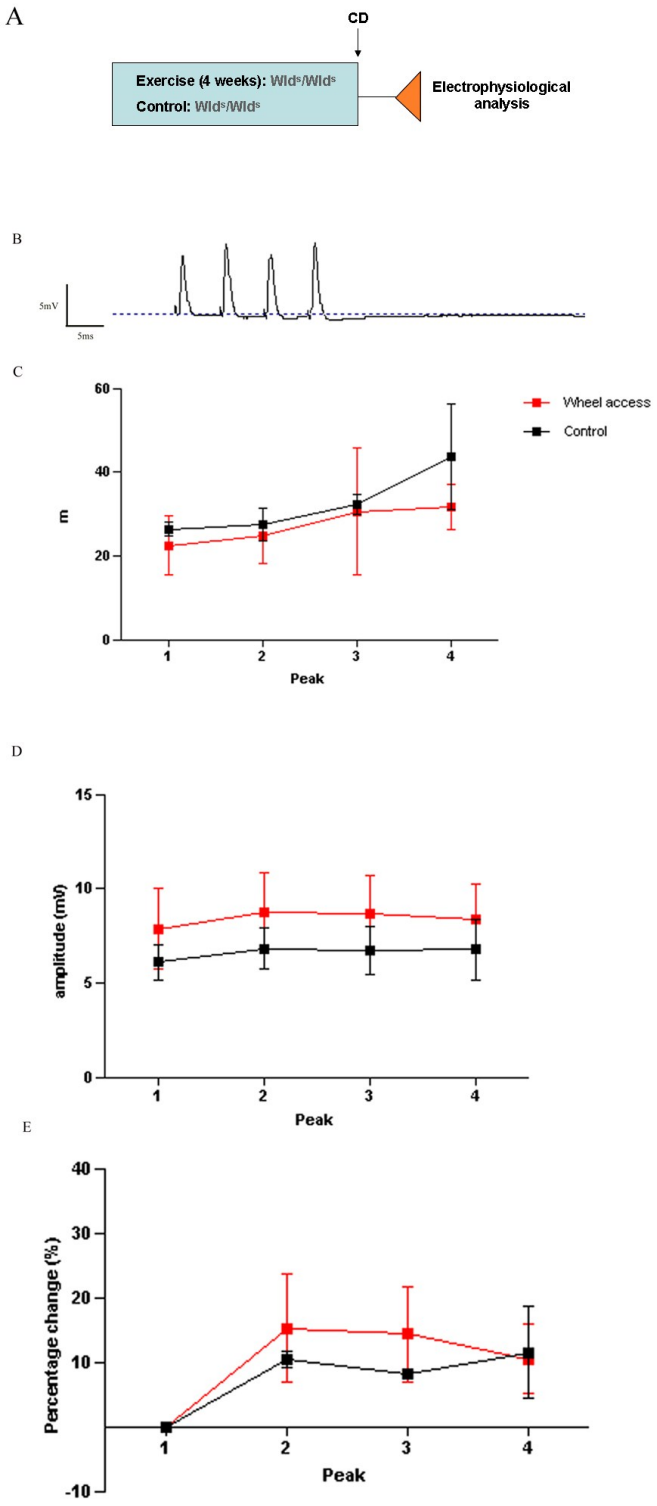
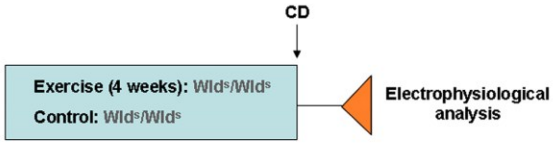

A
CD
Exercise (4 weeks): Wlds/Wlds
Control: Wlds/Wlds
Electrophysiological analysis
B
5mV
5ms
C
Wheel access
Control
m
Peak
D
amplitude (mV)
Peak
E
Percentage change (%)
Peak

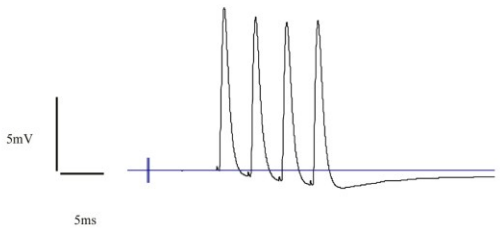
Figure 4.9: Trains of stimuli at 50Hz reveal a significant reduction in QC in exercised mice

Averaging the QC from each EPP in a train of four at 50Hz (A) across 30 trains revealed a significant reduction in QC in FDB muscles from mice exercised for four weeks (n=5) compared to controls with no wheel access (n=4, B, C, $p<0.05$, two-way ANOVA).

A



B



C

50Hz	Exercised			Control		
Peak number	Mean	SEM	n	Mean	SEM	n
1	26.55	5.87	5	35.44	14.13	4
2	28.94	7.82	5	41.63	6.01	4
3	29.86	6.92	5	41.01	3.68	4
4	27.47	5.76	5	42.79	7.91	4

D

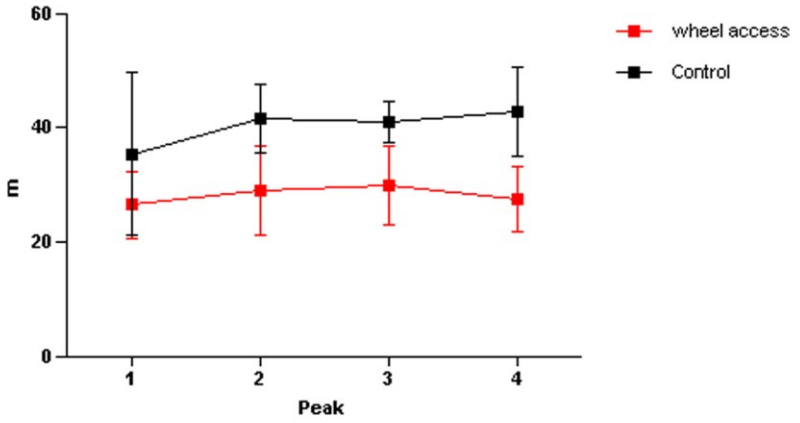
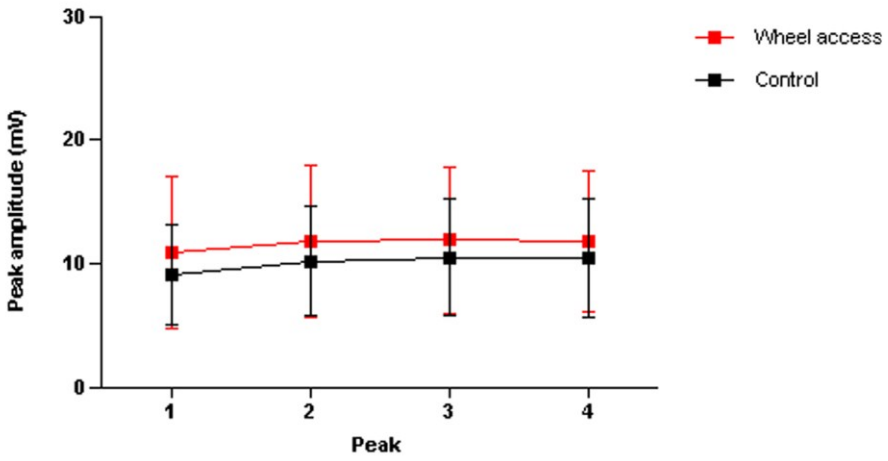


Figure 4.10: Trains of stimuli at 50Hz demonstrate no adaptation of EPP amplitude in exercised muscles

EPP amplitude was averaged for each EPP in a train of four at 50 Hz. No significant difference in EPP amplitude was noted in muscle fibres from mice with wheel access for four weeks compared to those with no wheel access (A, n=5, 4 respectively). There was also no significant difference in the percentage change in peak amplitude between groups, although there was a significant facilitation in both (B, $p<0.05$, two way ANOVA).

A


Wheel access
Control
Peak amplitude (mV)
30
20
10
0
1
2
3
4
Peak

B

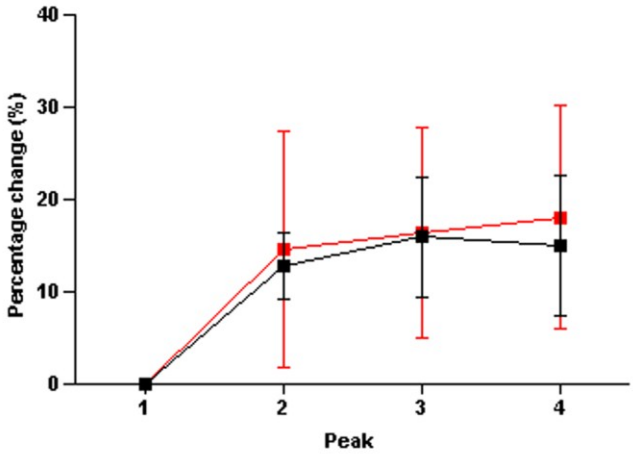
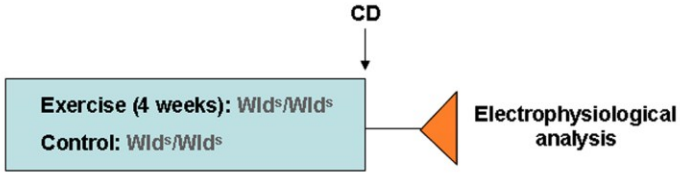

Percentage change (%)
40
30
20
10
0
1
2
3
4
Peak

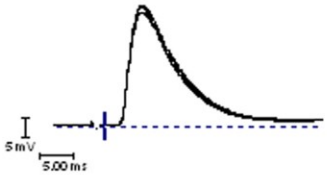
Figure 4.11: FDB muscle fibres from exercised mice show no alteration in ion sensitivity

A. Following four weeks of voluntary running-wheel activity, animals were killed by cervical dislocation and electrophysiological analysis of FDB muscles carried out.
B. In MPS with normal Mg^{2+}/Ca^{2+} concentration, muscle fibres respond with robust EPPs, whereas when $[Mg^{2+}]$ was increased up to 3mM and $[Ca^{2+}]$ reduced to 1mM, EPPs display stepwise fluctuations in amplitude ('quantal jumps') and failures of transmission (C), indicative of a reduced QC (D). The reduction in QC was no different between FDB muscles from mice with four weeks of wheel access (n=5) compared to controls (n=5).

A


CD
Exercise (4 weeks): Wlds/Wlds
Control: Wlds/Wlds
Electrophysiological analysis

B


2mM Ca
1mM Mg
5 mV
5.00 ms

C


1mM Ca
3mM Mg
5mV
500 ms

D

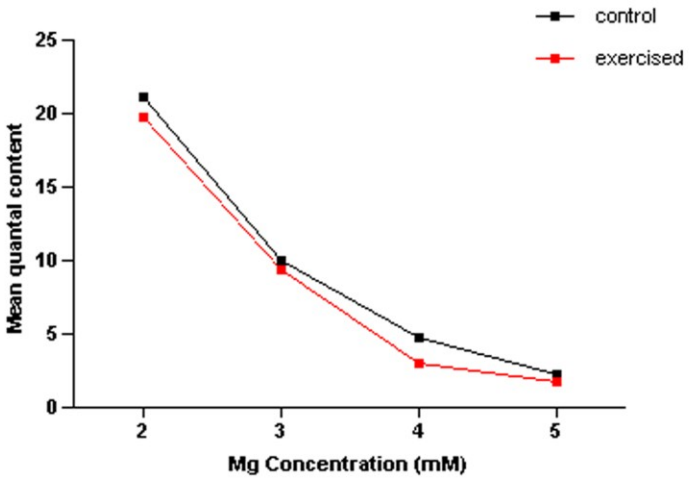

control
exercised
Mean quantal content
25
20
15
10
5
0
2
3
4
5
Mg Concentration (mM)

Figure 4.12: Muscle fibres from exercised mice show an adaptation in MEPP amplitude

A. Following four weeks of voluntary running-wheel activity, animals were killed by cervical dislocation and electrophysiological analysis of FDB muscles carried out.
Trains of miniature endplate potentials (B) were recorded from mice with running wheel access (C, n=5) and controls (D, n=4). MEPP amplitude was averaged for each muscle (E). Four weeks of voluntary wheel access increased the amplitude of MEPPs compared to controls ($p<0.05$, unpaired t-test).

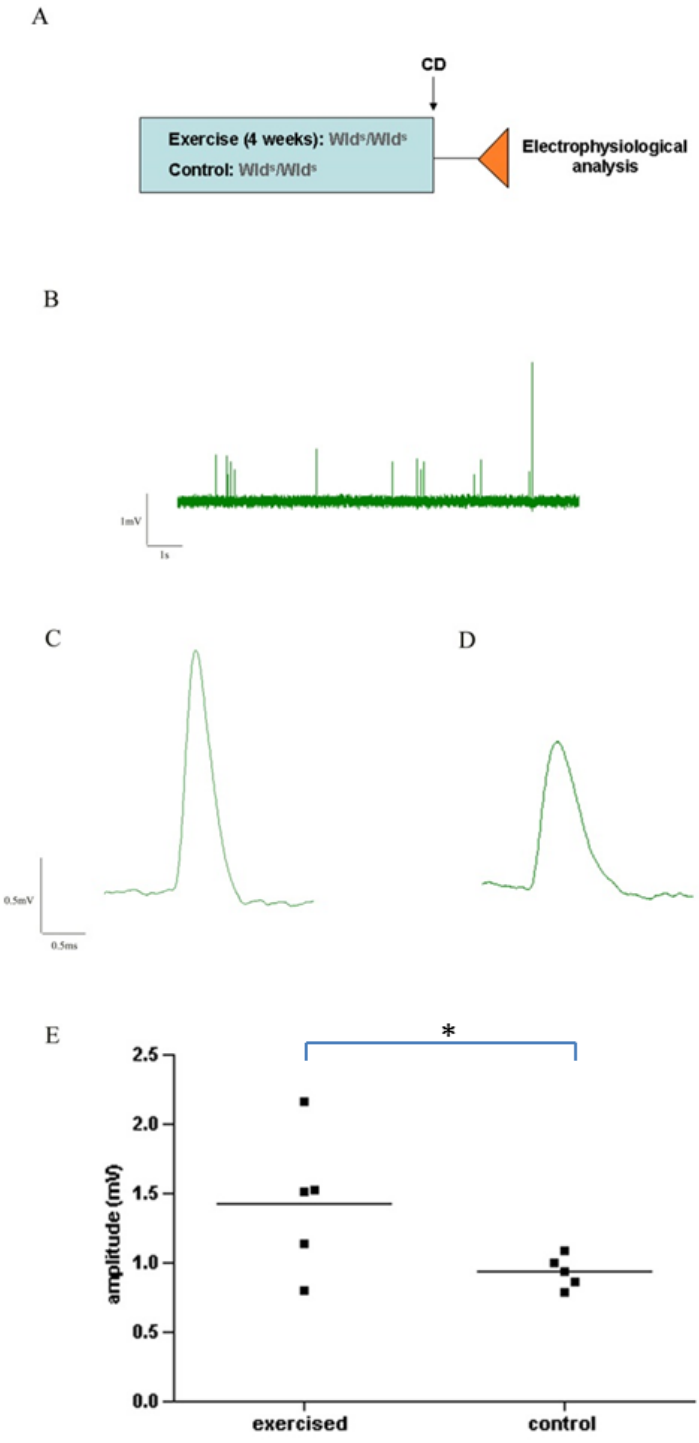
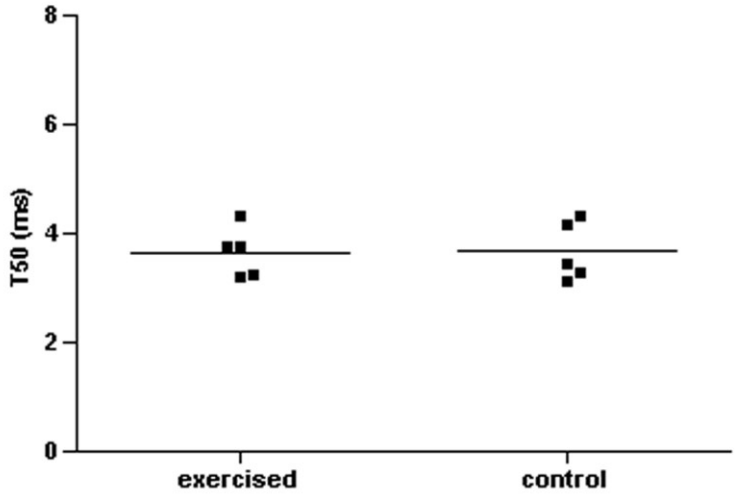

A
CD
Exercise (4 weeks): Wlds/Wlds
Control: Wlds/Wlds
Electrophysiological analysis
B
1mV
1s
C
D
0.5mV
0.5ms
E
*
amplitude (mV)
2.5
2.0
1.5
1.0
0.5
0.0
exercised
control

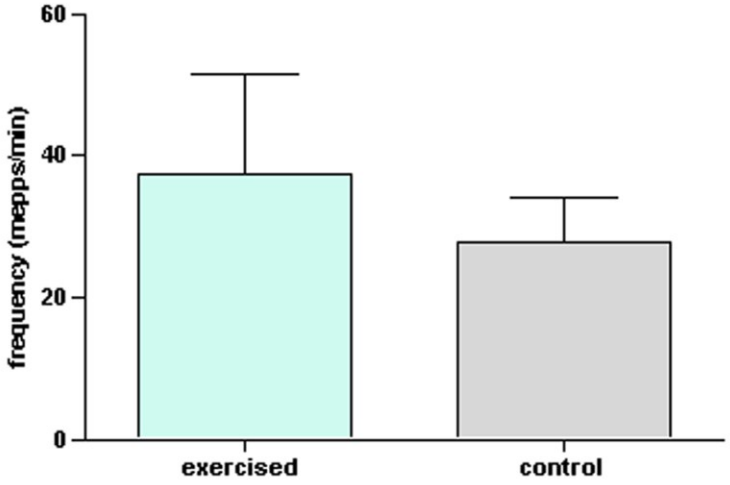
Figure 4.13: Voluntary wheel running does not alter the decay time or frequency of MEPPs in FDB muscle fibres

The half decay time of MEPPs, averaged for each muscle, was unaffected by four weeks of voluntary wheel running (A, n=5 exercised, n=4 controls, $p>0.05$). MEPP frequency was also unaffected by exercise (B, $p>0.05$, unpaired t-test).

A


8
6
4
2
0
T50 (ms)
exercised
control

B


60
40
20
0
frequency (mepps/min)
exercised
control

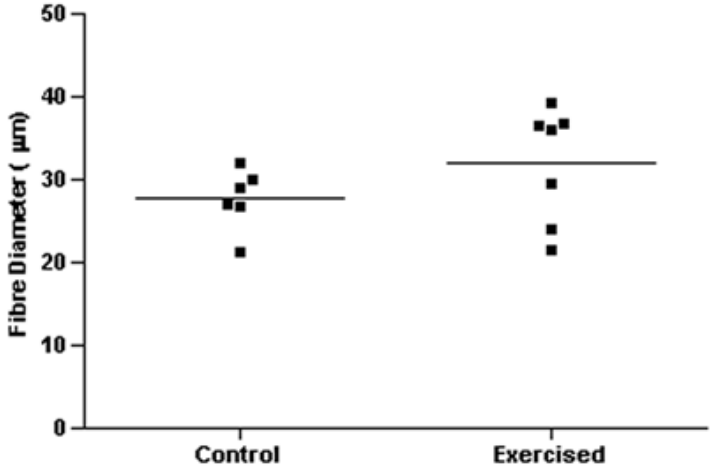
4.3.6 Morphology of the NMJ

In a subset of mice, the effect of four weeks running activity on fibre diameter and endplate diameter, area and perimeter length were analysed. As shown in figures 4.14 and 4.15, no significant effects of wheel running were found in any of the parameters analysed. Fibre diameter was an average of 27.57±1.53µm (n=6) in controls, and 31.86±2.64µm (n=7) in exercised muscles (figure 4.14). Similarly, endplate diameter remained unchanged, with an average of 19.26±2.97 (n=5) in control muscles compared to 22.55±3.12 in exercised muscles. Exercise also failed to affect endplate area (295.60±23.12µm^2 vs 318.60±20.63 µm^2) or endplate perimeter length (66.11±5.26 µm vs 73.96±4.16 µm) in control or exercised animals respectively (figure 4.15).

Figure 4.14: Four weeks of voluntary wheel running does not alter FDB muscle fibre or endplate dimensions

Muscle fibre endplates were stained with Tritc-α-BTX to allow the measurement of endplate dimensions. The measurements were averaged for each muscle. A. Four weeks of voluntary exercise (n=7), when compared to controls (n=6), did not alter muscle fibre diameter ($p>0.05$), or endplate diameter (B, $p>0.05$ unpaired t-test).

A


50
40
30
20
10
0
Fibre Diameter (µm)
Control
Exercised

B

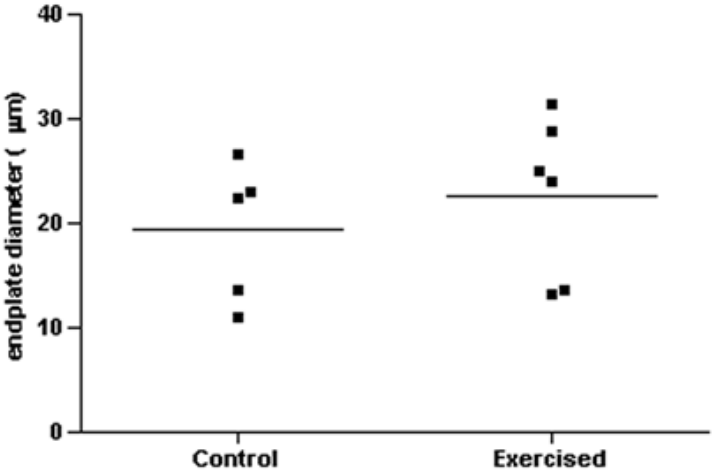
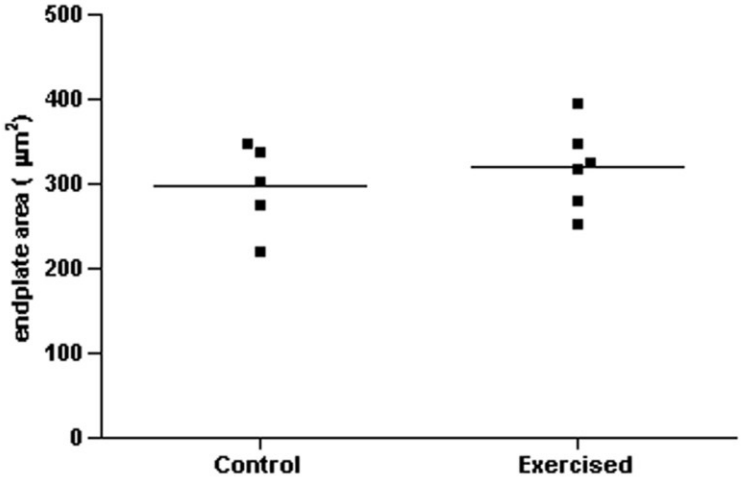

40
30
20
10
0
endplate diameter (µm)
Control
Exercised

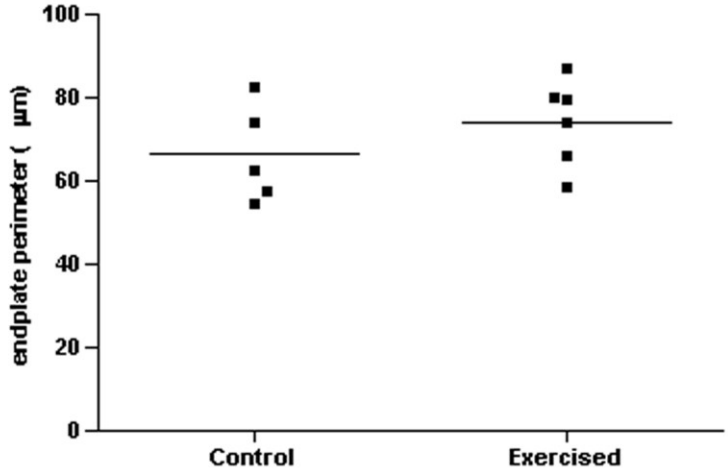
Figure 4.15: Four weeks of voluntary wheel running does not alter FDB endplate area or perimeter length

Muscle fibres were stained with Rh-BTX and endplate dimensions averaged for each muscle. Endplate area (A) and perimeter length (B) were no different between muscles from exercised mice compared to controls ($p>0.05$, $n=7$, 6 respectively, unpaired t-test).

A


500
400
300
200
100
0
endplate area (µm²)
Control
Exercised

B


100
80
60
40
20
0
endplate perimeter (µm)
Control
Exercised

4.4 Discussion

I hypothesised that by removing the variability of onset of neurodegeneration seen in disease models and studying the role of activity in a more defined model of synapse degeneration I would have a sensitive assay for detecting any effect of activity on this process.

Thus, I exposed *Wld*^{s} mice to up to four weeks of voluntary wheel running in order to test the hypothesis that the rate of synapse degeneration is determined in part by properties of the axon and synapse that can be modulated by neuromuscular activity. This hypothesis was based on the popular conception that synaptic use mitigates degeneration ("use it or lose it"); on multiple accounts of synaptic modulation by exercise and by my observations reported in chapter 3 that paralysis accelerated degeneration. Several papers report changes to electrophysiological properties and morphological parameters (see introduction). The results of testing the effects of activity on synaptic degeneration, in homozygous and heterozygous *Wld*^{s} mice, and also on the physiological and morphological properties of NMJ in the FDB muscle, are discussed below. To summarise, voluntary exercise altered some properties of synaptic transmission in the FDB muscle, but had little effect on the rate of synapse degeneration induced by axotomy.

4.4.1 Wld^{s} mice display a normal circadian rhythm

First, I observed that *Wld*^{s} mice display a circadian rhythm that is not discernibly different from that found in C57/Bl6 mice. Mice are mostly nocturnal, with only short bouts of activity during the day. Their activity on the running wheels was confined also mainly to the hours of darkness (De Bono et al., 2006), with an average distance of 6225m/24hr. This is comparable to the level of activity noted in previous studies (Allen et al., 2001, Pellegrino et al., 2005). Therefore the activity level is

comparable to the distance run by mice in which various physiological and morphological changes were noted.

4.4.2 Four weeks, but not two weeks, of voluntary running activity may have a slight detrimental effect on synaptic survival after axotomy

Neither two nor four weeks of voluntary exercise induced any significant alterations in the number of fibres giving an evoked response to stimulation at 5 days post-axotomy in homozygous *Wld*^{s} mice when compared to *Wld*^{s} mice with no such environmental enrichment. In other words, the mean number of axotomised nerve terminals that persisted and produced an evoked electrophysiological response was unchanged in the FDB. Thus, running activity was neither beneficial nor detrimental to the synaptic degeneration process in these mice. However, when the number of fibres giving an evoked response was combined with the number responding with only MEPPs the data revealed a significant reduction in innervated fibres in exercised muscles compared to controls. In other words, the number of fibres remaining capable of releasing neurotransmitter after axotomy was reduced after four weeks running activity. The physiological relevance of this is unclear; perhaps increased activity leads to a faster transition from the innervated to the denervated state. Alternatively, perhaps this subtle effect is an indicator that enhanced activity hastens synaptic degeneration, but that the wheel-running protocol is not robust or sensitive enough to evoke it. There is considerable scatter in the data, so the reliability of these interpretations is also uncertain. As *Wld*^{s} mice exhibit an age-dependent phenotype (Gillingwater et al., 2002), I ensured that all mice were 8-10 weeks at the end of the experiment. There was also no correlation between distance run and number of remaining innervated fibres (figure 4.5) so the variation cannot be explained by any difference in activity level between individual mice, and since all the mice were of the same genetic background the scatter may therefore just be a result of natural or random variation in the *Wld*^{s} phenotype.

Thus, perhaps a subtle effect of running activity on synaptic degeneration might have been covered by the natural variation in the degeneration rate in homozygous mice. In an attempt to resolve this, I then tested the hypothesis in heterozygous mice. Heterozygous mice show a time course of synaptic degeneration similar to WT mice, although degeneration of the axon is protracted almost as much as in young homozygous *Wld*^{s} mice (Wong et al., 2009). However, at 3 days post axotomy no innervated synapses remained. Therefore voluntary exercise was not sufficient in that instance to extend the axonal protection to the synapse either.

Previous studies demonstrated that increasing the animals' energy expenditure and exertion by increasing the load on the wheels can be more effective at inducing changes at the muscle and NMJ than unloaded wheels (Ishihara et al., 1998, Ishihara et al., 2002, Legerlotz et al., 2007). I tested this on a group of mice. However, this also produced no effect on the rate of synapse degeneration. Therefore if strenuous exercise is capable of inducing any protective benefits against synaptic degeneration, it is not detectable after 4 weeks of voluntary exercise in heterozygous *Wld*^{s} mice.

Mice ran 6225 metres per day, an average similar to that in studies that have previously demonstrated exercise induced changes in the muscle (Rodnick et al., 1989, Allen et al., 2001, Pellegrino et al., 2005), and in fact, although no effect on synaptic degeneration was detected, we were able to identify an effect of our exercise protocol on synaptic transmission in the FDB.

4.4.3 Four weeks of voluntary wheel running produces discernible alterations in synaptic transmission in the FDB

The responses of exercised fibres to train-of-four stimulation showed some deviation from controls. A significant decrease in quantal content was observed during a 50Hz stimulation train. This conflicts with the findings of other studies which showed an increase in QC following exercise (Fahim, 1997, Desaulniers et al., 2001). Some

studies have also demonstrated alteration in peak amplitude in response to trains of stimuli, but I did not confirm this finding either (Dorlochter et al., 1991).

The simplest explanation for a decrease in quantal content without an increase in peak amplitude would be an alteration in MEPP amplitude; therefore I analysed the MEPPs in the same muscles. The FDB shows an increase in MEPP amplitude in response to voluntary wheel running, which correlates with the decrease in quantal content but no change in overall EPP amplitude found under train-of-four stimulation, and also with previously reported increases in MEPP amplitude (Fahim, 1997).

There are various properties which can cause variability in MEPP amplitude. Fibre diameter and in turn input resistance of the membrane are implicated (Katz and Thesleff, 1957). Large diameter fibres have a low input resistance, which in turn leads to small MEPPs, whereas small diameter fibres have a higher input resistance and larger MEPPs (Katz and Thesleff, 1957; Harris and Ribchester 1979). These factors are also correlated with MEPP frequency (Kuno et al., 1971). Large diameter fibres produce small MEPPs but at a high frequency, whereas the frequency is lower in small diameter fibres. However, I found no alteration in MEPP frequency, tying in with the lack of change in fibre diameter or endplate measurements. However, MEPP amplitude was increased, indicating that perhaps although there was no change in fibre diameter there may be a change in fibre input resistance.

With regards to mechanisms, it has previously been reported that inactivity leads to modulation of components of the presynaptic nerve terminal. For example, inactivity in hippocampal slices results in stronger, larger synapses, with an increased number of vesicles and active zone size (Murthy et al., 2001). This indicates that, through homeostatic regulation of synaptic function, inactivity leads to signaling mechanisms that in turn strengthen the synapse and enhance transmitter release in order to sustain synaptic activity at a stable level (Pozo and Goda, 2010, Vitureira et al., 2011).

To determine whether these alterations were correlated to an alteration in Ca^{2+}/Mg^{2+} sensitivity, I measured the response of exercise and control FDBs to varying concentrations of these ions. The arrival of the action potential at the nerve terminal

triggers the opening of voltage dependent channels which allow the influx of calcium. The increase in intracellular calcium triggers the release of synaptic vesicles, and in turn the muscle EPP (Nudler et al., 2003). The relationship between the endplate potential and the concentration of calcium suggests that four calcium ions at least are required for vesicle binding and exocytosis of neurotransmitter (Dodge and Rahamimoff, 1967a). However, quantal release can be modulated by both calcium and magnesium ions. These ions are known to have opposing effects on transmission at the NMJ. Whereas increasing calcium increases the size of the EPP (Del Castillo and Stark, 1952, Hutter and Kostial, 1954), an increase in magnesium reduces transmitter release (Del Castillo and Engbaek, 1954, Del Castillo and Katz, 1954a). In fact, increasing calcium concentration relieves the blocking effects of magnesium (Del Castillo and Engbaek, 1954, Hutter and Kostial, 1954). If exercise modulates properties of the NMJ, we hypothesised that it may also alter its response to varying concentrations of calcium and magnesium. An increased QC in the presence of high magnesium has already been reported in mice subjected to exercise training by Dorlochter *et al.* (1991). However, any effect our exercise protocol might have had on transmitter release was not detectable under magnesium block. Reducing calcium and increasing magnesium content/concentration produced a decrease in quantal content that was not significantly different from that detected in the FDB muscles of control mice.

4.4.4 Four weeks of running activity induced no morphological alterations in the FDB

The area of the endplate and the diameter of the muscle fibre have previously been shown to be correlated, and quantal content is positively correlated with endplate size (Kuno et al., 1971, Harris and Ribchester, 1979, Costanzo et al., 1999), so we might have expected the endplate dimensions to change with our decreased quantal content. Also, the observed increase in MEPP amplitude may suggest an alteration in fibre diameter (Katz and Thesleff, 1957, Harris and Ribchester, 1979). However, in this experiment fibre diameter and endplate dimensions were unchanged, which was

surprising given the substantial evidence for the ability of exercise to modify both the muscle fibre and endplate (Waerhaug et al., 1992, Ishihara et al., 1998, Ishihara et al., 2002, Deschenes et al., 2006). In fact, short duration, low intensity activity has been shown previously to induce alterations in nerve terminal morphology (Tomas et al., 1997, Allen et al., 2001), although it has been reported that high intensity regimes are required to produce more substantial changes (Deschenes et al., 1993).
However, the method of fibre teasing is subject to methodological issues, for example it is possible that the fibres were squashed slightly when put on to the slides or damaged during preparation, which would affect the measurements. It may be possible to detect some slight changes using, for example, measurements of cross sectional area. It is also possible that relationship between fibre diameter and MEPP properties is not always strictly true, so it may be possible that this short duration of activity can alter one without resulting in a change in the other (Wilkinson et al., 1992).

As discussed previously, the input resistance of the muscle was not measured. Given the increase in MEPP amplitude with a lack of effect on muscle fibre diameter and also MEPP frequency, it would be of interest to measure input resistance to resolve the relationship between these changes. It would also be of interest to unravel any other alterations to activity that may have occurred, for example by studying active zones, in which protein composition and structure have previously been reposted to change in response to altered activity levels (DiAntonio et al., 1999, Murthy et al., 2001, Pozo and Goda, 2010).

4.4.5 Limitations and mechanisms in the present study

It is argued that activity may be detrimental in neurodegenerative disorders, as it may induce excitotoxicity and free radical production (Itoh et al., 1998, Liebetanz et al., 2004) which can trigger degeneration in motor neurons that are already vulnerable. However, there are arguments for beneficial effects of activity, in terms of the "use it or lose it" hypothesis (Shors et al., 2011) and reported increased muscle oxidative capacity (Kirkendall and Garrett, 1998). It seems the voluntary exercise protocol I utilised produces slight adaptations in synaptic transmission and degeneration, but not to overt morphological changes. However, the basic design of this experiment has its limitations. Despite the benefits of using *Wld*^{s} mice as a model of synapse degeneration that I have discussed previously, their use is limited by the age-dependency of their phenotype. As such, we were unable to provide wheel for any more than four weeks. The efficacy of the *Wld*^{s} gene starts to decline at around two months old (Gillingwater et al., 2002), and so the experiment was completed when the animals were no more than 10 weeks old. The inability to provide a longer exercise paradigm similar to those used in other studies begs the question of whether or not an extended protocol might in fact have some effect after all. It has previously been suggested that either load or duration must be reasonably high to induce measurable modifications at the muscle (Kariya et al., 2004) but our attempts to increase the load also produced no effect on degeneration.

Whether or not a longer protocol could in fact elicit any changes in synaptic degeneration remains unclear. Four weeks of low intensity treadmill training induced morphological changes at the rat NMJ (Tomas et al., 1997), and an alteration in muscle fibre type and size in mice that had also had voluntary wheel access for four weeks (Allen et al., 2001). However, it has been stated that it takes at least four weeks for any significant changes to occur in the muscle of exercising mice (Allen et al., 2001), and most exercise protocols have a much longer duration. Such regimes, as previously noted, have induced various changes on the structure and physiology of the muscle and NMJ (see introduction). However, the overall aim of these

experiments was to determine the effects of activity in a controlled model of degeneration as a test bed for disease models, in which longer exercise regimes show variable protective benefits, possibly due to the varying times of symptom onset between animals. We therefore have a 'catch-22'. *Wld*^{s} provides us with a good test bed for analysing effects of activity on synaptic degeneration as the onset of degeneration is user controlled, but four weeks of exercise produces no effect. However, we cannot extend the protocol as the *Wld*^{s} phenotype is age-dependent.

One way to overcome this may be to use ΔNLS-Wld^{s} mice, in which the ability of Wld^{s} to locate to the nucleus is reduced, therefore increasing the cytoplasmic localisation (Beirowski et al., 2009). Synapse protection is significantly extended in these mice, and they also seem not to display the same age-dependent phenotype seen in normal *Wld*^{s} mice. These therefore might provide a more effective means for testing the effect of exercise on degeneration as the lack of age-dependency of the phenotype means that these mice could be exposed to a much longer exercise protocol.

Alternatively, it may be possible that four weeks of exercise is sufficient to alter degeneration, but that this does not occur in the FDB. Most other exercise studies using rodents study muscles such as the soleus, extensor digitorum longus or tibialis anterior. Despite its assets for electrophysiological recording, the FDB has not been widely studied in terms of its fibre type composition etc. To my knowledge, there are few papers that have addressed this, and what has been published is conflicting. According to one paper (Zuurveld et al., 1985), the growth and development is unlike the soleus and EDL muscles, as it retains a high percentage of intermediate fibres and few fast and slow fibres, which did not change up to 90 days. However, another paper states that the FDB in the rat is composed of homogenous fast twitch type IIa fibres (Carlsen et al., 1985).

It remains to be determined whether we could have found any alteration in the EDL or soleus muscle following the exercise protocol I implemented. The effects previously documented do appear to be highly dependent on the type of muscle. For example, Allen and colleagues found a shift in fibre size but not in muscle fibre type in the gastrocnemius, but no significant effect in the TA following four weeks of exercise. Therefore an important next step could be to investigate the *Wld*^{s} phenotype

in various muscles and then study the effects of exercise within these muscles. It may be that the FDB is just not very responsive to low intensity short duration voluntary exercise.

It could also be argued that a voluntary running regime is not as controlled as something such a treadmill regime, and therefore open to more variables. Perhaps it may be necessary for us to use more resistance type training. It has been reported that the most substantial changes occur in the EDL muscles of treadmill trained animals compared to voluntary runners (Jeneson et al., 2007). Treadmill training has also been reported to be more effective than voluntary running in a model of stroke (Hayes et al., 2008), and voluntary wheel running was of no benefit either before or after injury in a model of spinal cord injury (Erschbamer et al., 2006). However, according to Ke *et al.* (2011), voluntary activity enhanced motor performance in a rat model of stroke whereas treadmill training did not. Voluntary exercise was also found to be more beneficial than treadmill running in a mouse model of Alzheimer's disease (Yuede et al., 2009). Therefore it seems that the effect of exercise is variable depending on the type, duration and muscle type amongst other things. Nonetheless, in my study voluntary wheel running evoked neither a beneficial or detrimental effect on synapse degeneration, suggesting overall that although this form of exercise may fine-tune transmission at the NMJ, it does not influence the rate of synapse degeneration.

4.4.6 Summary

I have carried out the first study of the effects of voluntary wheel running exercise on synapse degeneration in the *Wld*^{s} mouse and this is also the first study to investigate the effect of exercise on synaptic transmission in the FDB. Environmental enrichment by provision of voluntary access to a running wheel for 2-4 weeks is insufficient to affect the rate of degeneration at the axotomised NMJ in the FDB muscle, although there was an indication that four weeks of exercise causes synapses to degenerate more rapidly after axotomy when including fibres responding only spontaneously upon stimulation in the analysis of the number of remaining functional

synapses. In my opinion, this subtle change is not sufficient to imply that any significant effect of voluntary running should be noted in models of neurodegenerative disorders. However, the limitations of this model suggest it should be extended to a more rigorous model of increased synaptic activity over a longer time scale, including for example a test of the effects of age and exercise duration on the maintenance of synaptic transmission following nerve injury.

5: An organotypic explant assay as a test-bed for modulators of synaptic degeneration.

5: An organotypic explant assay as a test-bed for modulators of synaptic degeneration.

5.1 Background

In the previous two results chapters I presented evidence indicating that a low level of neuromuscular activity is essential for preventing rapid neuromuscular synaptic degeneration in *Wld*^{s} mice, whereas voluntary access to running wheels had little effect on the rate of synapse loss. When considering the results of chapter 4, it may be the case that the voluntary running paradigm employed is not robust enough to elicit an effect at the mouse NMJ. This has been argued in several other studies (Kariya et al., 2004, Jeneson et al., 2007). An alternative method of increasing neuromuscular activity would be to electrically stimulate the nerve *in vivo*. There are arguments in favour of using an electrical stimulation protocol to enhance activity in rodent models. The benefits of electrical stimulation in comparison to exercise are discussed by Pette and Vrbrová (1999). In essence, the electrical paradigm allows reproduction of specific, controlled stimulation protocols, compared to exercise regimes which depend entirely on the animals cooperation. Greater activation levels can be obtained than in unforced paradigms such as voluntary wheel running. In addition, stimulation can be targeted at just one area rather than increasing the activity of the whole system. However, stimulation *in vivo* is not without its disadvantages. For example, it is difficult to determine whether the level of stimulation is above the normal activity level of the animal, especially if the animal is anaesthetized during the procedure. It is also technically challenging to maintain electrodes within a freely moving animal (Pette and Vrbova, 1999).

However, electrical stimulation has been used successfully in models of synapse elimination (Brown et al., 1980). In a neonatal model, Ridge and Betz (1984) report that stimulated motor units had a greater competitive advantage than unstimulated motor units. Thompson (1983) also used stimulation to reveal that the effects of increasing activity on this process are dependent upon the stimulation frequency during neonatal synapse elimination (Thompson, 1983, Ridge and Betz, 1984).

To circumvent the difficulties of *in vivo* stimulation some groups have tested effects of stimulation *in vitro*. Culture systems have been used to test the effects of stimulation on synapse elimination (Nelson et al., 2003), and stimulating muscle fibres in culture has been shown to alter the fibre type composition (Barjot et al., 1998). Stimulating in culture allows reproducible protocols for increasing activity to be employed, whilst avoiding the difficulties of maintaining stimulating electrodes in a free-moving animal (Pette and Vrbova, 1999). I therefore developed an assay that enabled me to test the effects of electrical stimulation on synapse degeneration in isolated FDB nerve-muscle preparations.

In order to stimulate FDB muscles, I devised a method of sustaining nerve muscle preparations in a manner suitable to separate the degeneration time course of WT and *Wld*^{s} NMJs, and that further would allow me to easily remove the muscles from the culture system and conduct electrophysiological analysis. Various studies have successfully cultured *Wld*^{s} and WT nerves and demonstrated the difference in degeneration rates (Tsao et al., 1994, Tsao et al., 1999, Beirowski et al., 2004). Parson *et al.* 2004 cultured Wld^{s} and WT FDBs with their intact nerve supply. Similar to the situation *in vivo*, they found that WT NMJs were degenerated by 24h, with only a few surviving at the 16h time point. However, *Wld*^{s} NMJs survived up to 72h in culture (Parson et al., 2004).

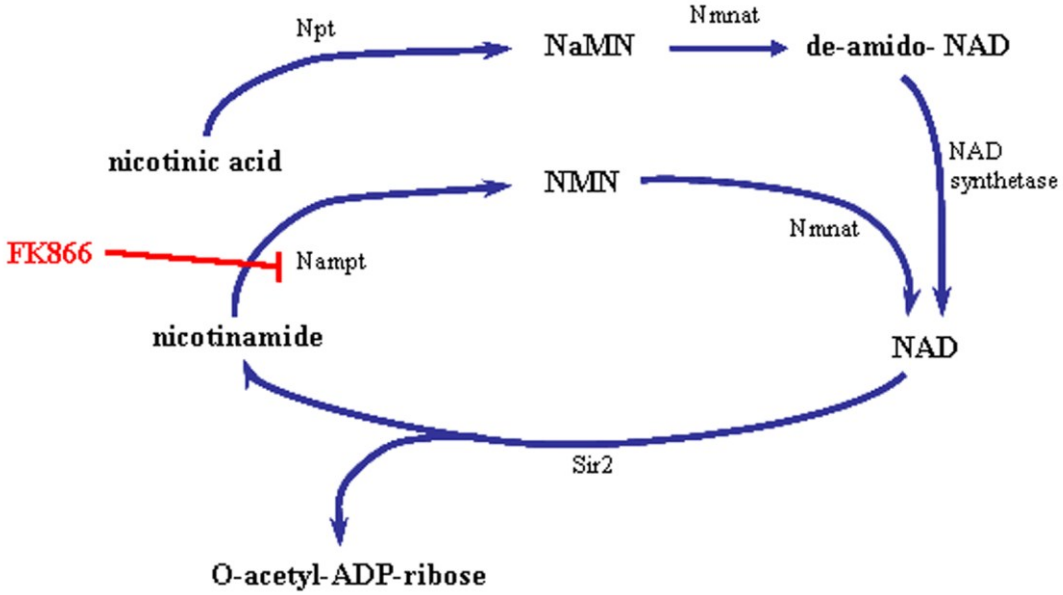
My aim was to develop a simple method that could sustain both the muscle and the nerve and reveal the effects of treatment. The physiological saline used for electrophysiological recordings, MPS, maintains both WT and Wld^{s} nerve-muscle preparations for at least 16 hours at room temperature. By contrast both WT and Wlds preparations degenerate rapidly if the temperature is maintained at or above 37ºC (R. Ribchester, personal communication). Using MPS would also be practical in terms of transferring the muscles to complete the electrophysiological analysis. In the first part of this chapter I investigate the potential for 'culturing' isolated FDB's in MPS. I then extend to testing a range of stimulation protocols and examine their effect on the rate of degeneration of the FDB muscles within this system.

An additional aim for this study was to find a suitable preparation in which the mechanisms behind the enhanced degeneration seen in TTX pretreated muscles might be elucidated (see chapter 3). To unravel the molecular basis of this effect *in vivo* may prove difficult. I therefore aimed to reproduce in vitro the enhanced degeneration in muscles blocked with TTX for one week *in vivo*. Finally, I tested the enzymatic inhibitor FK866 and evaluated its effects on NMJ survival in this culture system. FK866 inhibits the action of nicotinamide phosphoribosyltransferase (Conforti et al., 2009, Formentini et al., 2009) and is therefore of interest when considering the NAD pathway and its involvement in Wallerian degeneration. In fact, FK866 has previously been reported to reduce the level of protection seen in *Wld*^{s} culture (Conforti et al., 2009, Wang and He, 2009) and is also undergoing analysis for cancer treatment (Hasmann and Schemainda, 2003, Bruzzone et al., 2009, Houtkooper et al., 2010, Bi et al., 2011) as inhibiting Nampt kills cells that rely on high NAD turnover, such as cancerous cells. It has previously been shown that Nmnat, which makes NAD, is essential for axons (Gilley and Coleman, 2010). FK866 inhibits NMN synthesis, and NMN is required for NAD formation. Therefore, FK866 should decrease NAD levels, which would result in enhanced degeneration.

This assay proved effective for demonstrating the difference in degeneration rates for WT and *Wld*^{s} muscles over a 48 hour period. Furthermore I was able to use it to detect a detrimental effect of high frequency stimulation on nerve terminal integrity. However, the assay did not replicate the enhanced degeneration following TTX treatment I observed *in vivo*. Finally, I tested a chemical modulator of degeneration, FK866, which - contrary to initial expectations - proved to be effective in extending the level of synaptic protection in *Wld*^{s} muscles.

Figure 5.1: FK866 selectively inhibits Nampt

The mammalian NAD biosynthesis pathway. FK866 reduces NMN levels by selectively inhibiting the action of Nampt, the enzyme which converts Nicotinamide, the major substrate of this pathway, to NMN. Based on Revollo et al. (2004).


Npt
NaMN
Nmnat
de-amido- NAD
nicotinic acid
NAD
synthetase
NMN
Nmnat
FK866
Nampt
nicotinamide
NAD
Sir2
O-acetyl-ADP-ribose

5.2 Methods

5.2.1 Establishing the Assay

These experiments were conducted on Wld^s (N=46), C57 Bl6 (N = 16) and heterozygous Wld^s (N=8) mice.

To establish the assay I measured the levels of degeneration at different time points in the FDB muscles three different genotypes of mice: C57 Wld^s (n=29), Heterozygous Wld^s (n=15) and C57 Bl6 (n=23). FDB muscles were dissected as described previously (see section 2.4), and pinned onto small sheets of Sylgard. These were then placed in sterile 20ml tubes containing filtered MPS (see section 2.4), connected to a supply of medical gas (95% oxygen, 5% CO_2) and maintained at 32°C in a water bath. The muscles were bubbled in fresh MPS for 20 minutes prior to electrophysiological estimation of the number of surviving fibres (see section 2.5) at designated time points up to 48 hours.

5.2.2 Investigation of the effects of electrical stimulation on the degeneration of isolated FDBs

Only C57 Wld^s mice were used in this part of the experiment.
To enable stimulation of muscles I developed a stimulation chamber which consisted of two silver dissecting pins connected to plastic coated copper wires and attached to the base of a 60mm Petri dish. This was then lined with Sylgard, ensuring that enough silver wire was still protruding to maintain contact with the nerve.
The FDB was pinned to the Sylgard lining and the nerve placed in contact with the silver wires. The entire dish was then placed in oxygenated MPS and maintained as before, at 32˚C in a water bath. Initially muscles were stimulated at a frequency of 1Hz continuously (n=12). The level of degeneration was estimated

electrophysiologically at various time points between 24 and 48 hours and compared to unstimulated controls (n=12).
A 30 hour time point was then selected to investigate the effects of two further stimulation protocols – 10Hz for 1 second every 10 seconds (n=4), and 100Hz for 1 second every 100 seconds (n=5).

5.2.3 Investigation of the effects of inactivity on the rate of degeneration in vitro

To establish whether the effects of pre-blocking with TTX were transferable to this culture system, unilateral hind limb paralysis was initiated in three *Wld*^{s} mice using 15mM TTX as described in chapter 3. Following one week of block the FDB muscles (n=3) were dissected and placed into the culture system. The level of degeneration was estimated by electrophysiological recording at 26 hours and compared to the unblocked contralateral controls (n=3). The incubation was at 32ºC.

5.2.4 Modulation of synaptic degeneration by application of FK866

Initially, 1μM FK866 was added to either WT (n=4) or *Wld*^{s} (n=5) FDB muscles, and 10μM FK866 added to a further five WT muscles in the culture system. The amount of synaptic degeneration was measured 16-20h later. This was compared to controls maintained in normal MPS.
As the level of protection at this time point for *Wld*^{s} was maintained in the presence of 1μM FK866, I then investigated the effect of 10μM FK866 on *Wld*^{s} muscles (n=8) for 42-48h. The incubation was at 32ºC.

5.3 Results

5.3.1 Establishing a suitable culture system

The assay was tested initially at 32ºC. The number of responsive fibres in WT muscles was found to decay rapidly, from an average of 36.39±16.02% at 5-15 hours, to 10.77±5.30% at 15-25 hours (figure 5.2). Eventually, at 25-50 hours, only 1.25±1.25% of fibres produced a response. A similar decay was found in heterozygous *Wld*^{s} mice, which *in vivo* also demonstrate a similar time course of synaptic loss to WT (Wong *et al.* 2009). At 5-15 hours, the average number of responding fibres was 87.33±10.35%, compared to 15.84±5.671% at 15-25 hours ($p<0.001$) and 8.33±4.37% at 25-50 hours ($p<0.001$).

However, *Wld*^{s} muscles were found to be 89.61±2.93% responsive at 15-25 hours in solution, which declined to 70.42±7.06% at 25-30h. This number was significantly reduced to 45.72±11.90% at 35-45h ($p<0.001$). Eventually the number of innervated fibres reduced to 2.5±1.71% at 45-50h ($p<0.001$, Figure 5.2).

The same pattern of degeneration was established when including fibres giving only MEPPs in the analysis. The response of *Wld*^{s} muscles decayed from 99.24±0.52% at 5-15 hours to 12.00±12.00% at 45-50 hours ($p<0.001$, figure 5.3). Heterozygous *Wld*^{s} muscles degenerated much more rapidly, with 99.33±0.67% fibres responding at 5-15 hours, and only 13.33±5.78% responding at 25-50 hours ($p<0.001$). WT muscles again degenerated rapidly, with an average of 46.89±14.52% responsive fibres at 5-15 hours in comparison to 13.75±13.75% at 25-50 hours, though again the change in responsive fibres over time was not significant ($p>0.05$, figure 5.3).

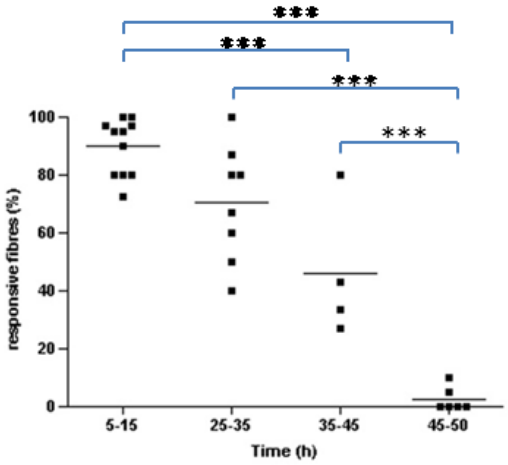
Although this system does not fully replicate the prolonged degeneration seen *in vivo* in *Wld*^{s} mice, NMJs were still functional for twice as long as WT or heterozygous *Wld*^{s} mice. Therefore this method is suitable for investigating methods of modulating the synaptic protection seen in the *Wld*^{s} muscles.

Figure 5.2: The extended time course of homozygous *Wld*^{S} NMJ degeneration compared to WT can be replicated in vitro

The mean percentage of muscle fibres responding to stimulation was calculated for each muscle after the given times in vitro. The overall mean for each time point is denoted by a bar. A. At 32°C in MPS, *Wld*^{S} muscles (n=29) maintained some fibres giving an evoked response to stimulation for up to 50 hours. B. Heterozygous *Wld*^{S} (n=15) NMJs were mostly degenerated by 30 hours, as shown by a high percentage of unresponsive fibres. C. A similar pattern of degeneration was noted in WT muscles (n=23), with most fibres incapable of responding to stimulation by 30h, though the reduction in responsive fibres over time was not significant ($p>0.05$, one way ANOVA).

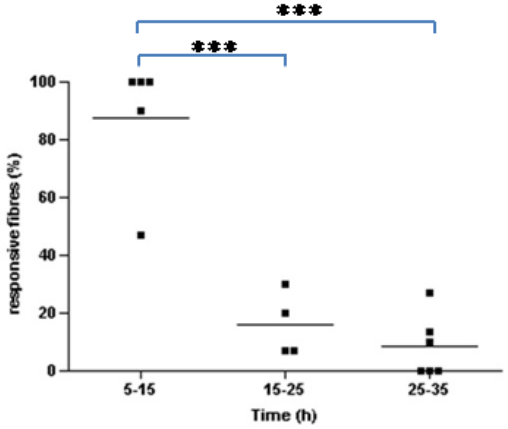
A

EPPs only Wld^S/Wld^S



B

EPPs only WT/Wld^S



C

EPPs only WT

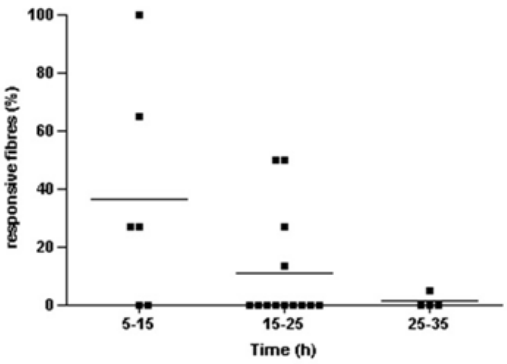
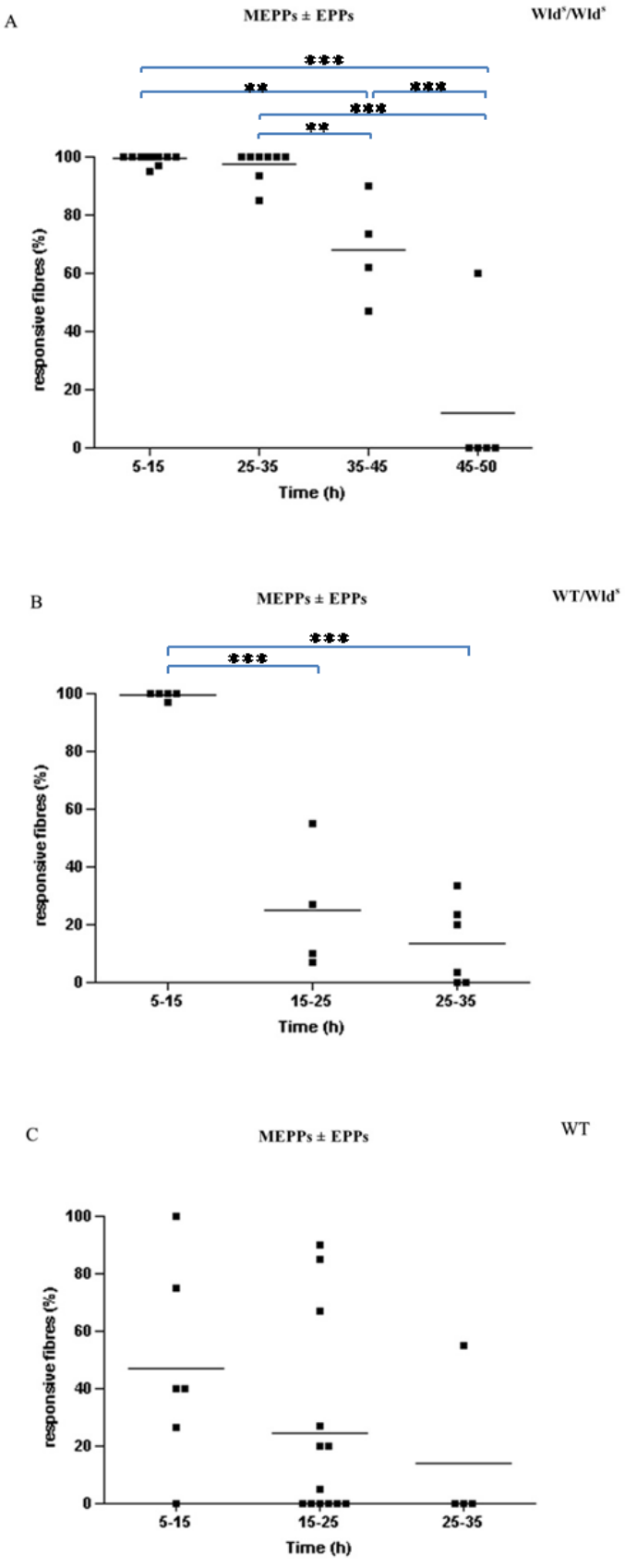


Figure 5.3: Analysis of the number of FDB fibres responding to stimulation with EPPs and/or MEPPs reveals that the extended time course of homozygous *Wld*^{S} NMJ degeneration compared to WT can be replicated in vitro

Analysis of the number of fibres that respond to stimulation with EPPs and/or MEPPs revealed a similar time course of degeneration to analysis of fibres giving EPPs only (figure 5.1). Each point represents the mean percentage of fibres responding with EPPs and/or MEPPs for each muscle. A. *Wld*^{S} FDB muscles (n=29) remained functional for up to 50h, whereas both heterozygous *Wld*^{S} (B, n=15) and WT (C, n=23) NMJs had degenerated by 30h, though the reduction in responsive WT fibres over time was not significant ($p>0.05$, one way ANOVA).


A
MEPPs ± EPPs
Wld^S/Wld^S
***
**
***
***
**
100
80
60
40
20
0
responsive fibres (%)
5-15
25-35
35-45
45-50
Time (h)
B
MEPPs ± EPPs
WT/Wld^S
***
***
100
80
60
40
20
0
responsive fibres (%)
5-15
15-25
25-35
Time (h)
C
MEPPs ± EPPs
WT
100
80
60
40
20
0
responsive fibres (%)
5-15
15-25
25-35
Time (h)

5.3.2 Investigating the effects of stimulation

The initial experiment involved stimulating the isolated *Wld*^{s} FDB at a continuous frequency of 1Hz. Analysis of the number of responsive fibres at several time points revealed no significant effect on the rate of synaptic degeneration, either beneficial or detrimental, although a slight trend towards an enhanced protection was noted at 30h, but this was not significant (figure 5.4). When including fibres responding with only MEPPs and no evoked response in the analysis, the two curves are virtually indistinguishable (figure 5.4), though in both cases there was a significant reduction in the number of responsive fibres over time ($p<0.05$, two way ANOVA). Thus, a 1Hz continuous stimulation is insufficient to modulate synaptic degeneration, at least in this context. Therefore two additional stimulus protocols were applied. These were tested at the 30h time point, which allowed detection of any additional or reduced synaptic protection.

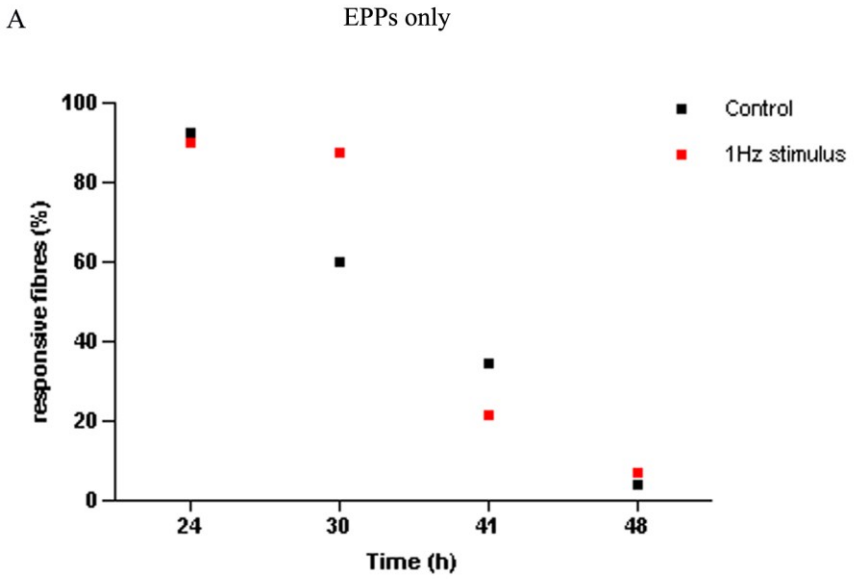
A stimulation protocol with the same total number of stimuli but patterned at 10 Hz for one second every 10 seconds results in an average of 52.50±11.17% fibres, which is not significantly different from the average percentage remaining in the control muscles (53.75±10.28%, figure 5.5). However, stimulating at a higher frequency of 100 Hz for 1 second every 100 seconds resulted in a decrease in synaptic protection, with the average number of remaining functional fibres dropping to 32.67±6.86%. This significantly reduced compared to the muscles stimulated at 1Hz continuously (78.75±11.21%, $p<0.05$).

However, this effect was not noticeable when including fibres with MEPPs only in the total for analysis, with the mean number of responsive fibres increasing to 68.67±6.96% after 100Hz intermittent stimulation. Unstimulated controls gave an average of 93.33±3.12% responsive fibres, and a similar number of fibres responded after 1Hz or 10Hz stimulation (86.25±6.61%, 77.50±9.47% respectively, figure 5.5). Thus, high frequency stimulation impaired evoked rather than spontaneous transmission.

Figure 5.4: The time course of *Wld*^{s} synaptic degeneration in vitro was not altered by 1Hz continuous stimulation

Analysis of the overall mean of the number of responsive fibres from *Wld*^{s} muscles *in vitro* revealed that continuous stimulation at 1 Hz did not alter the degeneration rate of *Wld*^{s} NMJs when measuring fibres giving an evoked response (A, $p>0.05$) or including those with only spontaneous MEPPs (B, $p>0.05$), though there was a significant reduction in responsive fibres in both groups over time ($p<0.05$, two way ANOVA, n=12 per group).

A


EPPs only
Control
1Hz stimulus
responsive fibres (%)
100
80
60
40
20
0
24
30
41
48
Time (h)

B

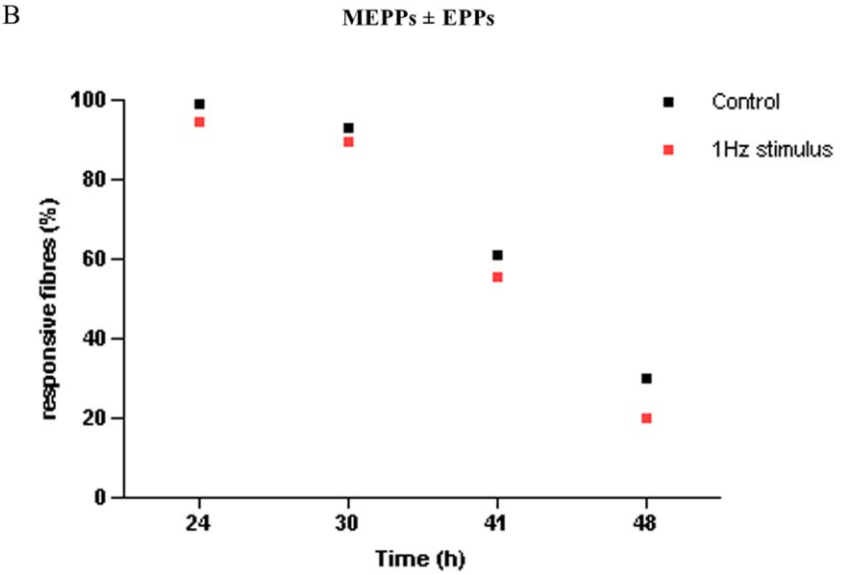
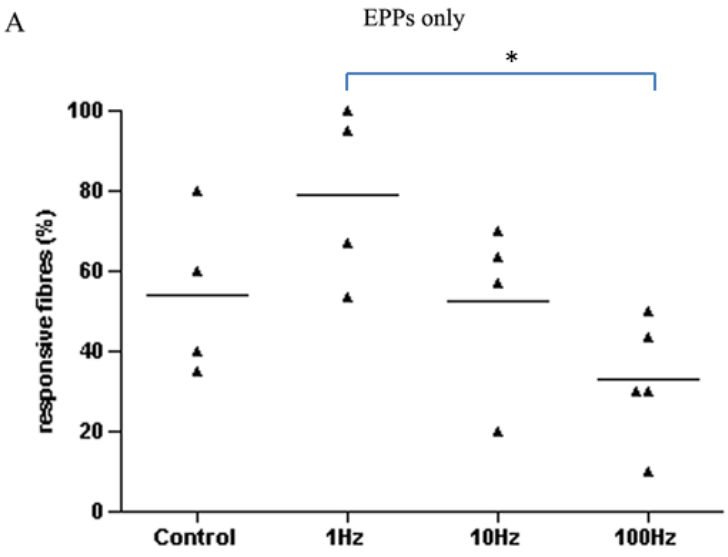
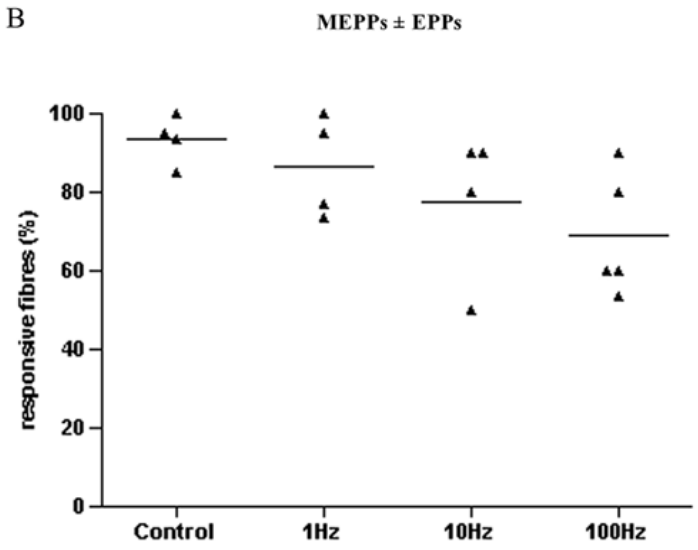

MEPPs ± EPPs
Control
1Hz stimulus
responsive fibres (%)
100
80
60
40
20
0
24
30
41
48
Time (h)

Figure 5.5: High frequency stimulation is detrimental to *Wld*^{S} synaptic degeneration *in vitro*

The mean number of responsive points was scored for each muscle after receiving either 1Hz continuous stimulation (n=4), 10Hz (n=4) or 100Hz (n=5) intermittent stimulation, or no stimulation (n=4) for 30h in MPS. A. Intermittent 100Hz pulses significantly reduced the number of functional synapses remaining after 30h ($p<0.05$, one way ANOVA). B. However, when fibres exhibiting only MEPPs were included in the analysis intermittent stimulation at neither 1, 10 nor 100Hz did not alter the amount of synaptic degeneration after 30h in MPS (B, $p>0.05$, one way ANOVA).


A
EPPs only
*
100
80
60
40
20
0
responsive fibres (%)
Control
1Hz
10Hz
100Hz


B
MEPPs ± EPPs
100
80
60
40
20
0
responsive fibres (%)
Control
1Hz
10Hz
100Hz

5.3.3 Extending the effects of TTX block from an in vivo to an in vitro assay

To determine whether the reduced synaptic efficacy noted in axotomised FDB's that had been previously paralysed with TTX *in vivo* was maintained in an *in vitro* system, I applied unilateral TTX block to 3 mice. All mice were successfully blocked, and one week later the FDBs were dissected and placed in the culture system. However, the enhanced synaptic loss seen *in vivo* was not replicated in this system. At 26h, control *Wld*^{s} muscles have an average of 67.78±10.60% functional muscle fibres remaining compared to 75.56±11.11% in those pre-blocked with TTX (figure 5.6). When including fibres with only MEPPs in the analysis, 100±0% of the terminals were retained in controls compared to 94.45±1.11% in TTX blocked preparations.

5.3.4 FK866 as a modulator of synaptic degeneration

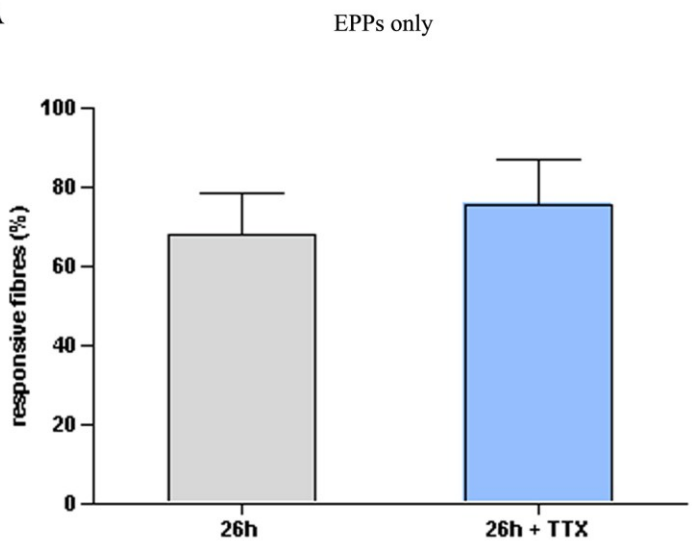
There was no significant difference in the level of degeneration between WT and heterozygous *Wld*^{s} muscles, though both show a significantly greater level of synaptic degeneration at 16-20h than *Wld*^{s} (10.77±5.30% n=13, 15.84±5.67% n=4, 89.53±3.22% $p<0.001$, n=9 respectively, figure 5.7). 1μM FK866 had no detrimental effect on *Wld*^{s} muscles after 16-20h in culture when compared to untreated *Wld*^{s} muscles (81.33±8.86% n=5, 89.53±3.22% n=9 respectively, $p>0.05$). However, there was evidence for a dose-dependent enhanced protection in WT muscles, though this did not reach statistical significance ($p=0.095$ WT vs WT 10μM FK866). WT muscles treated with 1μM FK866 had a mean of 23.33±19.15% responsive fibres, and 10μM FK866 resulted in an average of 43.00±17.72% responsive fibres. The increase in responsive fibres seen in WT muscles treated with 10μM FK866 compared to untreated WT muscles became significant when including fibres with spontaneous transmitter release only in the analysis (69.24±13.19% vs 24.10±9.39% respectively, one way ANOVA $p<0.01$, figure 5.7).

To test whether FK866 could also enhance protection in *Wld*^{S} muscles, I examined the level of degeneration at 42-48h with 10μM FK866. Interestingly, the level of synaptic protection was two to three-fold greater compared to untreated Wld^{s} muscles at this time point (56.25±9.01% vs. 19.79±8.35% respectively; $p<0.01$ unpaired t-test figure 5.8). This difference was maintained when analysis of fibres showing spontaneous MEPPs but no evoked transmitter release were included in the analysis (85.42±4.36% vs. 39.19±11.22% $p<0.01$ unpaired t-test).

Figure 5.6: TTX-induced facilitation of synaptic degeneration seen in vivo was not replicated in vitro

FDB muscles paralysed TTX block of the sciatic nerve for one week *in vivo*, then placed in MPS at 32°C did not show any reduction in the number of fibres giving an evoked response to stimulation compared to controls (A, $p>0.05$, $n=3$ per group). Including fibres giving MEPPs only also revealed no significant effect on the number of responsive fibres (B, $p>0.05$, unpaired t-test).

A


EPPs only
responsive fibres (%)
100
80
60
40
20
0
26h
26h + TTX

B

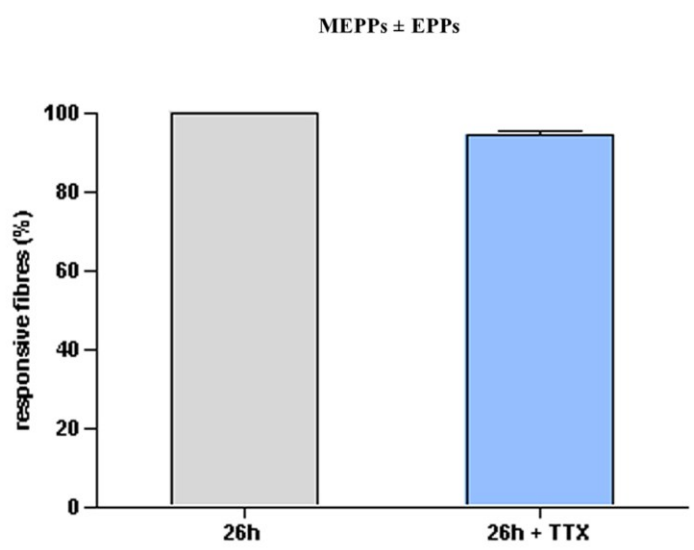

MEPPs ± EPPs
responsive fibres (%)
100
80
60
40
20
0
26h
26h + TTX

Figure 5.7: FK866 delays WT synaptic degeneration in vitro

A. 1μM FK866 did not confer any additional degeneration in *Wld*^{S} muscles (n=9 control, n=5 treated, $p>0.05$), but there was evidence of a dose-dependent enhanced protection in WT muscles with 10μM FK866, though this was not significant (B, n=13 WT, n=4 heterozygous *Wld*^{S}, n= 4 WT + 1μM FK866, n= 5 WT + 10μM FK866, $p>0.05$, one-way ANOVA). When considering also the fibres showing only spontaneous transmitter release, there remained no effect of 1μM FK866 on *Wld*^{S} muscles (C, $p<0.05$), however 10μM FK866 significantly increased the number of functional NMJs in WT muscles (D, $p<0.05$, one way ANOVA).

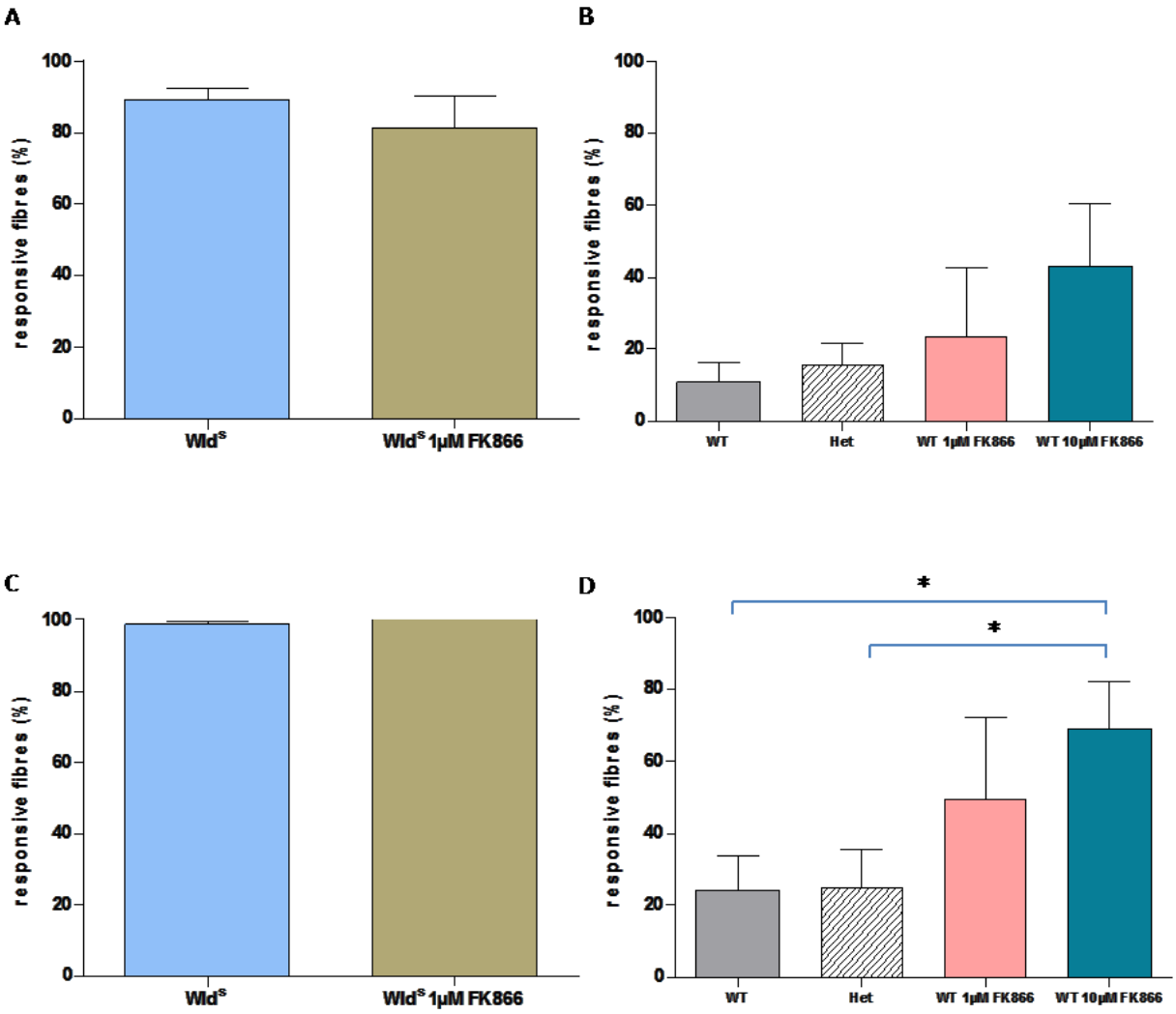
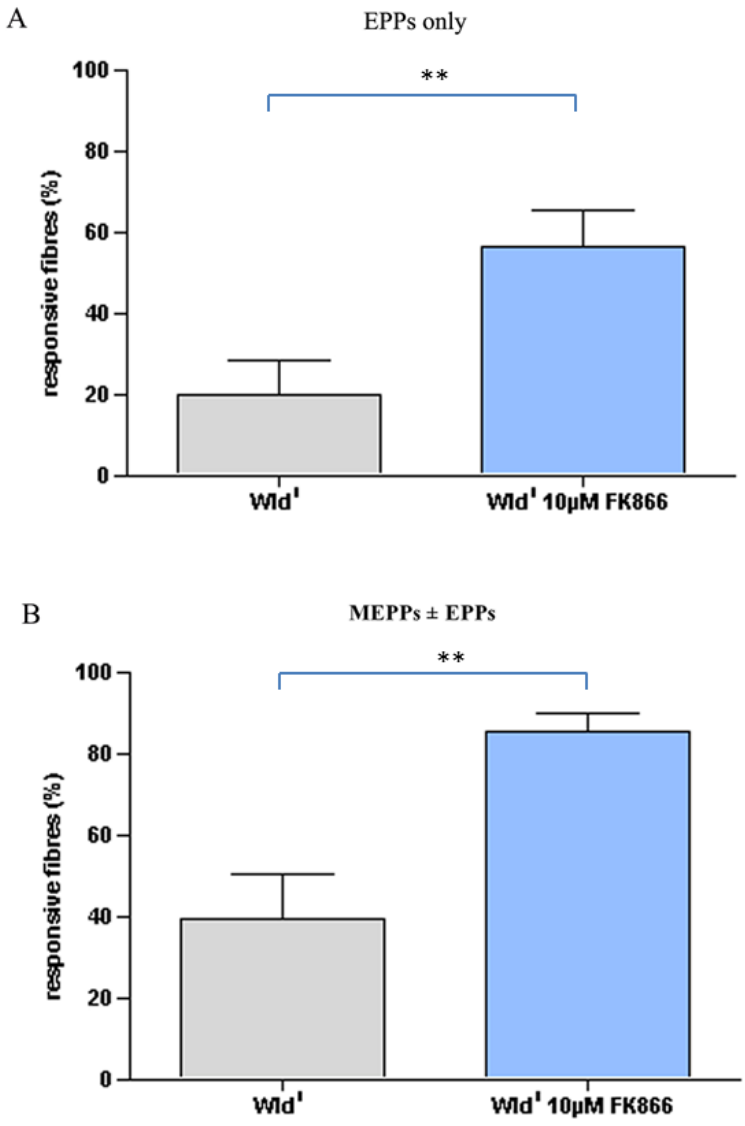

A
B
C
D
responsive fibres (%)
100
80
60
40
20
0
Wld^S
Wld^S 1μM FK866
WT
Het
WT 1μM FK866
WT 10μM FK866
*
*

Figure 5.8: FK866 delays *Wld*^{S} synaptic degeneration in vitro

At 42-48h 10µM FK866 delayed synaptic degeneration in *Wld*^{S} muscles (A, N=8, $p<0.01$), which was also significant when including fibres with only spontaneous transmitter release (B, $p<0.01$, unpaired t-test).


A
EPPs only
**
100
80
60
40
20
0
responsive fibres (%)
Wld'
Wld' 10μM FK866
B
MEPPs ± EPPs
**
100
80
60
40
20
0
responsive fibres (%)
Wld'
Wld' 10μM FK866

5.5 Discussion

The aim of these experiments was primarily to develop a suitable assay for testing the effects of stimulation on FDB muscles. First, I developed a method of sustaining isolated FDB muscles whilst being able to differentiate between the different time courses of *Wld*^{s} and WT NMJs. At 32ºC I was able to clearly distinguish the time course of neuromuscular synaptic degeneration in *Wld*^{s}, heterozygous *Wld*^{s} and WT FDB muscles. Next, I utilised this to determine the effects of stimulation or paralysis on degeneration of motor nerve terminals in FDB muscles. Finally, I investigated the effects of Nampt inhibitor FK866 on synaptic degeneration.

5.5.1 Establishing the Culture System

The time course of degeneration for *Wld*^{s} muscles was noticeably faster than *in vivo* indicating the inability of the *ex vivo* preparation to fully replicate the *in vivo* conditions. Others have also found that degeneration is faster in culture. Tsao *et al.* (1994) maintained *Wld*^{s} nerve explants for 7 days compared to 21 days following transection *in vivo* in young *Wld*^{s} mice. When culturing FDB nerve muscle preparations, Parson *et al.* (2004) only managed to maintain them for up to 72h based on the morphological integrity. However, it has been previously reported that deterioration of synaptic transmission occurs prior to cytological evidence of denervation in axotomised Wld^{s} mouse muscles (Gillingwater et al., 2002).

Based on the physiological methods I used, *Wld*^{s} nerve muscle preparations only survive for up to 48 hours. It is possible that this time course could be extended by refining the culture conditions. One important consideration when studying degeneration is temperature, which can enhance, or slow down, the rate of this process (Birks et al., 1960b, Wang, 1985, Sea et al., 1995, Tsao et al., 1999). For example, cultured nerves survive longer when temperature is reduced from 37˚C to 25˚C (Tsao et al., 1999). Therefore this culture system may be refined by modulation of temperature.

The capacity of this system to separate the time course of WT and *Wld*^{s} degeneration make it a potential test bed for investigating different factors that might mediate or

mitigate synaptic degeneration. Since the focus of my thesis is to investigate the potential of activity-dependent plasticity to modify the degeneration of neuromuscular synapses, I initially attempted to modify the degeneration rate of cultured FDB muscles via electrical stimulation.

5.5.2 High frequency stimulation is detrimental to Wld^{s} NMJ survival

Various studies have demonstrated the ability of stimulation to preserve muscle function following denervation (Al-Amood and Lewis, 1987, al-Amood et al., 1991). Furthermore stimulation has been shown to accelerate developmental synapse elimination (Thompson, 1983, Ridge and Betz, 1984, Busetto et al., 2000, Favero et al., 2010). In these experiments, neither 1 Hz continuous nor 10Hz intermittent stimulation altered the rate of degeneration, though the 1Hz protocol produced a greater variation towards a higher level of survival at 30h. However, it seems that high frequency (100Hz) intermittent stimulation reduced synaptic protection, although this was not apparent when including fibres capable of MEPPs only in to the analysis. Therefore it seems that 100Hz stimulation increases the number of fibres responding with only spontaneous transmitter release in relation to the number of fibres giving an evoked response, which is reduced. Therefore, theoretically, there is a reduction in the number of innervated fibres, but also a greater number in an intermittent stage.

Thus it seems that maintaining a moderate level of activity is not detrimental, yet bursts of high frequency activity are capable of enhancing the rate of synapse degeneration. When considering the results of chapter 3, it seems that either too much or too little activity has a negative effect of NMJ degeneration. In fact, a similar situation is seen with NMDA receptors, with too much or too little activity being detrimental (Hardingham and Bading, 2003).

It has been previously reported that electrical stimulation hinders recovery following sciatic nerve crush (Gigo-Benato et al., 2010) and intermittent high-frequency stimulation does not prevent denervation-induced changes (Nix, 1990). However

long-term stimulation at 20Hz, or acute stimulation is apparently beneficial to recovery following denervation (Ashley et al., 2007, Beaumont et al., 2009, Alrashdan et al., 2010, Kim et al., 2011). Interestingly, detrimental effects of high-frequency stimulation have been reported in the SOD1 rat model of motor neuron disease. Hyperstimulation of the phrenic nerve of SOD mice alters disease progression and shortens the lifespan of these rats, whilst having no adverse effect on control animals (Lepore et al., 2010). This demonstrates the sensitivity of injured or diseased motor neurons to over activity.

There is much variation in the effects of stimulation, that seem to derive from the level of stimulation, frequency and also which factors are studied. For example, in one study stimulation increased the cross sectional area of denervated muscles, particularly in protocols with high frequency bursts of stimuli (Salmons and Jarvis, 2008). However, there was no improvement of fatigue resistance and an incomplete reversal of structural abnormalities resulting from the denervation. In fact, in some cases the fatigue resistance was worsened. Therefore, interpretation of the beneficial or detrimental effects of stimulation must consider the different aspects of functional recovery.

5.5.3 TTX-mediated enhanced synaptic degeneration is not replicated in vitro

In chapter 3 I presented results that show that pre-blocking with TTX enhances degeneration in the FDB and lumbrical muscle. In an attempt to unravel a mechanism for the process, I tested the outcome of removing FDBs following one week of TTX block, placing them in the culture setup and monitoring degeneration. Though I successfully replicated the different time course of degeneration for *Wld*^{S} and WT FDBs, I was unable to reproduce the effects of TTX block in the culture system. Thus it seems that maintaining the muscle in the *in vivo* environment is essential for the increased rate of degeneration to occur, as the rate of degeneration detected in culture was not different from that detected in unblocked *Wld*^{S} preparations. However, the degeneration rate in this assay is already much faster than *in vivo*, so it may be that it is not possible to detect any further enhancement of the

degeneration rate. Therefore investigation of the potential mechanism of TTX mediated enhanced degeneration must be carried out on muscles maintained in vivo, as discussed in chapter 3.

5.5.4 FK866 provides added protection to degenerating FDB muscles

To establish whether the overnight assay was suitable for testing potential pharmacological modulators of synaptic degeneration, I evaluated the effects of FK866. Published data suggest that FK866 is detrimental to *Wld*^{S}, acting by reducing NAD levels (Conforti et al., 2009, Wang and He, 2009). FK866 inhibits Nampt, and so inhibits the formation of NMN and therefore conversion of NMN to NAD via Nmnat (Revollo et al., 2004). It has been demonstrated previously that NAD and NMN levels are reduced by FK866 via its action on Nampt (Formentini et al., 2009, Sasaki et al., 2009). However, it is suggested that FK866 has no effect on Nmnat action as adding NMN to the U937 cell culture in the presence of FK866 increases NAD to the extent seen without FK866. Thus it seems FK866 is a selective inhibitor of Nampt (Formentini et al., 2009).

Interestingly, I found that 10μM FK866 significantly delayed synaptic degeneration in *Wld*^{s} muscles, and there was a trend towards a dose-dependent enhanced protection in WT muscles, which became significant when including analysis of fibres giving MEPPs only upon stimulation. These results appear superficially to conflict with previous findings (Conforti et al., 2009, Wang and He, 2009). However, they are in agreement with one previous study, which reported that cultured DRG neurons displayed a modest level of protection 24h after axotomy in medium containing FK866 (Sasaki et al., 2009). However, the degeneration continued and was complete by 72h. A further conclusion from these studies was that NAD is not required for axonal protection, as cytNmnat1 neurons (in which Nmnat is located in the cytosol and axon) treated with FK866 displayed strong axonal protection. Further evidence for this was provided when Nampt expression was knocked down. Axonal

protection was still conferred by cytNmnat1 and Nmnat1, despite the absence of Nampt and therefore reduction in NAD (Sasaki et al., 2009).

One potential explanation for the mechanism of FK866 mediated protection is that it prevents the formation of NMN. In injured axons it is possible that an accumulation of NMN is toxic and causes rapid degeneration (Sasaki et al., 2009). In *Wld*^{S} muscles, the potential prolonged activity of Nmnat (Gilley and Coleman, 2010) should delay this accumulation and in turn delay axonal degeneration. The effects of FK866 *in vivo* have yet to be determined, but this notion is supported by work that indicates that adding FK866 to WT DRG cultures provides protection against injury (Conforti *et al.* 2011 unpublished observations).

5.5.5 Summary

In summary, incubation of isolated nerve-muscle preparations did not fully replicate the *in vivo* time course of neuromuscular synapse degeneration of *Wld*^{S} mice *in vivo*, nor did it reproduce the effect of pre-blocking with TTX (chapter 3). However, it successfully reproduced differences in degeneration of WT and *Wld*^{S} over a 48hour time frame, and furthermore I have shown the potential of this assay to be an effective test bed for pharmacological and activity-dependent modulators of synaptic degeneration. This assay can be further refined to extend the *Wld*^{S} time course, and to test the effects of alternative modulators of synaptic degeneration. The work should also be extended to morphological studies, for repeating these experiments with YFP expressing mice could enable us to determine whether NMJs in this assay degenerate in a similar manner to that seen *in vivo*, and provide further evidence in support of the physiological data.

6. General Discussion

6. General Discussion

The data presented in this thesis may have an important bearing on the treatment or prevention of neurodegenerative disorders such as MND, Alzheimer's and Huntington's disease. For instance, MND, in which both upper and lower motor neurons are lost, progresses rapidly and eventually leads to death normally from respiratory muscle failure. The incidence of MND in Europe alone is approximately 2 in 100,000 people (Kiernan et al., 2011, Naganska and Matyja, 2011). Over the past few years it has become apparent that MND and other neurodegenerative disorders such as AD share common features. Developing a better understanding of one such disorder may therefore lead to effective treatment of other neurodegenerative diseases of this type. As yet, however, while there are multiple therapeutic strategies, none has yielded a cure or treatment that substantially prolongs the lifespan of patients (Carlesi et al., 2011).

There is substantial controversy over the role played by activity, not only in susceptibility to the disease but also in disease progression (Francis et al., 1999, Dalbello-Haas et al., 2008, Kiernan, 2009). Certain exercise regimes are stated to be beneficial to patients (Milner-Brown and Miller, 1988), yet there is significant evidence that physical activity is a risk factor for developing MND (Longstreth et al., 1991). Studies of activity in an animal model of familial MND, the SOD mutant, have failed to reach a definitive conclusion, and much of the evidence produced is conflicting (Bruestle et al., 2009, Deforges et al., 2009, Carreras et al., 2010). Furthermore the relevance of the SOD mouse model has been questioned in recent years (Scott et al., 2008).

A common mechanism of degeneration is thought to be shared by a group of neurodegenerative disorders (Coleman et al., 2005, Dadon-Nachum et al., 2011, Marcuzzo et al., 2011). The degenerative process leading to loss of synapses and nerves is thought to be related to the process of Wallerian degeneration, first described by Augustus Waller in 1850. Waller showed that after nerve cut, the distal

synapses and axons undergo degeneration (Waller 1850, Pearce *et al.* 2000, Stoll *et al.* 2002). Knowledge of this process has progressed and been shown to be compartmentalised, with the synapses degenerating prior to the main body of the axon (Gillingwater and Ribchester, 2001). This is also true of some neurodegenerative disorders and as such they are known as 'Wallerian-like' or 'dying back' disorders (Raff et al., 2002, Fischer et al., 2004). Therefore further study of the process of WD is essential to our understanding of such disorders, as without the knowledge of the basic mechanisms of WD, our ability to manage and treat neuromuscular disorders is hindered. This has been aided by the discovery of the *Wld*^{s} mouse, in which the process of WD is slowed 10 fold (Lunn et al., 1989, Lyon et al., 1993). Through the study of this mutation we are not only beginning to unravel the process of WD but also the molecular mechanisms involved in the process (Coleman and Freeman, 2010, Gilley and Coleman, 2010).

In this study, I have utilised this model and attempted to determine whether activity can alter the rate of Wallerian degeneration. I focussed on understanding the effect of activity on the degeneration of the NMJ, as the NMJ is the first entity to degenerate in some neurodegenerative disorders (Fischer et al., 2004). The NMJ shows a variety of adaptations to alterations in its pattern of use (Thompson, 1983, Tsujimoto et al., 1990, Desaulniers et al., 2001), although the effect of activity on its degeneration has not been fully investigated.

In the first results chapter, I provide evidence that strongly suggests removing neuromuscular activity mitigates rapid NMJ loss following nerve injury. Pre-treatment with TTX substantially increased the rate of synapse degeneration. Although this may appear surprising in light of the effect of paralysis on polyneuronally innervated NMJs, in which TTX apparently stabilises these connections (Brown et al., 1982, Costanzo et al., 2000), it is likely that synapse withdrawal and synaptic degeneration are two distinct processes (Parson et al., 2004).

The exact mechanism for this finding is not yet clear. It is not likely that TTX interferes with factors that are essential for axonal survival, such as Nmnat (Coleman and Freeman, 2010, Gilley and Coleman, 2010), as application of TTX alone does

not cause degeneration. TTX also does not cause this response by blocking axonal transport (Lavoie et al., 1976, Pestronk et al., 1976a, Czeh et al., 1978). Furthermore, the effect of TTX is specific to the synapse, indicating an enhancement of the mechanisms that causes synapses to degenerate prior to the nerve. It has already been established that TTX causes alterations in enzymatic activity (Carr et al., 1998), gene expression (Tang et al., 2000), muscle fibre (Bray et al., 1979, Labovitz et al., 1984, Buffelli et al., 1997) and AChR expression (Lavoie et al., 1976, Pestronk et al., 1976a, b) similar to that caused by denervation, without actually causing denervation itself (Lavoie et al., 1976, Betz et al., 1980). For example, TTX alters the activity of genes involved in apoptosis (Tang et al. 2000). Perhaps by inducing such changes, TTX enhances susceptibility to insult (Cangiano et al., 1984a). It will be of interest to determine whether inactivity can enhance susceptibility to other forms of injury, such as hypoxia, or in models of neurodegeneration such as the SOD1 mouse. Although complete pharmacological block of activity can be argued to be unphysiological, these results enhance our knowledge of the activity dependence of synaptic degeneration, and imply that maintaining activity is important in preventing rapid NMJ degeneration.

I also tested whether subjecting *Wld*^{s} mice to voluntary wheel running activity for 2-4 weeks would enhance or delay synaptic degeneration after axotomy. Neither two nor four weeks of wheel running conferred any discernible effect on the number of fibres giving an evoked response upon stimulation 5 days post-axotomy. Thus exercise, or at least environmental enrichment, did not produce any substantial effect on synaptic degeneration, suggesting that activity, at least in this experimental paradigm is neither beneficial nor harmful in its effects on synapse degeneration. As discussed in chapter 4, the use of the *Wld*^{s} mouse is limited by the age-dependency of its phenotype (Gillingwater et al., 2002). However, recently developed mouse models with extended synaptic protection could be used to overcome this problem such as the ΔNLS-Wld^{s} mouse, in which synapse protection is significantly extended by reducing the nuclear localisation of Wld^{s} (Beirowski et al., 2009). These mice also do not display the same age-dependent phenotype seen in normal *Wld*^{s} mice, and therefore might extend the paradigm and provide a more versatile test-bed for

investigating the effect of exercise at the NMJ, since the lack of age-dependency of the phenotype means that these mice could be exposed to a much longer exercise protocol, which may be more likely to elicit an effect at the mouse NMJ (Allen et al., 2001, Kariya et al., 2004). It may also be necessary to consider an alternative protocol such as treadmill training or electrical stimulation, which allows a more controlled exercise protocol, which may produce more robust changes. However, this running wheel protocol did elicit some alteration in FDB NMJ function. Despite no morphological alterations, exercised muscles displayed alteration in QC and MEPP amplitude confirming that four weeks of voluntary exercise can evoke some neuromuscular plasticity (Fahim, 1997, Desaulniers et al., 2001).

These two studies, paralysis and wheel-running, demonstrate the adaptability of synaptic function to altered activity levels. Although the exact mechanisms behind these alterations are unclear, it is known that altering activity levels leads to homeostatic regulation of synaptic function, capable of maintaining synaptic activity at a stable level (Davis, 2006, Pozo and Goda, 2010, Vitureira et al., 2011). It seems that alteration of activity levels detected at the postsynaptic site leads to retrograde signaling that alters overall activity levels (Davis, 2006, Pacifici et al., 2011). For example, altering activity input alters the amount of transmitter released. Inactivity at hippocampal synapses results in an increase in the number of vesicles in the synapse and an increase in active zone size (Murthy et al., 2001). Further insight has been provided by Drosophila models. Alteration of activity levels at the postsynaptic site via genetic modification of postsynaptic glutamate receptor expression leads to an increase in the number of vesicles in the ready releasable pool, alteration in quantal size, along with enhanced expression of some active zone proteins such as bruchpilot (DiAntonio et al., 1999, Weyhersmuller et al., 2011).

Despite the lack of effect of voluntary running activity, using an *in vitro* stimulation paradigm, I showed that high frequency intermittent stimulation reduced the number of fibres capable of producing an evoked response to stimulation, indicating that both paralysis and overstimulation exacerbate synaptic degeneration. Lower frequency stimulation had no effect on the level of survival, although 1 Hz continuous

stimulation seemed to increase protection, though this was not significant. From this we are able to hypothesise a model of activity-dependent synaptic degeneration, with either too much or too little activity being detrimental. Thus it seems that the level of activity sustained after nerve damage ought to be carefully considered. However, to fully investigate this conclusion the results must be replicated *in vivo*. In previous studies, treadmill running has been reported by some to be detrimental (Mahoney et al., 2004), whereas moderate running wheel activity had no effect on disease progression (Liebetanz et al., 2004). Carreras *et al.* (2010) found that high intensity exercise slightly hastened disease onset, whereas moderate exercise slightly delayed it. Medium intensity exercise also showed an early increase in the density of motor neurons in the spinal cord compared to controls, but this was lost by 120 days. This highlights the importance of the intensity and type of exercise in determining the progress of degeneration.

In this study, I was unable to identify an activity-based paradigm that would be of unequivocal or complete benefit to neuromuscular synaptic survival. However, when I extended this study to pharmacological modulators of synaptic degeneration, the results were more promising. It is now thought that Nmnat2 acts as a survival factor for axons, and its presence is essential for their survival. Following injury, the levels of Nmnat2 fall, leading to degeneration of the axon and synapses. Wld^s is thought to slow this process and extends the survival of axons and NMJs by substituting for Nmnat2 (Gilley and Coleman, 2010).

The results of applying FK866, a selective inhibitor of Nampt, demonstrated not only the protective effects of this process, but also provided further supporting evidence that the NAD cycle is involved in neuronal degeneration. It seems that it is essential to maintain the correct balance of various components of the NAD cycle. As others have suggested, in degenerating synapses perhaps a toxic level of NMN builds up after injury, and Wld^s removes this toxic substrate by sustaining Nmnat activity (Sasaki et al., 2009). According to this model, FK866 causes a depletion of NMN (Formentini et al., 2009), which results in protection against degeneration.

We must investigate this further by measuring NMN, NAD and Nmnat activity in FK866 treated preparations in comparison to normal and axotomised preparations. It

would also be of interest to study the effects of other potential pharmacological modulators of synaptic degeneration, such as other modulators of the NAD cycle. The insight into this mechanism, supported by work from our colleagues in Babraham (manuscript in preparation) could substantially benefit the field of therapeutics. It also seems to be of importance to further investigate the sub-cellular organelles and processes involved in the axonal and synaptic protection mediated by *Wld*^{S}.

In summary, the results in this thesis suggest that maintaining a moderate level of activity is essential for preventing rapid loss of synaptic transmission after denervation. It also seems that it is possible to enhance synaptic protection with chemical compounds, and through novel methods of testing their efficacy this could lead to the identification of treatments or target pathways that could benefit the victims of neurodegenerative disorders.

7. Bibliography

7. Bibliography

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