

Protection of neuromuscular sensory endings by the Wld^S gene

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Declaration

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

Oyinlola Oyebode

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I would like to say a big thankyou to my supervisor Richard Ribchester who found me a project, was encouraging all the way through and gratifyingly fell for an April fool in the middle. I would also like to acknowledge members of the Ribchester and Coleman laboratories, past and present, for protocols and technical assistance, with special thanks to Derek Thomson for keeping all the mouse records and knowing where everything is, Jasjot Singhota for piloting the spindle teasing and Adrianna Teriakidis for introducing me to neuroinformatics, the DTC and making lab-work so much fun.

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Abstract

The compartmental hypothesis of neurodegeneration proposes that the neurone, long recognized to consist of morphologically and functionally distinct compartments, also houses distinct degeneration mechanisms for the soma, axon and nerve endings. Support for this hypothesis is provided by the phenomenon of the *Wld^S* (for Wallerian Degeneration, slow) mouse, a mutant in which axons survive several weeks after transection, rather than degenerating within 24-48 hours as in wild type mice, by virtue of expression of a chimeric Nmnat1/Ube4b protein. In this thesis I used the *Wld^S*-mouse to re-examine and extend the theory of compartmental neurodegeneration by focusing specifically on sensory axons and endings; and finally by considering a fourth compartment, the dendrites.

The first part of this thesis reports that Ia afferent axons and their annulospiral endings are robustly protected from degeneration in *Wld^S* mice. Homozygous or heterozygous *Wld^S* mice crossbred with transgenic mice expressing fluorescent protein in neurones were sacrificed at various times after sciatic nerve transection. Fluorescence microscopy of whole mount preparations of lumbrical muscles in these mice revealed excellent preservation of annulospiral endings on muscle spindles for at least 10 days after axotomy. No significant difference was detected in the protection with age or gene copy-number in contrast to the protection of motor nerve terminals, which degenerate rapidly in heterozygote and aged homozygote *Wld^S* mice.

In an attempt to explain the difference in motor and sensory protection by *Wld^S*, examination of three hypotheses was undertaken: a) differences in protein expression, tested by western blot and immunohistochemistry; b) differences in the degree of neuronal branching, tested through examination of γ -motor axons and endings which have a degree of branching intermediate to motor and sensory neurons; and c) differences in the activity in the disconnected stumps, through primary culture of the saphenous and phrenic nerve, selected because they comprise largely pure sensory and motor axons respectively. The data suggest that none of these hypotheses provides a sufficient explanation for the difference between sensory and motor protection by *Wld^S*.

The last part of this thesis attempts to extend the theory of compartmental degeneration. I examine a system for investigation of *Wld^S*-mediated protection of dendrites. In preliminary experiments retinal explants from transgenic mice expressing YFP in a subset of retinal ganglion-cell neurones were cultured. The dendritic arbours of these cells were shown to be amenable for repeated visualization and accessible to injury and monitoring of degeneration.

Overall the data in this thesis suggest that the level of *Wld^S*-mediated protection conferred to an axon or axonal endings varies between different neuronal types. This has implications for the potential applications of *Wld^S* research to clinical problems. Specifically, the data imply that sensory neuropathies may benefit more than motor neuropathies from treatments based on the protective effects of *Wld^S*. These findings in sensory neurones also challenge some of the assumptions made about *Wld^S*-mediated protection of neurones, for example the extent of the age-effect on axonal endings. Further investigation of *Wld^S*-mediated protection in the CNS could give renewed impetus to attempts to discover targets for treatment in common neurodegenerative diseases. Finally, a system for investigation of dendritic degeneration has been piloted, suggesting that molecules involved in the degeneration of dendrites or in protection from this degeneration may be amenable to investigation in this system, prospectively extending the compartmental hypothesis of neuronal degeneration.

Abbreviations

| | |
|------------|--|
| AChR | Acetyl Choline Receptor |
| AD | Alzheimer's Disease |
| ALS | Amyotrophic Lateral Sclerosis |
| ANCOVA | Analysis of covariance |
| ANOVA | Analysis of variance |
| APAF-1 | Apoptotic Protease-activating Factor-1 |
| APS | Ammonium persulphate |
| ATP | Adenosine Triphosphate |
| BTX | Bungarotoxin |
| CNS | Central Nervous System |
| DISC | Death-inducing signalling complex |
| DRG | Dorsal Root Ganglia |
| EAE | Experimental Autoimmune Encephalomyelitis |
| FDB | Flexor Digitorum Brevis |
| FITC | Fluorescein Isothiocyanate |
| <i>Gad</i> | Gracile axonal dystrophy |
| HD | Huntingdon's Disease |
| NAD | Nicotinamide Adenine Dinucleotide |
| Nmnat | Nicotinamide mononucleotide adenylyl transferase |
| HIV | Human Immunodeficiency Virus |
| NMJ | Neuro Muscular Junction |
| PBS | Phosphate Buffered Saline |
| PCD | Programmed Cell Death |
| PD | Parkinson's Disease |
| PFA | Paraformaldehyde |
| <i>Pmn</i> | Progressive motor neuropathy |
| PNS | Peripheral Nervous System |
| RGC | Retinal Ganglion Cell |
| SCG | Superior Cervical Ganglia |
| SDS-PAGE | Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis |
| TEMED | Tetramethyl ethylene diamine |
| TRITC | Tetramethylrhodamine Isothiocyanate |
| TNF | Tumour Necrosis Factor |
| Ube4b | Ubiquitination Factor E4b |
| UPS | Ubiquitin Proteasome System |
| VCP | Valosin-containing protein |
| WT | Wild Type |

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I Introduction

Understanding neural degeneration is one of the most important issues in clinical neuroscience.

Classical neurodegenerative diseases include those diseases defined by progressive and irreversible loss of neurones from the nervous system. Neurodegenerative diseases are responsible for a major portion of the economic burden of ill health (Waldmeier and Tatton, 2004). These diseases effect tens of millions of people worldwide and dementias associated with neurodegenerative disease are expected to double between 2001 and 2040 in developed nations, and to increase by more than 300 percent in India and China (statistics from the Alzheimer's Research Trust).

Alzheimer's disease (AD), the most common of the neurodegenerative diseases, is a progressive neurodegenerative disorder associated with loss of cells from the cortex and hippocampus. Other common neurodegenerative diseases of the Central Nervous System (CNS) include Parkinson's disease (PD), a neurodegenerative movement disorder characterized pathologically by the loss of dopaminergic neurones in the substantia nigra pars compacta and Huntington's disease (HD), a genetic autosomic disease characterized by select degeneration of neurones in the striatum (reviewed in Okouchi et al, 2007).

Amyotrophic lateral sclerosis (ALS) is a motor neurone disease characterized mainly by the degeneration of the anterior horn cells of the spinal cord (or motor neurones, historically known as lower motor neurones) and cortical neurones that project through the corticospinal tract to the anterior horn cells (historically known as upper motor neurones). In addition some ALS patients show degenerative signs in the front-temporal cortex, compounding their motor symptoms with dementia (Phukan et al, 2007).

Neurodegeneration also has a key role in the pathogenesis of diseases not normally classed as neurodegenerative. For instance, while demyelination is responsible for the early signs of multiple sclerosis, neurodegeneration may play a role in

irreversible damage that occurs as the disease progresses (Bjartmar and Trapp, 2003). Neurodegeneration can also be a complication associated with diabetes (Toth et al, 2007) and infection with human immunodeficiency virus (HIV) (Ozdener, 2005). Decreased hippocampal volume in patients with depression suggests there may even be a role of neurodegeneration in psychiatric disorders (Brink et al, 2006).

Neurodegenerative diseases usually progress over an extended time frame, at the shortest 1-2 years (in the case of ALS) up to several decades (in AD, PD or HD). This extended time frame provides opportunity for intervention between diagnosis and death. Research into ways to delay or arrest degeneration in the nervous system could have a major impact on many diseases and therefore the quality of many peoples' lives.

Given the hallmark of neurodegenerative disease is loss of neurones; much research has focused on preventing neurones from undergoing programmed cell death. This continues today with a raft of drugs that interfere with neuronal death being trialed (Waldmeier and Tatton, 2004). However recent evidence suggests that before the neurone dies, changes occur, which may, more closely correlate with disease progression. Decreased neuronal size, atrophy of neuronal dendrites and reductions in axonal terminal fields have also all been identified in neurodegenerative disease (Waldmeier and Tatton, 2004). In AD there is evidence of synaptic alterations that predate neuronal death (Coleman et al, 2004; Selkoe, 2002; Terry et al, 1991; deKosky and Scheff, 1990). HD and HIV associated dementia also appears to begin with synaptic pathology and this pathology is closely correlated to disease progression (Smith et al, 2005; Bellizzi et al, 2006). Neuromuscular terminal and Axonal degeneration are major pathological features of ALS and occur before the death of motor neurones (Frey et al, 2000, Fischer et al, 2004; Fischer and Glass, 2007).

In the face of increasing evidence that degeneration of other neuronal compartments precedes cell death, the benefits of a focus on drugs that act to preserve neuronal cell bodies alone become questionable. Indeed, a number of experimental studies demonstrate that improving survival of neurones does not have an effect on the onset

of disease, for example in ALS (Sagot et al, 1995), or maintain neuronal function (Iyirhiaro et al, 2008). This suggests that therapies that concentrate on protection of the axon, dendrites and synapses in the nervous system must also be developed.

I.1 The somatic Peripheral Nervous System

All the spinal nerves that innervate the voluntary muscles and the skin are part of the somatic peripheral nervous system (PNS), on which this thesis is focussed. This comprises sensory (afferent) and motor (efferent) axons carrying information between the central nervous system (CNS) and the body. Here I will briefly review these components of the somatic peripheral nervous system, whose maintenance and degeneration are examined further in the following thesis.

I.1.1 Overview

In mammals, the skeletal musculature is innervated by somatic motor neurones which have cell bodies located in the ventral horn of the spinal cord grey matter. The axons of motor neurones bundle together to form ventral roots. The cell bodies of sensory neurones are located within the dorsal root ganglia (DRG), the axons of these unipolar neurones branch in dorsal root, one branch projecting into the spinal cord and the other projecting peripherally. Each ventral root joins a dorsal root to form a spinal nerve that exits the cord between the vertebrae. Sensory and motor neurones project to specific muscles.

Motor neurones can be divided into two categories, according to morphology and function, as α - and γ -motor neurones. Sensory neurones can also be classified according to morphology and function. The smallest sensory neurones have unmyelinated axons (C-fibres or Group IV afferents) and receptors which mainly detect noxious stimuli, perceived as pain. Several intermediate myelinated axon sizes exist which respond to noxious or temperature stimuli ($A\delta$ fibres, Group III), or non-noxious touch sensations for example pressure ($A\beta$ fibres, Group II) or vibration ($A\alpha$, Group I). The largest sensory neurones with large myelinated axons supply the

muscle spindle organs. The innervations of muscle spindle organs is a focus of the research described in much of this thesis.

I.1.2 Peripheral nerve

Axons travelling in peripheral nerve are protected by three layers. The endoneurium contains the axons and their associated Schwann cells along with endoneurial fluid and connective tissue. The perineurium is a dense connective tissue formed by up to 15 layers of flat perineurial cells, interspersed with collagen fibrils and elastic fibres. This groups bundles of axons together to make fascicles and forms a diffusion barrier that protects the endoneurial space. The epineurium is another connective tissue layer which encircles both the fascicles and the entire nerve.

Within the endoneurium, all axons are associated with Schwann cells. The myelin of each myelinated axon is formed from the plasma membrane of Schwann cell which is wrapped multiple times around the axon. A series of Schwann cells will be associated with an axon along its length and at the gap between adjacent Schwann cells is a gap known as the node of Ranvier which allows saltatory conduction along the myelinated axons. Unmyelinated axons are also enveloped by Schwann cell cytoplasm and plasma membrane, but a single Schwann cell may associate with several unmyelinated axons (reviewed in Topp and Boyd, 2006).

I.1.3 α -motor neurones

α -motor neurones innervate large diameter extrafusal muscle fibre and are therefore directly responsible for the generation of force by muscle. One α -motor neurone and all the muscle fibres it innervates are called one motor unit. Motor unit sizes range from a few to several hundred muscle fibres. Muscle contraction results from the individual and combined action of these motor units.

The α -motor neurone causes muscle contraction through its innervations of muscle fibres at specialised chemical synapses known as neuromuscular junctions. Each muscle fibre is innervated by a single motor axon. At this site the membrane of the muscle fibre is invaginated forming the junctional folds (Roberts, 1963; Sanes and

Lichtman, 2001). Nicotinic acetylcholine receptors (AChR) are concentrated at the peaks of each fold. In the troughs are voltage-gated sodium channels responsible for triggering a muscle action potential in response to local depolarisation produced by activation of the AChRs (Wood and Slater, 2001). The terminus of the motor axon lies in register directly, opposing the junctional folds. Specialised active zones in the presynaptic terminal are in perfect alignment with the AChRs, separated by a 50nm gap, the synaptic cleft. The active zones are foci for clusters of synaptic vesicles, each packed with 1 quantum, or approximately 5000 molecules, of acetylcholine (ACh). The tight co-alignment of pre-synaptic motor axon terminal and post-synaptic junctional folds in the muscle fibre membrane ensures high fidelity transmission between the terminals of a motor neurone and contraction of the muscle fibres (Wood and Slater, 2001).

I.1.4 Sensory innervation of muscle

Muscle spindle organs are specialised to detect changes in muscle length and the associated sensory neurones conduct this information to the CNS where it is used in proprioception, the sense of where the body is in space. This organ is made up of specialised muscle fibres, known as intrafusal muscle fibres, supplied by one or more sensory neurones and γ -motor neurones mentioned below [Figure I.1].

There are 2 distinct types of intrafusal muscle fibre known as nuclear bag and nuclear chain fibres. These are contained in a fibrous capsule which is swollen and filled with endolymph in the equatorial region, conferring the characteristic shape of a spindle for which it is named. The muscle fibres are morphologically distinct from the extrafusal muscle fibres that make up the bulk of the muscle. Bag fibres contain a large cluster of nuclei in the equatorial region of the fibre, and chain fibres have them distributed along their length (Banks et al, 1978).

Two types of sensory neurone innervate the muscle spindle; Group Ia and Group II. Group Ia neurones have larger axons (8-14 μ m diameter) which terminate in the equatorial region, forming annulospiral endings that wind around the intrafusal muscle fibres. These terminations are described as annulospiral. Group II neurones

have slightly smaller axons (6-12 μm diameter) which terminate adjacent to the primary ending. The II termination is commonly described as having a flower spray appearance on contact with the intrafusal muscle fibres. Classical physiological studies show that Ia afferents respond partly to muscle length but respond more powerfully to changes in length. II afferents have a response that correlates to muscle length only (Barker and Scott, 1990; Banks et al, 1982).

When a muscle is contracted the intrafusal muscle fibres change in length due to the forces exerted on them by the extrafusal muscle fibres. The sensory receptors signal this change, responding to the length or change in length of the muscle as they are specialised to do. If a muscle is stretched and then held at the new position, the primary endings discharge at high frequency during the change in length, while secondary endings will demonstrate a less marked increase in discharge during stretch. Once the muscle is being held at one length the primary ending adapts and the discharge returns to somewhere near baseline (although not quite, primary endings do have some sensitivity to muscle length). However, secondary endings will continue to fire at an increased rate while the muscle is held in a stretch (Kucera et al, 1991; Bear et al, 2001).

As well as contributing to proprioception and kinesthesia, the muscle spindle organ can also regulate motor control. Ia and II afferents input on to the α -motor neurones supplying the same (ie: homonymous) muscle that contain the spindle. In the case of Ia afferents this input is direct, through one synapse in a pathway known as the monosynaptic reflex arc. Ia afferents can therefore powerfully excite the homonymous α -motor neurones. This is the basis of the classical stretch reflex where extension of a muscle causes a reflex contraction. Spindle afferents also monosynaptically and disynaptically innervate motor neurones that are synergistic in function to the homonymous motor neurone, and to interneurons that then synapse with antagonistic motor neurones (Windhorst, 2007; Hunt, 1990).

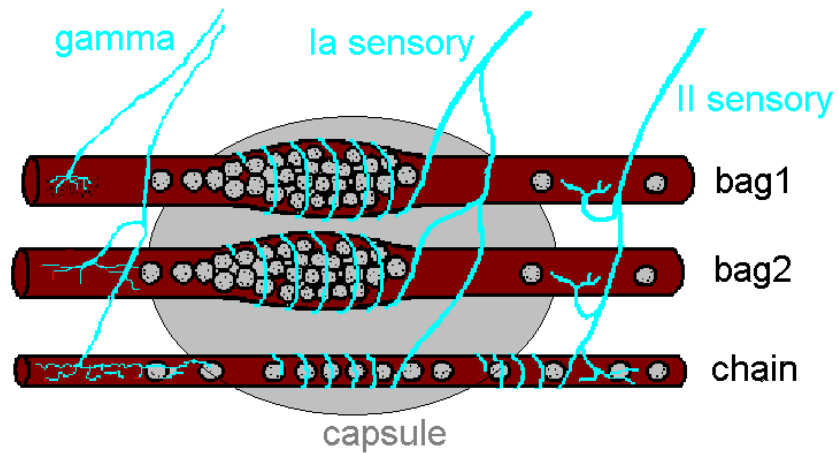


Figure I.1 Diagram of a Muscle Spindle

A diagram showing a simplified representation of a muscle spindle. Each component is shown; Bag and chain intrafusal fibres, with their characteristic shape and nuclear localisation, a Ia sensory axon with annulospiral termination within the capsule, a II sensory axon terminating with a flowerspray or incomplete annulospiral morphology beside the primary ending and γ -motor axons which terminate at the poles of the intrafusal muscle fibres.

I.1.5 γ -motor neurones

In addition to the afferent sensory innervation of the intrafusal muscle fibres there is also efferent motor innervation by the γ -motor neurones. These small (3-8 μ m diameter), myelinated axons innervate intrafusal muscle fibres at neuromuscular junctions located at the poles of the muscle spindle. This input from the highly specialised small motor neurones is also known as fusimotor innervation.

The γ -motor innervation of the muscle spindle provides additional control of α -motor neurones and muscle contraction. For instance, if the muscle spindle becomes slack as a consequence of α -motor discharge and muscle shortening, the afferent discharge is reduced and the spindle can no longer report subtle changes in the length of the muscle. Activation of the γ -motor neurones brings about contraction of the poles of the spindle, restoring spindle tone and sustaining sensitivity of the equatorial regions to changes in muscle length. There may be many other more complex roles that fusimotor innervation of the muscle spindles plays which are still to be investigated (Hunt, 1990).

I.2 Wallerian degeneration

In 1850 Augustus V. Waller published the paper "Experiments on the section of the glossopharyngeal and hypoglossal nerves of the frog, and observations of the alterations produced thereby in the structure of their primitive fibres." He had shown that when an axon was disconnected from the neural cell body, the distal portion underwent degeneration, while the proximal segment remained intact.

Three days after a lesion to the innervation of the tongue of the frog, Waller noted that there was a loss of muscular contraction and sensation. Examination of the nerve stump revealed discontinuous, fragmented and granulated axons (Waller, 1850). This axonal degeneration was subsequently named Wallerian degeneration, after him.

The histology of Wallerian degeneration is well established, although various factors can affect the speed of its completion. These factors include, for example, temperature (Tsao et al, 1999a) and the calibre of the axon including length, diameter and myelination (Lubinska, 1977; Weddel and Glees 1941; Friede and Martinez, 1970). The age of the animal also affects the rate of Wallerian degeneration with older animals demonstrating a slower rate than young animals (Cook et al, 1974). The following description is based upon the electron microscopic study of rat sural nerve, by Ballin and Thomas (Ballin and Thomas, 1969). The first abnormalities include accumulation of organelles, including mitochondria, noticeable at 12 hours and plainly evident at 24 hours. Microtubules fragment and lose their orientation before axonal continuity is interrupted at 30 hours. The first changes in the myelin surrounding the axon can be seen at 24 hours after injury. The myelin sheath collapses and remains within the parent Schwann cell cytoplasm where it begins to be degraded (Ballin and Thomas, 1969). Phagocytosis of the remnants of the degenerating distal axon is initially undertaken by activated endoneurial macrophages (Leonhard et al, 2002) and later by monocytes and macrophages recruited from the blood stream (Mueller et al, 2001; Hirata and Kawabuchi, 2002). In the peripheral nervous system (PNS) the remnants are cleared 4 or 5 days after the injury occurs. However, in the CNS, though axons disintegrate over the same time course as in the PNS, hematogenous macrophages are not recruited to the distal stump and the clear up can take months (Perry et al, 1987).

After Wallerian degeneration is complete, the severed neurones in the adult peripheral nervous system normally sprout new axons which will then re-establish connections. For this to occur, a growth permissive environment must be created (Kury et al, 2001). Schwann cells proliferate and align within basal lamina tubes forming what are known as Bands of Bungner. These appear to guide growing axons. Once the axons have contacted their targets, the proliferated Schwann cells align and re-myelinate them (Kury et al, 2001).

Waller's work led him to conclude that axons were dependent on the cell body for nourishment and that disconnection caused them to passively 'starve' to death. More

than 150 years later, this conclusion was challenged with the discovery of a mutant mouse in which axons survive several weeks after transection rather than degenerating with 24-48 hours as in wild type. This mouse is named *Wld^S* mouse for ‘WaLlerian Degeneration Slow’ (Lunn et al, 1989; Mack et al, 2001), although it was previously referred to as “Ola” mouse, before the mutant gene was mapped (Lyon et al, 1993).

Initially it was suggested the reason for the slow progression of Wallerian degeneration was due to a disruption in the recruitment of myelomonocytic cells to the distal portion of the axon (Lunn et al, 1989). However, transplant experiments (Perry et al, 1990a), graft experiments (Glass et al, 1993) and tissue culture experiments (Perry et al, 1990a; Deckwerth and Johnson, 1994; Glass et al, 1993) demonstrated that it was an intrinsic property of the axons/neurites.

The discovery of inherently slow degeneration of axons in the nerves of the *Wld^S* mouse indicated that Wallerian degeneration is not simply a passive wasting away of the axon because it has been disconnected from its source of ‘nourishment’. Rather this suggests that Wallerian degeneration is a self-destructive mechanism that the disconnected axon undergoes in response to injury. Similarities between this process and another well known mechanism for self-destruction suggest that Wallerian degeneration may be similar in principle to apoptosis (Gillingwater and Ribchester, 2001) [See I.7].

I.2.1 Mechanisms and molecules of Wallerian degeneration

Little is known about what signals to the axon to self-destruct or how this self-destruction occurs.

Severed axons maintained in EGTA, a calcium buffer, degenerate more slowly than those in high-calcium media. Calpains, calcium dependent proteases, have been demonstrated to cleave the major axonal proteins (Schlaepfer and Bunge 1973). Using murine dorsal root ganglia in cell culture, George et al (1995) examined the role of calcium and the calpains in Wallerian degeneration.

When extracellular calcium levels were reduced by adding EGTA to the culture media after axotomy, the axons developed a beaded appearance but failed to degenerate within a week after axotomy, as opposed to complete degeneration by 48-60 hours. Both A23187 and ionomycin, which elevate intra-axonal calcium, produce degeneration of uncut axons when added to dorsal root ganglia cultures. George et al demonstrated that the calcium entry in Wallerian degeneration is not simply due to disruption of the membrane, by showing that cobalt and manganese, non-specific calcium-channel inhibitors, effectively delayed axonal degeneration after axotomy. Conotoxin does not block axonal degeneration indicating that calcium enters through channels other than N-type calcium channels during axonal degeneration. Both leupeptin analogues and oxiranes, two classes of calpain inhibitor, preserve axotomised axons in a concentration dependent manner. This evidence gives a strong indication of an importance for calcium entry and calpain activity in Wallerian degeneration.

De Jonge et al have shown that components of the complement system are produced in healthy peripheral nerve and activated during Wallerian degeneration (De Jonge et al, 2004). Depletion of C3 or C5 (extinguishing the complement cascade) causes delayed Wallerian degeneration (Dailey et al, 1998; Liu et al, 1999) as does deficiency of C6, highlighting the Membrane Attack Complex as important for normal Wallerian Degeneration (Ramaglia et al, 2007). Recently, Ramaglia et al have investigated the effects of inhibiting all arms of the complement cascade with treatment with a soluble form of Complement Receptor 1 (sCR1), and inhibition of specifically the classical and leptin pathways with a C1 inhibitor (Cetor). Treatment was started 1 day before crush injury of the sciatic nerve. They report that 3 days after crush injury sCR1 treated nerves showed more neurofilament and myelin staining as well as more axon profiles in electron micrographs, whereas Cetor treated nerves, though they had some preserved myelin did not have axon profiles. Phosphate Buffered saline (PBS)-treated nerves showed loss of axons as well as collapsed and degraded myelin. Seven days after injury axons in all animals were

degenerated, demonstrating that Wallerian degeneration is delayed but not completely blocked in complement inhibited animals.

From the work described above, it would appear that calcium entry and calpain activation, followed by complement activation are necessary for Wallerian degeneration to proceed normally. However, what signalling originally causes the calcium entry is unknown.

1.3 The *Wld^S* phenotype

The slow Wallerian degeneration phenotype characteristic of *Wld^S* mice is caused by a spontaneous mutation in a line of C57/Bl6 mice originally supplied by commercial animal breeders and providers, Harlan-Olac laboratories. Superficially, these mice are not discernibly different from any other C57/Black mouse lines and behave and breed normally. What distinguishes them is the finding that Wallerian degeneration is significantly delayed in these mice after nerve injury. This phenotypic characteristic was discovered by chance during investigation into the role of the recruitment of myelomonocytic cells in Wallerian degeneration of mouse peripheral nerve by MC Brown, VH Perry and colleagues (Lunn et al, 1989).

Wld^S animals exhibit slow Wallerian degeneration after nerve injury in both the peripheral and central nervous system including optic nerve and striatum of the mouse as well as delayed degeneration of motor nerve terminals (Gillingwater et al, 2006b; Ludwin and Bisby, 1992; Ribchester et al, 1995). However, there are many subtleties to the phenotype which will be described below.

1.3.1 Effect of age on the *Wld^S* phenotype

Soon after the discovery of the *Wld^S* mouse, an effect of age on the phenotype was noted. During work to map the location of the gene through linkage testing, Lyon et al were confounded by the apparent shortage of mutants among the older animals. This weakening of the phenotype was investigated itself and described by Perry et al (1992).

Perry et al. reported that 5 days after section, sciatic nerve from 4 week old *Wld^S* mice exhibits compound action potentials, while sciatic nerve from 12 month old mice does not. They also report that using mice 8 weeks and older, they were only able to obtain neuromuscular transmission up to 2 days after sciatic nerve cut. However, in 4 week old animals they were able to elicit neuromuscular transmission 5 days after nerve section. Electron microscopy confirmed their findings, nerves fixed 5 days after axotomy showed almost no abnormality in sciatic nerve from 4 week old animals; however 27.3% and 36.3% of axons appeared abnormal in the nerves from 1 year old animals. (In any other mouse strain all axons would be completely degenerated by this point).

Two years later Tsao et al (1994) confirmed this finding noting that compound action potentials, as well as neurofilament immunoreactivity, were both utterly extinguished at 2 days post transection in wild type mice, but could still be detected at 21 days post-transection in 1 month old *Wld^S* mice. However, these were barely detectable at 14 days in 6 month old *Wld^S* mice or at 7 days in 12 month old *Wld^S* mice.

A systematic morphological, histochemical and immunochemical study was published contesting these findings (Crawford, 1995). Crawford et al reported no difference in the degeneration rates of axons in 1, 3, 6 and 16 month old mice. Mice of all ages had similar numbers of surviving axons at 7, 14 and 21 days after injury and they had a similar amount of total axon area. They also found no difference in the number of innervated neuromuscular junctions 5 days post axotomy in the muscles of 1 and 7 month old animals (in the gastrocnemius muscle).

The final published word on this (to date) is the paper by Gillingwater et al, published in 2002. Gillingwater et al reported that axonal protection is independent of age and therefore, agree with Crawford et al. However, they also provided compelling evidence that neuromuscular junctions are not protected in *Wld^S* mice over 7 months old, indicating some effect of age as previously described by both Perry, Tsao and colleagues. The Gillingwater et al (2002) paper does knit much of

the earlier data together as degeneration of the neuromuscular junctions could have hidden robust survival of axons if assessment of degeneration was done by neuromuscular transmission.

Taken together, the evidence favours some effect of age on *Wld^S*-mediated axonal and synaptic protection. *Wld^S*-protection appears to decrease with age with neuromuscular junctions being most susceptible to this possibly from as young as 8 weeks and certainly by 7 months. If age has an effect on axonal protection it is likely that this occurs much later in an animal's life than the effect on neuromuscular junction protection, probably not until at least 6 months of age and to no great extent even in animals older than that.

It appears to be the age of the synapse rather than the age of the animal which is the factor important for neuromuscular junction protection. Gillingwater et al (2002) compared synaptic preservation of immature synapses at regenerated neuromuscular junctions in old *Wld^S* mice. In mice aged between seven and twelve months, the sciatic nerve was crushed on one side. Regenerating axons were allowed to re-innervate the flexor digitorum brevis (FDB) muscle. Eight to ten weeks later 95% of fibres had regained synaptic activity from new synapses formed by the regenerated axons. Next the repaired synapses which could not have been more than eight weeks old were lesioned again, this time cutting the tibial nerve. The incidence of innervated FDB muscle fibres following the second lesion was then scored electrophysiologically and immunocytochemically. The first lesion produced rapid loss of synaptic transmission and morphology in these old mice; no fibres showed signs of activity three days post axotomy and fewer than 2% of endplates were contacted by nerve terminals. By contrast, synapses were consistently preserved after section of the regenerated axons: 33% of endplates still showed spontaneous miniature endplate potentials and or responded with end plate potentials to nerve stimulation. Immunocytochemistry labelled preparations showed that four or five days after axotomy 41% of endplates were contacted by nerve terminals. Therefore new synapses in old mice are better protected than the mature synapses innervating muscles without a prior conditioning lesion applied to the nerve.

I.3.2 Effect of Wld^S protein expression level on the Wld^S phenotype

The Wld^S protein concentration effect on axon degeneration was studied by Mack et al (2001) who produced 4 transgenic lines expressing the *Wld^S* gene, each with a different number of copies of the gene. The level of protection exhibited by these mutants was reported to correlate with the number of copies of the transgene. Axons persisted longer than functional motor nerve terminals in these mice (Mack et al, 2001). This result suggests that the degree of protection depends on the expression level of Wld^S protein. Mack et al, quantified this effect. They found that it is necessary for the expression level of Wld^S to reach a threshold level to exert a significant protective effect ie: the hemizygous mutant with the lowest copy number, though demonstrating detectable levels of Wld^S, exhibited Wallerian degeneration indistinguishable from wild type mice. Over the range tested they found no plateau of protection at the highest expression levels. Axons persisted longer than functional motor nerve terminals in these mice (Mack et al, 2001).

Heterozygote *Wld^S* animals demonstrate a phenotype similar to the aged phenotype described by Gillingwater et al (2002). The axons are robustly protected; however the neuromuscular junctions degenerate at the same rate as those in wild type animals (Li Fan PhD Thesis, University of Edinburgh; F. Wong et al, in preparation).

This finding is relevant to the future application of Wld^S-mediated protection therapeutically. It suggests that unless the critical threshold is reached, so that both axons and axon terminals are protected, clinical benefits may not be seen. Also, raising Wld^S expression could bring about even longer preservation of axons and their endings.

The findings also provide information about the mechanism. The more Wld^S present in the cell, the greater the protection of the axon. This suggests that whatever Wld^S does to interfere with Wallerian degeneration, the 'substrates' or the other component molecules involved are present in abundance. Ie: Wld^S may regulate the rate limiting step in the production of a protective factor. Or if it is acting through a

dominant negative mechanism, whatever molecules it inhibits with must be present in ample quantities.

Finally, this “gene-dose” effect, in conjunction with the age-effect has a third implication. In both heterozygotes and old homozygote *Wld^S* mice, the neuromuscular junction appears to degenerate despite the presence of *Wld^S* at levels able to fully protect the axon. This may suggest that the axon terminal has its own mechanism for destruction (see I.10).

I.3.3 Sensory and motor differences

The possibility of a difference between sensory and motor axon protection in *Wld^S* mice was first reported by Brown et al (1994). These authors found that the degeneration of axons in the saphrenous nerve, comprising only sensory axons, is slower than that in the phrenic nerve which largely comprises motor axons (Brown et al, 1994). This difference was apparent in mice aged 4 weeks of age.

An alternative explanation for the data reported by Brown et al is that there are inherent difference in the strength of protection in axons of the saphrenous and phrenic nerves, independent of their respective sensory and motor functions.

In 1995, Fruttiger et al reported a distinct difference in the speed of Wallerian degeneration between muscle and cutaneous branches of the femoral nerve in 16 week old *Wld^S* mice as opposed to 6 week old *Wld^S* mice. They used an antibody against L2/HNK-1 to identify motor nerve associated myelinating Schwann cells. They report that in 16 week old *Wld^S* mice, 5 days post axotomy, 11 % of all L2/HNK-1-positive Schwann cells displayed neurofilament immunoreactivity in the centre of their myelin sheath. In contrast, 57% of all L2-HNK-1-negative Schwann cells showed neurofilament immunoreactivity. I.e.: 5 days post lesion almost all motor axons had degenerated but more than half of the sensory axons were still present (Fruttiger et al, 1995).

The issue of sensory/motor differences in *Wld^S*-induced axon protection is further examined in the present thesis.

I.3.4 Other phenotypic differences

Given the profound effect of the mutation on the response of neurones to injury, it is remarkable that there is apparently very little else to say about the constitutive phenotype of *Wld^S* mice. The mice have no overt behavioural abnormality which is why the axon-protection phenotype may have remained undiscovered until Brown and Perry's serendipitous discovery in 1989. However, the following differences have been published.

Brown et al (1991) reported that the soleus muscle in the mutant has an increase in intrinsic tension, fewer macrophages per muscle fibre and lower levels of Acetyl choline sensitivity. Tsao et al (1999b), report that there is altered cerebral metabolism.

Two phenotypic characteristics reported to be specific to *Wld^S* mice, which I have examined further in the appendix of this thesis are decreased pain sensitivity (Zhong et al, 1999) and increased hair loss, which has not been published, but can be seen regularly in laboratory mice.

The functional significance of these characteristics or the role of *Wld^S* in their direct causes remains unknown. However other characteristics, which the mutation is likely to have a role in, have also been published.

Wishart et al (2008) report that *Wld^S* expression in mouse cerebellum and HEK293 cells alters a number of cell-cycle related genes and proteins and suggest this may result in abnormal cell-cycle control in *Wld^S* animals.

Brown et al (1994) investigated slow regeneration of axons in the peripheral nerve of *Wld^S* mice after crush injury. This appears to be due to the inappropriate environment that axonal sprouts find themselves in due to the persistence of the axonal stump. As

long as the myelinated axons in the distal stump remained intact, regeneration of axons did not occur, in spite of vigorous production of sprouts at the crush site. In addition, myelinated sensory axons regenerate very poorly in *Wld^S* mice, even in the presence of unmyelinated axons. However, this failure to regenerate is also explained by adverse local conditions confronting sprouts, as the cell bodies in the DRG respond normally to injury and if the saphrenous nerve is forced to degenerate more rapidly by multiple crush lesions, sensory axons regrow successfully.

The lack of obvious complications to living with the *Wld^S* phenotype further increases the interest in this gene as a potential molecular basis for therapeutic intervention in diseases in which axonal degeneration is important.

I.4 *Wld^S* and axonal degeneration

Wld^S protects axons from degeneration following axotomy. This has been demonstrated in the PNS, and also following injury in the CNS (Ludwin and Bisby, 1992; Gillingwater et al, 2004). However, axonal degeneration does not only occur in response to nerve transection. Synaptic and axonal degeneration are recognised as early stages of many neurodegenerative diseases, for example in motor neurone diseases (Fischer and Glass, 2007) and Alzheimer's disease (Selkoe, 2002). Axon degeneration can also occur as a result of exposure to neurotoxins such as taxol (Lipton et al, 1989), or cisplatin (Krarup-Hansen et al, 2007), agents which are used therapeutically, for instance in treatments for cancer. This raises the possibility that the downstream molecular action of the *Wld^S* gene might be exploited to prevent or alleviate neurodegeneration in these instances.

Wld^S has been successfully used to protect axons challenged with neurotoxic chemicals in both cell culture studies and in vivo. Cultured superior cervical ganglion (SCG) neurites from *Wld^S* animals are resistant to degeneration induced by vinblastine, a micro-tubule disrupting agent (Ikegami and Koike, 2003). Cultured sympathetic neurones from *Wld^S* mice have neurites that are resistant to nerve growth factor deprivation (Deckwerth and Johnson 1994). Mice receiving three taxol

injections over the course of a week develop a highly reproducible sensory neuropathy. *Wld^S* mice are resistant to this neuropathy, as assessed by behavioural, electrophysiological and pathological means (Wang et al, 2002b).

There has also been protection from neurotoxic insult in the CNS. 6-hydroxydopamine (6-OHDA) is a catecholaminergic neurotoxin that, when injected into the forebrain, results in the loss of dopamine fibres in the nigrostriatal pathway. This is used as a model for Parkinson's disease, where the same fibres are affected. To assess any *Wld^S*-mediated rescue of dopamine fibres, the nigrostriatal pathway of *Wld^S* mice was lesioned with 6-hydroxydopamine (6-OHDA), a catecholaminergic neurotoxin. Young *Wld^S* mice showed remarkable dopamine fibre protection in the striatum. Drug-induced rotational behaviour confirmed the nigrostriatal fibre ability to release dopamine (Sajadi et al, 2004).

Results from crossbreeding *Wld^S* into other mouse models of disease have been mixed. The strongest *Wld^S* protection has been found in the progressive motoneuropathy mouse (*pmn* mouse), a model of early-onset motor neurone disease. Ferri et al, crossed *pmn* mice with *Wld^S* mice and found the *Wld^S* gene product attenuates disease signs, extends life span, prevents axon degeneration, rescues motor neurone number and size, and delays retrograde transport deficits (Ferri et al, 2003).

Another disease model that has demonstrated benefits after cross-breeding with *Wld^S* mouse is the Protein zero deficient mouse (P0 *-/-*), a genetic model for myelin-related axonopathy. Samsam et al show *Wld^S* expression reduces axonal degeneration in peripheral nerves of 6 week old and 3 month old P0 *-/-* mice, and increases muscle strength. However axon loss at 6 months is comparable between p0 mice with and without the *Wld^S* mutation (Samsam et al, 2003).

The promising results of neuroprotection against chemical injury in the CNS and PNS, and against disease in the *pmn* and P0 *-/-* mice indicate that there are some molecular or mechanistic similarities between the degeneration occurring in various neuropathies and the 'pure' Wallerian degeneration that occurs after a nerve is cut.

By studying *Wld^S* and Wallerian degeneration, other Wallerian-like degenerative processes and possible therapeutic targets may be better understood.

However, *Wld^S* has not provided successful protection in all mouse models of disease. For examples Fischer et al (2005) investigated the SOD1-G93A mouse, an extensively used model of familial ALS. At 80 days SOD/*Wld^S* animals had 32.9% more innervated endplates than SOD/WT animals and they lived on average, 10 days longer. But at time points beyond 80 days, there was no significant difference in innervation of neuromuscular junctions, and the mice had no phenotypic improvements as measured behaviourally by a rotorod test. The authors conclude that ‘The *Wld^S* gene modestly prolongs survival in the SOD1-G93A mouse’ (Fischer et al, 2005). When Vande Velde et al investigated *Wld^S* protection in two more SOD1 mutants, SOD1G37R or SOD1G85R and found that *Wld^S* did not slow disease onset, ameliorate mutant motor neurone death, axonal degeneration, or preserve synaptic attachments (Vande Velde et al, 2004). Similarly, Kaneko et al found only a modest delay in axon degeneration and onset of symptoms in EAE mice (Kaneko et al, 2006).

One fundamental problem in using *Wld^S* to try and treat neurodegenerative disease is the age-dependency of the phenotype. It is possible that the pmn mouse was the most successful case because this mouse model of disease displays signs of disease at a young age, when both motor axons and motor nerve terminals are protected by *Wld^S*. In disease models where symptoms do not appear until 12 weeks or more, *Wld^S* may be losing the capacity to protect neuromuscular junctions and so however long the motor axons remain intact, their function in mediating neuromuscular transmission and muscle contraction, will be lost.

The gene-dose effect on the phenotype provides an additional hurdle when studying protection against disease. If protection of axonal endings is important, sufficient copy numbers of the gene must be delivered to their cell bodies.

1.5 Mapping of *Wld^S*

The discovery of the *Wld^S* mouse suggested that Wallerian degeneration is not a passive wasting away of the axon as a consequence of disconnection from the cell body. Study of the mechanism of *Wld^S* protection from Wallerian degeneration might lead to insights about the mechanism and molecules involved in Wallerian degeneration. It could also lead to identification of new targets for drug development in neurodegenerative research.

Perry et al (1990b) studied the inheritance of the phenotype in genetic outcrosses and backcrosses with the *Wld^S* mouse and as a result showed that the mutation segregates as a single autosomal dominant gene. Lyon et al (1993) narrowed down the locus. They employed chromosome mapping techniques in which mice are crossed with stocks carrying visible markers covering 80-90% of the genome and linkage was found with the locus of *jerker* on chromosome 4. This allowed molecular mapping to position the gene more precisely in a probable order with various microsatellites and restricted fragment length variants at the distal end of chromosome 4.

An 85kb tandem triplication within the candidate region was discovered to be unique to *Wld^S* mice (Coleman et al, 1998). This 85 kb repeat sequence contains the exons of 3 genes, each of which could have accounted for the *Wld^S* phenotype (Conforti et al 2000). The first of these was named *Retinal binding protein 7* due to homology with other known retinoid binding proteins. The remaining two genes, originally described as *ubiquitin fusion degradation protein 2 (Ufd2)* and a novel gene *D4Cole1e*, were reported to span the proximal and distal boundaries of the repeat unit, forming a chimerical gene encoding a 43 kDa fusion protein. It has since been shown that the novel sequence *D4Cole1e* incorporates the complete sequence encoding nicotinamide mononucleotide adenylyl transferase-1 (*Nmnat-1*), one of the enzymes responsible for synthesising Nicotinamide Adenine Dinucleotide (NAD) (Emanuelli et al, 2001) and also incorporating an N-terminal sequence of 18 amino acids that is not normally translated in *Nmnat1*. The novel chimerical gene includes this full sequence of *Nmnat-1*. The *Ufd2* constituent is now referred to as Ubiquitination Factor E4 (*Ube4B*), which is the name given to the mammalian

homologue of the yeast *Ufd2*. Only a truncated N-terminal 70 amino acid coding sequence is included in the novel chimerical gene.

Rbp7 was not expressed at sufficient levels in nervous tissue to be suspected of contributing to the phenotype leaving the chimerical gene as the most likely candidate responsible for the neuroprotection. This prediction was strongly supported when transgenic animals, expressing the chimerical gene exhibited the same protective phenotype as the naturally occurring mutant mouse (Mack et al. 2001) [Figure I.2].

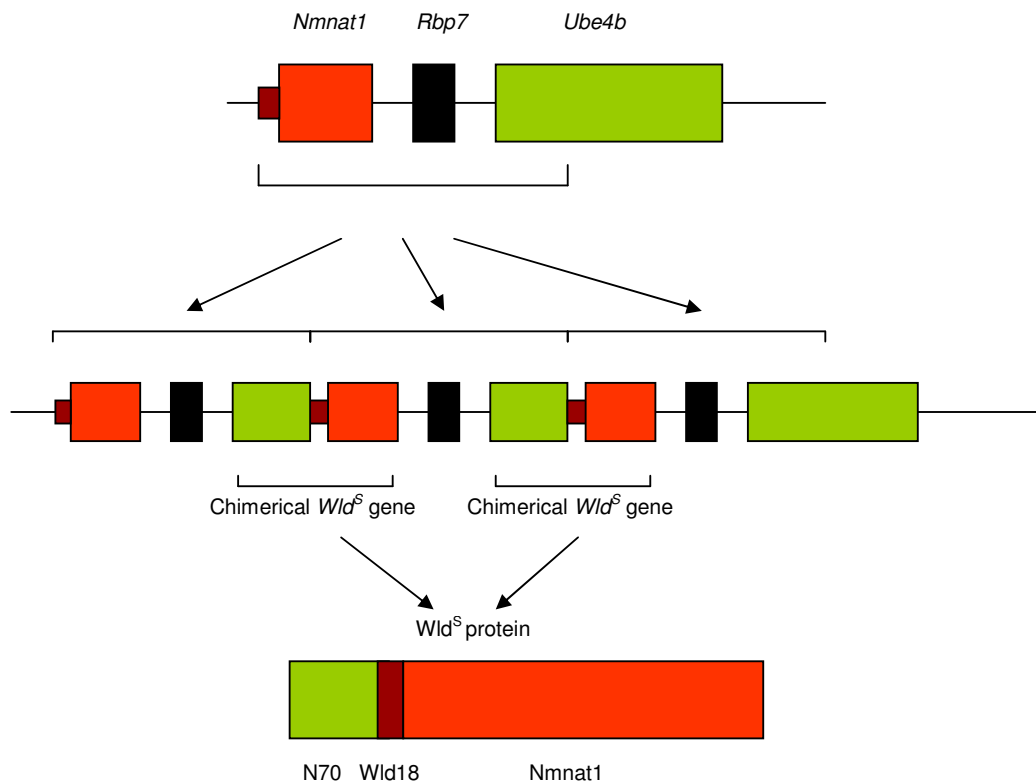


Figure I.2 *Wld^S* protein is produced by a chimerical gene

85 kb of DNA are triplicated in the *Wld^S* mouse giving rise to a chimerical gene. The gene contains 2 exons of Ube4B located within 30 kilobases of DNA, and the entire coding sequence of Nmnat1, which is four exons spanning about 11 kilobases of DNA. This translates to 70 amino acids of Ube4B and 302 of Nmnat1, including 18 amino acids which is normally untranslated, known as *Wld¹⁸*. A third gene is also triplicated in the mutant mice, this is known as Rbp7. However, transgenic mice expressing the chimera alone, demonstrate the *Wld^S* phenotype.

I.6 Mechanism and molecules of *Wld^S* Protection

Though the gene responsible for the phenotype has been identified, the mystery of how it interferes with Wallerian Degeneration remains. The *Wld^S* phenotype has been extended to neurones in both *Drosophila* and rats and it has been investigated in both CNS and PNS (Lunn et al, 1989; Mack et al, 2001; Adalbert et al, 2005; Gillingwater et al, 2006b; Hoopfer et al, 2006). This suggests the mechanism for Wallerian degeneration is ancient and conserved. However, we still have no conclusive evidence of molecules involved in the initiation of Wallerian degeneration or a mechanism by which *Wld^S* disrupts this process.

Wld^S appears to act upstream of the calcium influx, calpain activation and complement activation in Wallerian Degeneration. *Wld^S* neurofilaments degenerated following incubation in media containing Calcium ionophore A23187 and 10 mM Calcium ions at the same rate as BALB/c neurofilaments (Tsao et al, 1994) and Calpain activity can be demonstrated in *Wld^S* axons (Glass et al, 1994; Glass et al, 1998). This suggests that calpain activation and neurofilament degradation proceed as they do in wild type animals if the intra-axonal calcium is raised.

Mack et al, 2001 reported that the protein is predominantly localised to the nucleus, with none detectable in the axons of the sciatic nerve, or in end-bulbs of 24-hour transected CNS and PNS axons, which normally accumulate other axonal proteins. Given that the nucleus is spatially distinct from the axon and, importantly, completely disconnected from the axons when they exhibit the phenotype, it is reasonable to hypothesise that other factors must mediate the effect of *Wld^S* on the axon. According to this view, these factors must already be present in the axon at the time-point of injury.

If *Wld^S* somehow regulates other factors downstream, a further question is whether there is a specific domain of the *Wld^S* protein that is involved in this regulation.

Since the chimerical protein is made up of two proteins conserved in the normal animal; a plausible hypothesis is that one of these domains, either the full-length Nmnat1 or the 70 amino acids of Ube4B has assumed a new function. This issue has not yet been resolved so I shall briefly discuss the evidence that has come to light so far.

In the study by Mack et al, although it was demonstrated that brains from *Wld^S* mice show a fourfold increase in Nmnat1 enzymatic activity when compared to ordinary C57Bl6 mice, the amount of NAD in *Wld^S* nervous tissue was shown to be the same as that in wild type tissue (Mack et al, 2001). In addition, the ubiquitin-proteasome system has an important role in protein degradation and has been implicated in axon degeneration (see below), so the 70 amino acids of Ube4B seemed more promising to be causing the phenotype.

I.6.1 The N70 Hypothesis

The Ubiquitin Proteasome System (UPS) is responsible for degrading most cellular proteins and regulates a remarkably diverse group of cellular processes by conditionally degrading proteins that control or are essential to those processes (Glickman and Ciechanova, 2002). Deregulation of the UPS has been implicated in a wide range of neurodegenerative disorders.

The gracile axonal dystrophy (*gad*) mouse is an autosomal recessive mutant that shows sensory ataxia, followed by motor ataxia at a later stage. Pathologically, the mutant is characterized by 'dying-back' type axonal degeneration and formation of spheroid bodies in nerve terminals. The *gad* mutation is caused by deletion of 2 exons from the ubiquitin carboxy-terminal hydrolase isozyme (*Uch-11*), which is selectively expressed in the nervous system (and testes) (Saigoh et al, 1999). This mutation was published just 2 years prior to the *Wld^S* mutation along with the suggestion that altered function of the UPS directly causes neurodegeneration.

The 70 amino acids of Ube4B (N70) that makes up part of the chimerical *Wld^S* protein does not include any part of the U-Box (which is located towards the C-

terminus of the full length protein and is responsible for the polyubiquitination activity of Ube4b). Since this region is absent from *Wld^S*, it is unlikely that direct modulation of protein ubiquitination is responsible for the phenotype. However, N70 could interact with other molecules normally involved in interactions with full-length Ube4B and perhaps, therefore, have a dominant negative effect on functioning of the UPS.

There is evidence that inhibition of the UPS can delay Wallerian Degeneration. Zhai et al (2003), provide pharmacological evidence that UPS inhibition can delay Wallerian degeneration. They used two mechanistically different proteasome inhibitors: MG132, a peptide-based reversible proteasome inhibitor; and lactacystin, a synthetic irreversible proteasome inhibitor. Cultured superior cervical ganglia neurones were transected and control axons began developing signs of degeneration 8 hours later. Those treated with both MG132 and lactacystin did not show degeneration until 16 hr post-axotomy. 24 hours post-axotomy, when most of the axon debris has detached from the culture surface in control cultures, proteasome inhibitor-treated explants still retained many axons with few signs of degeneration. In vivo, gelfoams pre-soaked with MG132 and applied to optic nerve which had been lesioned, slowed the breakdown of microtubules and neurofilaments compared to vehicle soaked controls. These results suggest that inhibiting UPS activity is able to delay Wallerian degeneration both in vitro and in vivo. MacInnis and Campenot (2005) achieved similar results using sympathetic neurones cultured in compartmentalised dishes. Treatment with Mg132 protected transected axons from degeneration for at least 24 hours (up from 15-18 hours) and epoxomicin protected axons for 30 hours.

Although it may be that the N70 acts in a dominant negative fashion and hence inhibits the UPS, the evidence presented above is not on the scale of the protection conferred to axons by *Wld^S*. On the basis of this evidence it seems unlikely that N70 alone is responsible for the *Wld^S* phenotype. However, as described in the following section, it may be active in the phenotype.

I.6.2 The VCP hypothesis

During a screen for *Wld^S* binding partners Valosin-containing protein (VCP) was established as a binding partner for N70 (Laser et al, 2006). VCP is involved in many cell maintenance and cycling functions as well as endoplasmic reticulum protein degradation, nuclear envelope reconstruction and suppression of apoptosis.

Yeast Ufd2 is implicated in cell survival under stress (Koeogl et al, 1999). In Yeast Ufd2 interacts with *cdc48* (Koeogl et al, 1999). Mouse Ube4B interacts with VCP, an ortholog of yeast *Cdc48* (Kaneko et al, 2003), suggesting this association has been conserved through evolution and may be functionally important (Okumura et al, 2004). Kaneko et al suggest that the Mammalian Ube4B/VCP complex might function as an E3 chaperone in the response of neurones to stress (Kaneko et al, 2003).

Wld^S binding of VCP does not significantly alter steady-state levels of VCP in the brain; however it does alter the nuclear localisation of VCP. *Wld^S* clusters into intranuclear foci in some neuronal subtypes and cell lines. Laser et al found that *Wld^S* and VCP colocalised within these nuclear spots both in vitro (PC12 cells) and in vivo (in cerebellar granule cells and molecular layer neurones). In wild type cerebellum, there were no foci of VCP (Laser et al, 2006).

Subsequent pull-down experiments with truncated *Wld^S* demonstrated that VCP bound to the N-terminal 16 amino acids (N16). Cells transfected with *Wld^S* lacking N16 were not able to affect VCP distribution in the nucleus. Importantly, *Wld^S* lacking N16 still formed the intranuclear foci (Laser et al, 2006).

Though this interaction may be functionally important, which (if any) of VCP's functions is disrupted by *Wld^S* is unknown. RNAi of *Drosophila* VCP promoted an almost twofold accumulation of ubiquitinated proteins, a general indicator of inhibited UPS function (Wojcik et al, 2004). Perhaps *Wld^S* interaction with VCP decreases availability of the molecule for its normal interaction with Ube4B. This may result in inhibited UPS function.

Another possibility is that the high concentration of VCP in the intranuclear foci brings about a new function. Through the interaction with VCP, which binds long ubiquitin chains, *Wld^S* is also able to partially redistribute ubiquitin inside the nucleus (Laser et al, 2006). Perhaps VCP or ubiquitin are brought into proximity with other molecules (perhaps molecules involved in *Nmnat1* functions) at higher concentrations that would normally occur, and this gives rise to interactions which cause *Wld^S* protection.

I.6.3 The *Nmnat1* Hypothesis

In 2004, the possibility that it is *Nmnat1* over-expression (due to the triplication in *Wld^S*) which causes the phenotype, resurfaced with a paper published by Araki et al (2004) demonstrating that transfection of cultured DRG neurones with *Nmnat1* alone was sufficient to protect their axons from degeneration.

Araki et al found that *EGFP-Nmnat1* delayed axonal degeneration comparable to *Wld^S* gene itself whereas *N70* had no effect whether targeted to the nucleus or cytoplasm. They then used the crystal structure of *Nmnat1* to predict amino acids that might be mutated to alter substrate binding. This enabled them to create *Nmnat1* and *Wld^S* with a single codon mutation that caused an amino-acid substitution that severely limited the ability to synthesise NAD. Neurones expressing these enzymatically null mutants had no axonal protective effects.

They then attempted to recreate the phenotype with exogenously applied NAD. They found that 0.1-1µM NAD added 8 or more hours before transection was enough to delay axonal degeneration, although to a lesser extent than lenti-virus mediated *Nmnat1* expression.

Araki et al conclude that *Wld^S* protection is mediated by NAD, overproduced by the triplicated *Nmnat1* in *Wld^S* mice. Without the ability to produce NAD, there is no *Wld^S* protection. *Nmnat1* and NAD could drive protection based on known biochemical pathways. In the 2004 Araki paper these were investigated.

Sirtinol added at the same time as NAD blocked the protection suggesting mediation through the sir2-pathway [Figure I.3, Diagram based on Revollo et al, 2004]. They next performed knock-down to the 7 SIRT proteins with the Sir2 domain and discovered that a knockdown of SIRT1 blocked axon protection as effectively as sirtinol.

The same group claim in a later paper (Sasaki et al, 2006) to provide axonal protection with Nicotinamide phosphoribosyl transferase (Nampt) and nicotinic acid phosphoribosyl transferase (Npt), which convert the precursor molecules nicotinamide and nicotinic acid, to the respective mononucleotides. They also found that exogenous application of nicotinic acid mononucleotide (NMN) and nicotinamide riboside (NmR) promoted axonal protection to a similar extent as NAD itself. They conclude that stimulating the activity of the NAD biosynthetic pathway at multiple steps provides protection against axonal degeneration after axotomy.

Further work showed that lentivirus transfection with *Nmnat1* localised to different neuronal compartments also provides axonal protection. *Nmnat1* localised to the cytoplasm provided strong axonal protection indistinguishable from wild-type *Nmnat1*. *Nmnat3* expressed in the nucleus or in the mitochondria had the same effect. The changes had to be made 24 hours before transection or no protection was afforded. These results suggest that the subcellular location of *Nmnat* is not crucial for the phenotype (and therefore that the interaction with VCP is irrelevant).

This evidence comes together to suggest that *Wld^S* protection is afforded through the increased production of NAD acting through the Sirtuin pathway. As well as the practical evidence for this, theoretically it is an exciting prospect. It is known that the Sirtuin pathway is involved in survival during starvation in yeast (Fabrizio et al, 2005). Evidence is emerging that the Sirtuins have continued this function in multicellular organisms including *Caenorhabditis elegans* and *Drosophila melanogaster* (Michan and Sinclair, 2007). They are conserved in mammals and caloric restriction has extended the life of mice, and is currently being trialled in

primates who are already indicating delayed aging (Michan and Sinclair, 2007). It is easy to extrapolate that the sirtuin pathway may have been hi-jacked in the case of *Wld^S* axon protection. The sirtuins mediate a signal to produce adaptation in an organism facing calorie restriction. A severed axon is facing nutrient restriction, unable to receive the products of transcription produced in the cell body. Due to an over expression of NAD, working through the Sirtuins, biochemical changes that help a cell adapt to starvation might be present in *Wld^S* neurones that helps the isolated axon to survive for longer.

The Araki group are not the only authors to claim the *Wld^S* phenotype is dependent on NAD production. *Nmnat1* over expression in *Drosophila* can delay axon degeneration (Macdonald et al, 2006). Exogenously applied NAD or nicotinamide suppresses neurite degeneration induced through Zinc depletion (Yang et al, 2007). Suzuki and Koike (2007) found that basal levels of microtubule acetylation is increased in *Wld^S* granule cells and postulate that this acetylation is what increases axon stability. They found that they could increase the levels of acetylated α -tubulin through treatment with NAD.

However, some reports, though also aligning with the *Nmnat1* hypothesis, do not fully support Araki's conclusion. For example, Zhai et al report that enzymatically null *Nmnat1* can protect against retinal degeneration in *Drosophila*, caused by excessive neuronal activity (Zhai et al, 2006). Wang et al show that NAD levels decline in degenerating neurones and that application of NAD or nicotinamide can prevent degeneration of transected axons. However, the doses they use (up to 10mM) are high and maybe non-physiological, and they conclude that *Nmnat1*/NAD-mediated protection is mediated locally rather than through a nuclear pathway (Wang et al, 2005). Watanabe et al generated *Wld^S* or *Nmnat1*-overexpressing Neuro2A cell lines and induced neurite elongation with retinoic acid. The over expression of *Wld^S* delayed the neurite degeneration by vincristine, whereas that of *Nmnat1* did not delay it much. They conclude that *Nmnat1* is considerably weaker than *Wld^S* for protection from toxic injury in vitro (Watanabe et al, 2007).

The hint that the neuroprotection might be due to *Nmnat1* over expression has led to optimistic trials of drugs that interfere with the Nmnat1-NAD-Sirt metabolism in a variety of animal models of disease. It has been reported that nicotinamide, the precursor to NAD reduces hypoxic-ischemic brain injury in the newborn rat (Feng et al, 2006) and that it protects against ethanol induced neurodegeneration in the developing mouse brain (Ieraci and Herrera, 2006). However, these studies look at apoptosis rather than axon degeneration and may be investigating a different phenomenon altogether. Nicotinamide was also used to protect mice with experimental autoimmune encephalomyelitis (EAE) from axonal degeneration. The authors found a profound protective effect of nicotinamide on neurological disability in the EAE model as opposed to a modest reduction of neurological disability of EAE in *Wld^S* mice (Kaneko et al, 2006). However, the nicotinamide treatment affected inflammation and demyelination as well as axonal loss, suggesting that again, the phenomenon might be slightly different from *Wld^S* protection.

Despite the evidence and enthusiasm for the Nmnat1 hypothesis, there is significant data against a theory of axonal protection based purely on Nmnat1 overexpression. Conforti et al (2007) made *Nmnat1* transgenics and despite matching the Nmnat activity of *Wld^S* heterozygotes, which have a robust slow Wallerian degeneration phenotype, *Nmnat1* transgenic mice showed rates of Wallerian degeneration that were not discernibly different from wild type mice. They also demonstrated that Nmnat1 has significantly lower potency to protect neurites from traumatic or toxic injury in vitro. Although they do not rule out the possibility that Nmnat1 could delay Wallerian degeneration when expressed at very high levels, they conclude that the data demonstrates the ability of *Wld^S* to protect axons is greater than that of *Nmnat1* alone. Overexpression of *Nmnat1* cannot therefore be the complete explanation for the *Wld^S* phenotype (Conforti et al, 2007).

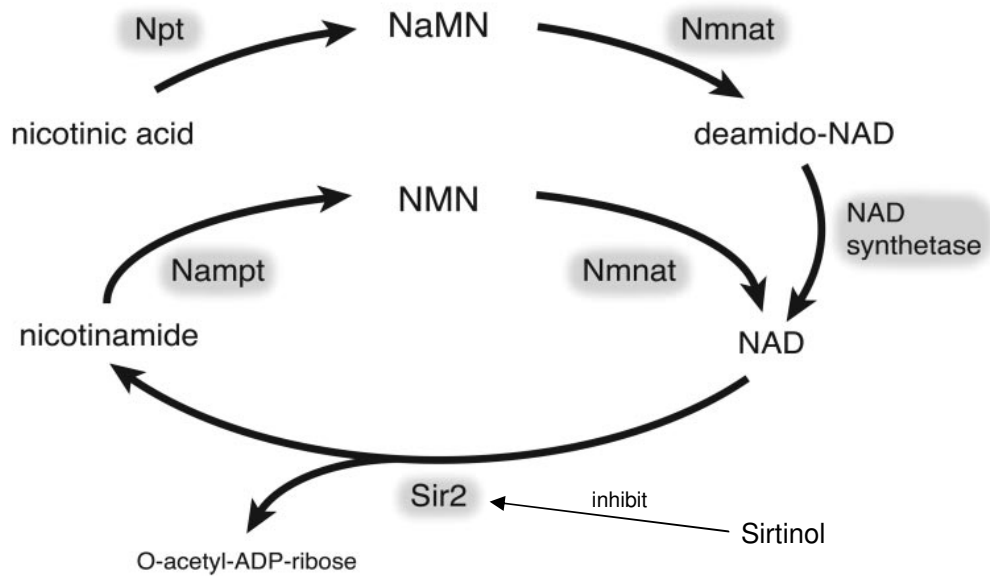


Figure I.3 The NAD biosynthesis pathways from nicotinamide, based on Revolla et al, 2004

Mack et al demonstrated a 4 fold increase in Nmnat1 enzymatic activity but no increase in the amount of NAD in the nervous system in *Wld^S* mice. The theory that *Wld^S* protection is mediated by increased Nmnat1 activity, argues that the increased Nmnat1 activity results in an increase in flux through the Sir2 pathway known to regulate survival during nutrient restriction in yeast.

I.6.4 Other mediators

While one approach has been to ask which part of the Wld^S molecule causes the phenotype, another approach has been to look at differences in gene and protein changes between Wld^S and wild-type mice in order to identify downstream messengers.

Gillingwater et al (2006a) performed a comparative gene expression profile (microarray) on three independently prepared and analysed preparations of mRNA extracted from Wld^S and wild type mice. Based on a stringency criterion of consistent two-fold or greater difference in expression level, 10 genes were identified, 6 upregulated and 4 down-regulated, of which the largest changes were upregulation of Erythroid differentiation regulator 1 like-EST (*Edr11-EST*) and down regulation of pituitary tumour transforming 1 (*Pttg1*). Gillingwater et al validated the microarray data using quantitative real time Polymerase Chain Reaction (PCR) to measure the mRNA expression levels of these candidate genes.

HEK293 cells, transfected with a Wld^S -expressing plasmid, demonstrated that *Pttg1* and *Edr11-EST* mRNA expression could be altered in human cells in vitro. They were also altered following transfection of the NSC34 neural cell line with the same construct. Exogenous application of NAD and expression of either of the Wld^S domains (*N70* or *Nmnat1*) alone could also cause some of the mRNA levels to change suggesting that different components of the Wld^S regulate bidirectional gene transcription changes.

There was no indication, from the data in the study by Gillingwater et al, as to how Wld^S regulates the mRNA expression of these other genes. It shows no homology to characterised transcription factors. The paper gives some preliminary evidence that Wld^S might regulate at least some of the genes through *Nmnat1*-NAD-sirtuin pathway. Down regulation of *pttg1* mRNA was abolished when Wld^S transfected HEK293 cells were treated with sirtinol.

There is some indication that, at least some of the specific genes discussed in the paper may not turn out to be involved in the protective phenotype. Null-mutant (knock-out) mice without *pttg1* do not show neuromuscular synaptic or axonal protection after axotomy (Gillingwater et al, 2006a). Interference in expression of the other genes has not yet been tested.

Following up these investigations, Wishart et al (2007) carried out a differential proteomics analysis of synaptosomal preparations from *Wld^S* and wild type mice. They prepared these synaptosomes from the striatum since these synapses show a robust neuroprotective phenotype after traumatic brain injury (Gillingwater et al, 2006b). This study resulted in identification of 16 proteins which had significantly different expression profiles in *Wld^S* and wild-type synaptosomes.

Eight of the proteins identified by Wishart et al (2007) are known to be localised to mitochondria. The other eight have links to known neurodegeneration and neuroprotection. The eight mitochondrial proteins that were modified showed either elevated or reduced levels indicating that the number of mitochondria remains the same in *Wld^S* mice, but perhaps their state is altered. Subsequent screening showed that 3 more mitochondrial proteins are also altered in *Wld^S* animals (Wishart et al, 2007). These changes may amount to priming the mitochondria to resist degeneration. How this comes about remains to be resolved.

There is other evidence suggesting that mitochondria might be central to the *Wld^S* phenotype. Ikegami and Koike showed that while neurites of wild-type neurones experienced an early loss of mitochondrial membrane potential and subsequent Adenosine Triphosphate (ATP) depletion, when treated with vincristine, *Wld^S* neurites maintained both the mitochondrial membrane potential and ATP content (Ikegami and Koike, 2003).

As well as highlighting mitochondria as a possible target in *Wld^S* protection. The authors also suggest some of the protein changes can be linked both to the *Nmnat1* and *Ube4b* components of the *Wld^S* gene. E.g.: One of the proteins identified in the

screen is UBE1. Changes in the expression levels of this gene (it was upregulated) could be seen to be linked to the expression of N70 in *Wld^S*. Another of the proteins is Aralar1, involved in the transfer of NAD(P)H into mitochondria. Alterations in the proteins in both NAD metabolism and the UPS indicate that both components of the *Wld^S* gene potentially affect the state of the synapses. However this does not indicate which of these changes are important for the phenotype.

Interestingly none of the proteins flagged by the proteomic analysis coincided with any of the genes strongly up or down regulated at the mRNA level. None of the 11 molecules found in the microarray were pulled up in the proteomics analysis. There are several possible explanations for this: Some of the proteins translated from the mRNAs maybe localised to the soma or axon rather than the synapse; due to the analysis of the 2D protein blot some proteins spots may have been occluded by larger protein spots or perhaps the spots were of too small an amount to be seen.

In summary, whatever the mechanism for affecting transcription, and whether or not the most dramatically altered genes or proteins are involved in the *Wld^S* phenotype, it is conclusive that expression of *Wld^S* in neurones affects transcription of other genes and translation of synaptic proteins. This constitutes compelling evidence that expression of *Wld^S* affects the constitutive baseline state of the neurone.

I.7 Apoptosis

Apoptosis is the well characterised (molecularly, mechanistically and morphologically) process by which cells are eliminated when they are damaged, dangerous or superfluous. It is an active, highly organised and regulated process characterised by cell shrinkage, chromatin condensation, DNA fragmentation, membrane blebbing and the formation of apoptotic bodies that are rapidly phagocytosed by macrophages (Wyllie, 1997).

Apoptosis can be triggered by either extrinsic or intrinsic signals. Both these pathways converge on activation of executioner caspases that cleave various substrates which bring about cell death.

1.7.1 Caspases

Caspases are cysteine proteases that target aspartate residues of various substrates and are the main effectors of apoptosis. Caspases are normally present in the cell as inactive zymogens. 14 mammalian caspases have been described. However, not all of these participate in apoptosis; caspase-1 and caspase-11 are predominantly involved in the processing of pro-inflammatory cytokines. Depending on their mode of activation, the other caspases are categorised as either initiator or effector caspases (Freidlander, 2003).

The initiator caspases are caspase-8 and caspase-9 which mediate the extrinsic and intrinsic pathways respectively. Protein complexes assembled in these pathways activate the initiator caspases; the death-inducing signalling complex (DISC) in the extrinsic pathway and the apoptosome, which consists of apoptotic protease-activating factor-1 (APAF-1) and cytochrome-c in the intrinsic pathway. Several models for activation of initiator caspase-8 and caspase-9 have been proposed.

Effector caspases such as caspase-3 and caspase-7 are activated by cleavage by initiator caspases and they subsequently cleave various substrates to induce cell death. Over 100 proteins have been identified as substrates.

1.7.2 The extrinsic pathway

The extrinsic or external pathway involves activation of a death receptor by a pro-apoptotic ligand. Death receptors belong to the Tumour Necrosis factor (TNF)/nerve growth factor receptor superfamily. Among the best characterised are Fas and TNF receptor 1. The ligands that bind these belong to the TNF superfamily of cytokines and include TNF α and Fas ligand (FasL). When a death receptor binds its ligand this brings about a conformational change which sets in motion a cascade of protein-protein interaction that leads to assembly of the DISC. Once the DISC activates

initiator caspases, apoptosis continues with or without involvement of the mitochondria (Okouchi et al, 2007).

I.7.3 The intrinsic pathway

The intrinsic pathway is activated by apoptotic stimuli such as DNA damage, reactive oxygen species or fas signalling. The central step involves the permeabilisation of the outer membrane of the mitochondria and subsequent release of pro-apoptotic factors that are normally based in the mitochondrial inter-membrane space, into the cytosol. Outer mitochondrial membrane permeability is governed by members of the Bcl-2 family (Danial, 2007; Wei et al, 2001).

The Bcl-2 family comprises over 30 proteins and can be divided into anti-apoptotic proteins (eg: Bcl-2) and pro-apoptotic proteins (eg: Bad, bak and bax). The pro-apoptotic proteins can be divided into multi-domain (eg: bad and bak) or Bh-3 proteins (eg: bax). The pro-apoptotic Bh-3 proteins require a multi-domain protein to induce apoptosis.

The anti-apoptotic proteins defend mitochondrial outer membrane integrity through interaction with the pro-apoptotic proteins. The susceptibility of a cell to apoptosis is directly related to the ratio of pro- and anti- apoptotic Bcl-2 family proteins. Cells that are deficient in both the two multidomain pro-apoptotic proteins, bax and bak, fail to release cytochrome-c and are resistant to all apoptotic stimuli that activate the intrinsic pathway (Wei et al, 2001). Similarly, over-expression of the anti-apoptotic protein Bcl-2 also protects cells from apoptosis (Martinou et al, 1994). However, once the mitochondrial apoptotic signalling process has been initiated, Bcl-2 proteins no longer exert any effect.

The pro-apoptotic proteins, normally resident in the mitochondrial inter-membrane space include cytochrome-c and AIF, both normally involved in the electron transport chain. Released into the cytosol, these molecules trigger caspase-dependent or independent apoptotic death pathways. The caspase-dependent mechanism is mediated by caspase 9 via the apoptosome formed through interaction of

cytochrome-c and Apaf-1. For the caspase independent mechanism molecules such as AIF translocate to the nucleus and can induce DNA fragmentation and chromatin condensation.

I.7.4 Apoptosis and neurodegenerative disease

Apoptosis has a role in the normal development of the nervous system. Terminally differentiated neurones retain the normal full apoptotic machinery and ability to undergo apoptosis in response to various apoptotic stimuli (Okouchi et al, 2007).

As described in I.1, what all neurodegenerative diseases have in common is the loss of neurones. Evidence for a role in apoptosis in the loss of neurones is extensive (Okouchi et al, 2007). For this reason many treatment strategies focus on preventing the neurone from undergoing apoptosis (Waldmeier and Tatton, 2004).

However there is growing evidence that neurodegenerative diseases are correlated more directly with degeneration of neuronal processes, which precede neuronal apoptosis (Coleman et al, 2004; Smith et al, 2005; Fischer and Glass, 2007).

Sagot et al (1995) investigated inhibition of apoptosis in the *pnn* mouse model of Motor Neurone Disease by crossing the *pnn* mouse with a transgenic mouse that over-expresses human *Bcl-2*. The *pnn* mouse is the same mouse model of ALS used by Ferri et al (2003) to test the potential protective benefits of *Wld^S*, as described in section I.4.

The overexpression of *Bcl-2* in the *pnn* mice rescued facial neurones, which demonstrated a normal soma size and expression of choline acetyltransferase. However, *Bcl-2* over expression did not rescue the mice. Their symptoms (atrophy of the hind limbs and loss of grasp activity in the back paws) appeared at the same time as *pnn* mice without *Bcl-2* over expression and progressed over the same time course, with death occurring in the sixth or seventh week. Sagot et al conclude that *Bcl-2* overexpression cannot prevent disease progression in *pnn* mice. They noted that *Bcl-2* over expression did not prevent degeneration of myelinated axons in the

facial and phrenic motor nerves. This contrasts sharply with the protective effects of *Wld^S* observed by Ferri et al and highlights the importance of the axonal impairment in the clinical manifestation of the disease.

Perhaps regulation of apoptosis can be used to counteract neurodegenerative disease. However, what the above experiment demonstrates is that without axonal protection, increasing survival of neuronal cell bodies may have limited use.

I.8 The compartmental theory of neurodegeneration

There is plenty of evidence against the same key molecules being involved in Wallerian degeneration and in apoptosis.

As described in I.7.4 Sagot et al found that protection of the cell body, through *Bcl-2* over expression, was not enough to retard onset or progression of one neurodegenerative disease (Sagot et al, 1995). This was explained through the observation that axon loss, which is more tightly correlated with clinical progression of the disease, occurred regardless, in these mice. Similarly, *Bcl-2* does not prevent colchine-induced neurite degeneration although it delays apoptosis of the cell soma in cerebellar granule cell neurones (Volbracht et al, 2001). *Bad* and *bax* null mice exhibit no protection from Wallerian degeneration in explants of sciatic and optic nerve, though their retinal ganglion cell bodies are completely protected from apoptosis (Whitmore et al, 2003).

Finn et al (2000) showed that caspase-3 is not activated in PNS or CNS axons during degeneration, although it is activated in the dying cell body of the same neurones. Caspase inhibitors (eg: zVAD-fmk) do not delay axon degeneration, although they inhibit apoptosis of the same neurones. The authors conclude that axon degeneration is molecularly distinct from caspase-dependent apoptosis in the same neurones.

Just as those genes and proteins known to be involved in apoptosis do not seem to have similar roles in Wallerian degeneration, the corollary is also true. *Wld^S* does not

protect against somatic apoptosis in conditions where it protects neurites from degeneration (Deckwerth and Johnson, 1994).

However, there are indications that Wallerian degeneration is *analogous* to apoptosis. Wallerian degeneration is a self destruction mechanism, executed in response to injury, which can be manipulated genetically. Where apoptosis is specific to the cell body, Wallerian degeneration is specific to the specialised processes of one cell type- the axon of a neurone.

The *pmn* mouse, demonstrated by Sagot et al, to show signs that correspond, not to the loss of cells, but to the loss of axons belonging to those cells, might be expected to take greater benefit from a therapy that protects axons. Indeed Ferri et al, crossed the *pmn* mouse with the *Wld^S* mouse, as described in I.6, and found the *Wld^S* gene product attenuates signs of disease, extends life span, prevents axon degeneration, rescues motor neurone number and size, and delays retrograde transport deficits (Ferri et al, 2003). By contrast *Bcl-2* over expression does not attenuate disease signs or increase life span in *pmn* mice (Sagot et al, 1995).

Wld^S also protects axons in explants of sciatic and optic nerve from Wallerian degeneration where *Bad* and *bax* knock-outs failed (Whitmore et al, 2003)

The compartmental neurodegeneration hypothesis, proposed by Gillingwater and Ribchester (2001), suggests that three compartments of a neurone, which we have long described as morphologically and functionally distinct, may also have distinct programmes of self destruction, governed by different molecular players. The controlled death of the cell body is regulated by a plethora of proteins such as the Bcl-2 family and the caspases. Wallerian degeneration of the axon is regulated by *Wld^S* and as yet unconfirmed additional molecules.

The third putative compartment is the axon terminal. As explained in I.3, *Wld^S* protection of axon terminals is not as complete as its protection of axons. Though the

latter is robust, the former is eliminated with age, and with lower gene dosage. The fact that axon terminals are able to degenerate in the presence of both *Bcl-2* upregulation and *Wld^S* expression suggests that they also have their own mechanism for self destruction, though the molecules responsible are not yet known.

I.8.1 Clinical relevance of the compartmental theory of neurodegeneration

The consequences of this theory are important for neurodegenerative research. What has been demonstrated is that protection of the cell body can not diminish the severity of diseases where axon degeneration is what correlates with symptoms, as in Motor Neurone Disease.

When an Alzheimer's disease patient dies, neuronal loss is a prominent feature. However, evidence suggests that, like Motor Neurone Disease, what actually correlates with disease progression is axon and synapse loss in the brain (Selkoe, 2002). Song et al (2006) describe a culture system in which the environment of the cell bodies and axons can be manipulated separately. Addition of amyloid peptide ($A\beta$), a peptide which accumulates and deposits in the brains of Alzheimer's disease patients, to the axon compartment induces axonal degeneration followed by neuronal cell body degeneration. Inhibition of caspase activation blocks $A\beta$ induced apoptosis but not the axonal degeneration. However, blocking the axonal degeneration with a calpain inhibitor blocks not only the axonal degeneration but also apoptosis of the cell body. This suggests that it might even be possible to prevent neuronal cell death through inhibition of axonal degeneration.

Ultimately to battle a disease in which multiple neuronal compartments are involved it may be necessary to invoke a cocktail of substances that can combat both apoptosis and Wallerian degeneration, as well as protecting synapses from degeneration.

I.9 Aims of the thesis

Many questions remain to be answered on the subject of *Wld^S* and Wallerian Degeneration. For instance, important questions remain about the molecular

mechanism of protection by Wld^S protein, including the roles of Nmnat1 enzymatic activity, the VCP binding domain in the N-terminus, the precise cellular locus of the protein's main effects, the signalling pathways involved and the ultimate molecular targets of the protective effects. However the phenotypic characterisation of Wld^S is also incomplete and arguably the unknowns here are at least as important to establish, since the phenotype defines the kinds of questions that must be addressed on a cellular and molecular level. For instance it is remarkable that a mutation having such a profound effect on the response of neurones to injury, apparently has no discernable constitutive phenotype, despite substantial accumulation of mutant protein in neurones. In general, bridging phenotype-genotype gaps in biology remains an important challenge in many areas of molecular genetics. This thesis, therefore, largely addresses the hypothesis of compartmental degeneration and the scope and utility of the Wld^S phenotype in exploring, testing and better defining it.

Currently the compartmental degeneration hypothesis is based on extensive work with motor neurones. It explains the degeneration of neuronal cell somas by apoptosis and how interference in that may protect them. It explains the degeneration of axons by Wallerian degeneration and how interference in that may protect them. It also explains that neither of these degeneration mechanisms account for the degeneration of axonal terminals, pre-synaptic compartments, which exhibit degeneration in the presence of Wld^S (known to protect axons) and *Bcl-2* (known to protect cell bodies). However, it is unknown if this compartmentalisation extends to all axonal endings, as most of the work has been carried out examining neuromuscular junctions. Therefore in the first part of this thesis, I ask:

Does Wld^S protection differentiate between sensory endings and axons, as it does motor endings and axons?

Should all axon terminals, or specifically pre-synaptic structures, be considered as a separate neurodegenerative compartment?

To answer this question I have studied the degeneration of Ia afferent endings on muscle spindle organs in the muscles of Wld^S mice.

Given the answer to this question, the second part of my thesis follows:

Why are sensory axons and endings so much better protected than motor axons and endings?

I have investigated hypotheses based on differences in protein expression through Western Blotting of dorsal root ganglia and spinal cord, and through immunostaining sensory and motor nerves.

I also examined whether a difference in the branching patterns might distinguish the protection between sensory and motor neurones. This was achieved through study of the specialised γ -motor neurones that innervate the intrafusal muscle fibres of the muscle spindle and that branch less than α -motor neurones. I compared the degeneration of the terminals of these neurones in *Wld^S* mice, to that of motor neurones with larger motor unit sizes, which innervate the extrafusal muscle fibres.

Finally, I tested whether a difference between motor and sensory axon stump could be due to endogenous the activity present. Tissue culture of sensory and motor nerve was used to eliminate this difference.

The last part of this thesis attempts to extend the theory of compartmental degeneration. Though descriptions of the degeneration of cell body, axon and axon terminal have been discussed, another classical compartment of the neurone, the dendrite, has not been examined. So my final question is:

Can *Wld^S* protect against dendritic degeneration?

Does this separate morphological and functional compartment have a self destruction programme involving the same molecules as Wallerian Degeneration?

M. Methods

M.1 Animals and animal care

A transgenic mouse line that has become central to the study of Wallerian degeneration is one in which fluorescent protein is expressed in neurones driven by the *thy1.2* promoter.

Green Fluorescent Protein (GFP) from the *Aequorea victoria* jellyfish has proved invaluable to biological research because it is a protein and can be harmlessly driven in cells in an organism of interest. This was first shown to be useful in eukaryotes by Chalfie et al in 1994. They demonstrated that expression of the DNA in *Caenorhabditis elegans* produced a fluorescent product. The gene sequence has been altered to produce spectral variants, for example cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) that emit different wavelengths of light. This can be visualised in a variety of ways, most commonly by fluorescence microscopy or confocal microscopy (Shaner et al, 2005).

In *thy-1.2-XFP* mice, the fluorescent protein, driven by the *thy-1.2* promoter, labels the neurone in its entirety, however, despite using identical constructs, different subsets of neurones are labelled in each of the 25 lines generated (Feng et al, 2000). Of these 25 lines, there are 3 which have been used extensively throughout my PhD. These are the CFP-23, YFP-16 and YFP-H lines [Figure M.1, adapted from Feng, et al 2000]. All DRG sensory neurones and spinal motor neurones are reported to be labelled in the CFP and YFP16 lines, but only about 5% of them in the YFP-H line. However, I found that γ -motor axons and their terminals were usually not labelled in the CFP line.

Mice from *thy-1.2-XFP* lines were mated with *Wld^S* homozygotes to produce *thy-1.2 XFP Wld^S* heterozygote lines. Backcrossing to *Wld^S* homozygotes produced *Wld^S* homozygotes which also expressed fluorescent protein in neurones. Mice of each

genotype and at a range of ages between 8 and 88 weeks were examined for this thesis.

All animals procedures reported in this thesis were performed according to Home Office regulations and with the necessary project and personal licences

| Line | Motor Axon | Retina | | SCG | | DRG | Cortex | Cerebellum | | |
|--------|------------|--------|-------|------|------|------|--------|------------|------|-------|
| | | RGC | INL | Pre | Post | | | Mossy | Purk | Molec |
| YFP-12 | All | Many | A | None | None | Many | 5,6 | All | Many | Few |
| -16 | All | All | A+B | All | Few | All | 2-6 | All | None | Few |
| -21 | All | All | A | All | Many | All | 2-6 | All | All | None |
| -A | Many | Many | A | Many | None | Many | 2-6 | All | Many | Few |
| -C | All | All | A+B+M | All | Few | All | 2-6 | All | None | None |
| -D | All | All | A | All | None | All | 2-6 | All | None | Few |
| -F | All | Many | A | Many | None | All | 2-6 | All | Few | Few |
| -G | All | Many | A | All | Few | All | 2-6 | All | None | None |
| -H | Few | Few | None | Few | Few | Many | 5 | Many | None | None |
| GFP-F | All | All | A+B+M | All | None | All | 2-6 | All | Many | Few |
| -G | All | All | A+B+M | All | Few | All | 2-6 | All | Many | Few |
| -H | All | Many | None | All | None | Many | 2-6 | Many | None | None |
| -I | All | Few | None | All | None | Many | 6 | All | Many | None |
| -J | All | All | A+B+M | All | None | All | 2-6 | All | Many | None |
| -M | Few | Few | None | None | None | Few | Few | Many | None | None |
| -O | All | Few | None | None | None | Few | 2-6 | All | Many | None |
| -S | Few | Few | None | None | None | None | Few | Few | None | None |
| CFP-4 | All | Many | None | None | Few | Many | 5,6 | All | Many | None |
| -11 | All | Many | None | Few | Few | Many | 2-6 | All | Few | Few |
| -23 | All | All | A | None | None | Many | 2-6 | All | None | None |
| -D | All | Few | None | None | None | All | 5,6 | All | All | None |
| -F | All | Many | None | All | None | All | 2-6 | All | None | Few |
| -H | All | Many | None | None | None | Many | 2-6 | Many | None | None |
| -I | All | All | None | None | None | All | 2-6 | All | None | None |
| -S | Few | None | None | None | None | Few | Few | Few | None | None |

Figure M.1 Table of expression of fluorescent protein in *thy1.2*-XFP mice based on a figure by Feng et al (2000)

Regulated by the *thy1.2* promoter, very different expression profiles of yellow, green and cyan fluorescent proteins produced were produced in the nervous system of mice. The mouse lines I use have been highlighted: the YFP16, YFP-H and CFP-23 lines.

M.1.1 Animal sacrifice

All animals were killed by either cervical dislocation or by exposure to increasing levels of CO₂, in accordance with schedule 1 of the licensing regulations provided by the UK Home Office.

M.2 Morphology

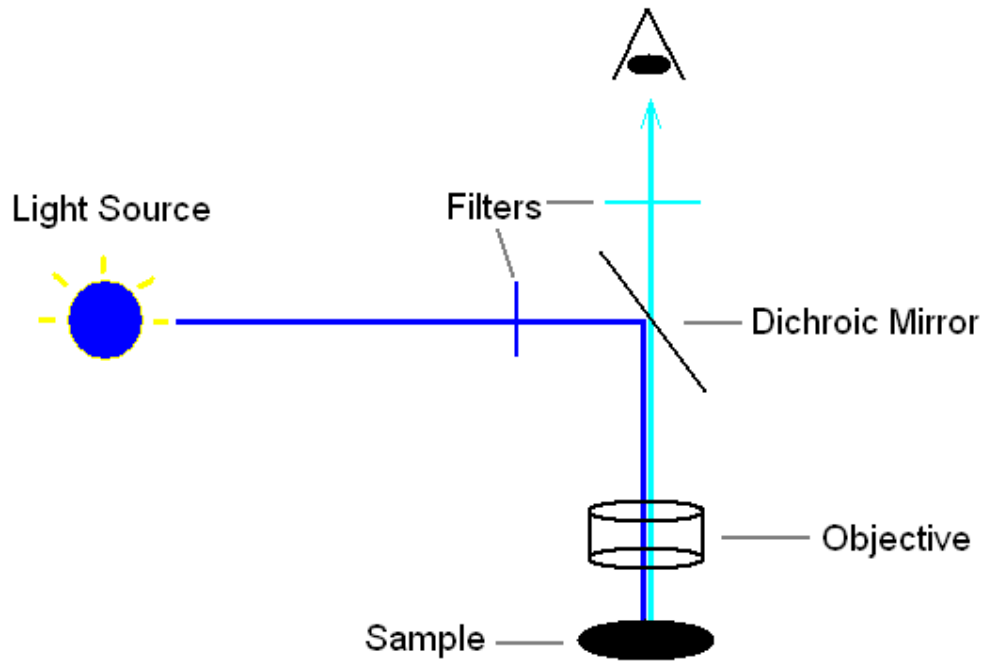
M.2.1 Fluorescence microscopy

Fluorescent molecules glow when their electrons are raised to an excited energy state by absorbing a high energy photon. Some energy is lost to other molecules as electrons return to their ground state. The rest of the energy is lost by emitting photons of light with lower energy (ie: a longer wavelength). The difference between excitation and emission wavelengths is called the Stokes shift and forms the basis for using separate emission, absorption filters and dichroic (or dichromatic) mirrors in fluorescent microscopy.

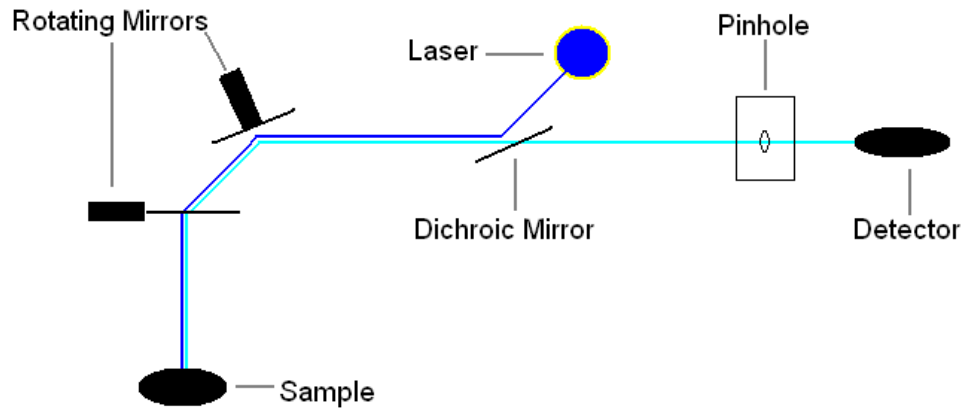
In fluorescence microscopy, light from a source (often a mercury lamp) is reflected off a dichroic mirror toward the specimen. A dichroic mirror reflects short wavelengths, but allows longer wavelengths to pass through. The returning emitted fluorescence, now at a longer wavelength, is allowed to pass through the dichroic mirror to the eye-piece [Figure M.2, based on a figure by Semwogerere and Weeks, 2005].

For confocal microscopy the mercury lamp is replaced by a laser. An argon laser is typically used to excite CFP, GFP or YFP; a helium-neon laser is used to excite red fluorescence, such as from rhodamine-conjugated proteins. Light from the laser is reflected on the dichroic mirror and is scanned across the specimen by scanning mirrors. Optical sectioning occurs after the light passes back through the dichroic mirror, when it enters a confocal pinhole on its way to the detector, comprising a sensitive photomultiplier. [Figure M.2, based on a figure by Semwogerere and Weeks, 2005]. The recorded photon energies are used to construct a two-dimensional

digital image. The confocal arrangement of excitation and emitted light maximises resolution and minimises blur from light emitted above and below the focal plane. Maximum brightness projection of a stack of confocal images obtained by systematic, sequential scanning through a vertical set of image planes in a specimen (Z-series) allows viewing of features of interest from different angles, rotating movies, and three dimensional rendering.



A



B

Figure M.2 Diagrams of fluorescent and confocal microscopy based on Semwogerere and Weeks, 2005.

A) In fluorescence microscopy, light is reflected off a dichroic mirror toward the specimen, this reflects short wavelengths, but allows longer wavelengths to pass through. The returning emitted fluorescence, now at a longer wavelength, is allowed to pass through the dichroic mirror to the eye-piece. B) For confocal microscopy the light source is replaced by a laser. The laser is reflected on the dichroic mirror and scanned across the specimen by the scanning mirrors. Optical sectioning occurs after the light passes back through the dichroic mirror, when it enters a pinhole on its way to the detector.

M.2.2 Sciatic Nerve Cuts

Mice were anaesthetised by Halothane inhalation (Merial Animal Health Ltd.) at 2-5% in Oxygen and Nitrous oxide (1:1). This was delivered using a Fluovac system (International Market Supply). Once a suitable level of anaesthesia had been achieved, examined by pinching the toe and noting the absence of a reflex response, the skin above the nerve was shaved and swabbed and the sciatic nerve on one side exposed. Approximately 1mm of the nerve was removed. Complete nerve transection was achieved with surgical scissors. The skin wound was closed with a single suture using 7/0 silk suture (Ethicon).

M.2.3 Lumbrical Preparation

Mice were sacrificed between 8 hours and 20 days after the sciatic nerve cut and the four deep lumbrical muscles from each hindfoot were dissected in oxygenated mammalian physiological solution (Ringer solution) with mM concentrations as follows: NaCl 120; KCl 5; CaCl₂ 2; MgCl₂ 1; NaHCO₃ 23.8; D-glucose 5.6. All chemicals used in the Ringer are purchased from VWR International Ltd and Fisher Chemicals, Fisher Scientific.

Dissected preparations were pinned out in Sylgard (Dow Corning) coated Petri dishes containing oxygenated Ringer solution using minuten pins (fine science tools).

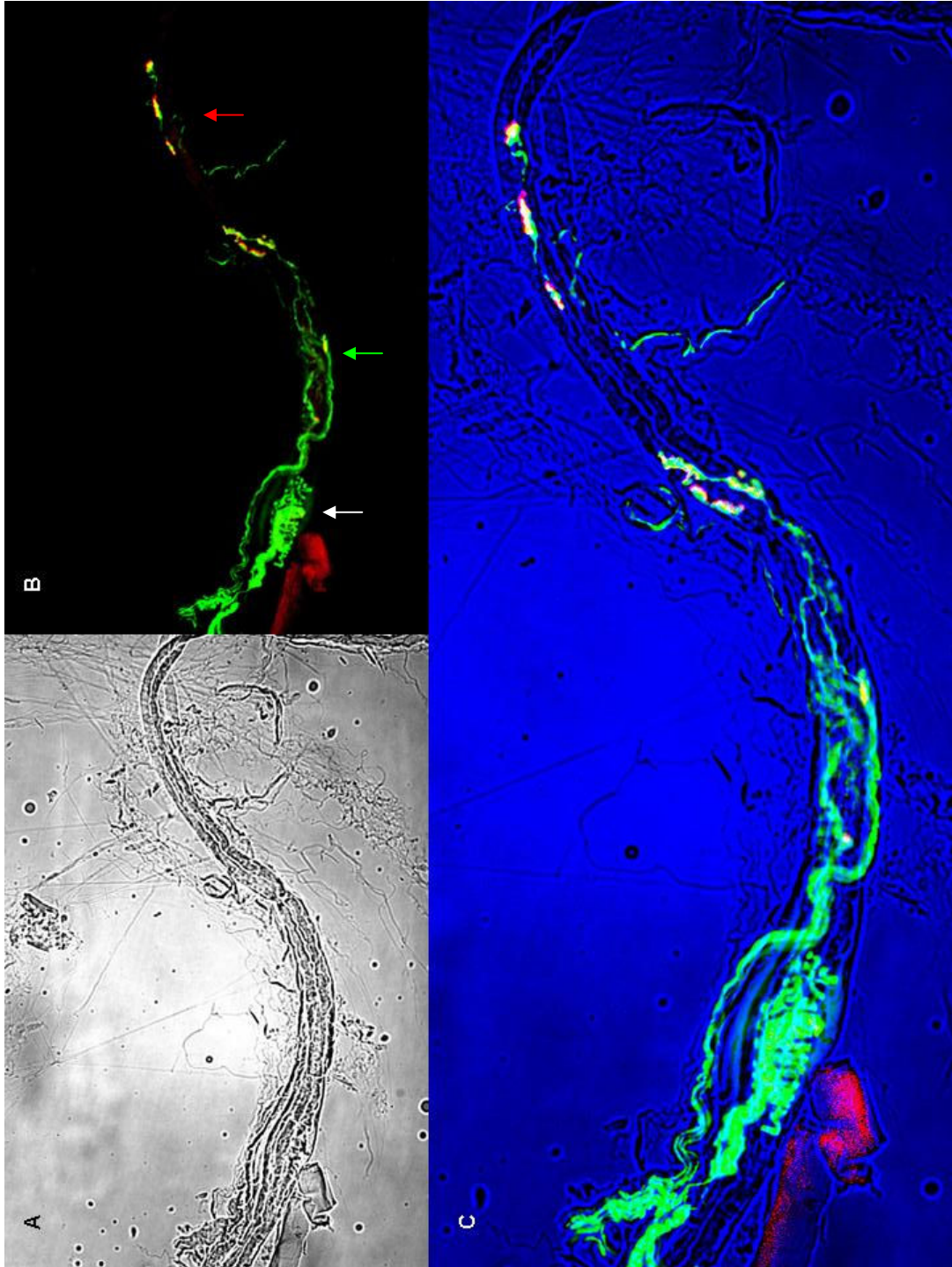
Lumbricals were incubated with Tetramethyl rhodamine isothiocyanate conjugated α -bungarotoxin (TRITC- α -BTX; purchased from either Molecular Probes or Cambridge Bioscience) for 10 minutes followed by washes in Ringer solution, then fixed in 4% paraformaldehyde (PFA; purchased from Fisher Scientific) for 20 minutes and washed in 1% phosphate buffered saline (1% PBS with the following micromolar concentrations; 137 NaCl, 2.76 KCl, 8.10 Na₂HPO₄ and 1.47 KH₂PO₄).

The solutions were kept in constant motion by placing the dish on a rotating platform during incubations.

Lumbrical preparations were mounted on glass slides in glycerol with 2.5% 1, 4-diazabicyclo [2.2.2] octane (DABCO) and covered with glass coverslips, to allow visualisation using fluorescence and confocal microscopy.

M.2.4 Spindle teasing

After dissection and preparation of lumbrical muscles, as described, the muscle was teased apart on a microscope slide in DABCO in glycerol. This was performed under the dissection microscope using a pair of pin vices. The position of the spindle was confirmed by visualisation of the preparation under a fluorescent microscope, as described below, during the process of teasing [Figure M.3]. When the intrafusal muscle fibres were isolated to the extent that their innervation was distinct from all the extrafusal innervation, a coverslip was applied to allow visualisation under the confocal microscope.



M.3 A teased muscle spindle

A teased muscle spindle from a YFP16 mouse with α -BTX used to stain γ -neuromuscular endplates: A) Transmitted light to show the intrafusal muscle fibres, B) Fluorescence microscope photo of the primary sensory ending (white arrow), secondary sensory endings (green arrow) as well as γ -motor innervation (red arrow) at the pole, C) Combined image.

M.2.5 FM1-43fx

Lumbrical muscles were dissected as described above. Muscles were subsequently pinned in the sylgard coated Petri dish and incubated with a fixable form of the original vital aminostyryl dye FM1-43fx (Molecular Probes) diluted in oxygenated Ringer solution to give a final concentration of 4-10 μ M. They were pinned under full tension for 20 minutes and then returned to resting length for a further 20 minutes. This stretch followed by a relaxation of the muscles was repeated 3 times giving a total time of submersion in the FM1-43fx of 2 hours. The solutions were kept in constant motion by placing the dish on a rotating platform during incubations. Muscles were then given a few quick washes in oxygenated Ringer solution and one or two short washes then fixed in 4% PFA for 20 minutes. After washing they were placed on slides in DABCO and glycerol.

M.2.6 Wld^S immunocytochemistry

Muscles, nerves and dorsal root ganglia were dissected in oxygenated Ringer solution. They were then fixed in 4% PFA for 20-30 minutes and washed in 1% PBS.

Tissue was then incubated in blocking solution (4% bovine serum albumin, 0.5% Triton-X in PBS) for 4 hours/overnight at 4°C. Primary antibody (Anti-Wld¹⁸, 2/1000 in blocking solution) was applied overnight at 4°C. The tissue was washed in 1% PBS for 5-8 hours and secondary antibody applied overnight at 4°C. After a final wash in 1% PBS the tissue was put on slides in DABCO and glycerol and a coverslip applied for visualisation.

M.2.7 Antigen retrieval method

In order to investigate whether Wld^S immunoreactivity was concealed by protein cross-linking caused by the fixation procedure, I examined whether it could be detected in axons or muscle treated with citraconic anhydride (Sigma Aldritch) and heat as described in Namimatsu et al (2005).

Tissue was treated as described above with an additional step. After fixation and before the application of the primary antibody, the tissue was heated to 98°C in 0.05% citraconic anhydride solution in PBS, for 45 minutes.

M.2.8 Visualisation and image capture

Slides were viewed under an Olympus BX50WI fluorescent microscope using Zeiss 10x, 20x and 40x objectives and a mercury lamp. Fluorescein Isothiocyanate (FITC) excitation/emission block (450-490 nm excitation filter, 510 nm dichoric mirror and 510-520 emission filter) was used for visualising YFP labelled axons and a customised excitation/emission block designed for visualisation of FM1-43 (435nm excitation filter, 455nm dichoric mirror and 515 IF emission filter) was used for visualising CFP labelled axons and FM1-43 labelled muscle spindles. A TRITC excitation/emission block (510-560nm excitation filter, 580 nm dichroic mirror and 590 emission filter) was used for visualising TRITC labelled α -bungarotoxin.

Images were captured using a colour chilled 3CCD camera (Hamamatsu C5810 or C4742-80) connected to an Apple G4 computer running Openlab (Improvision).

Slides were subsequently viewed under a Biorad Radiance 2000 confocal microscope via a Nikon Eclipse E600 microscope. A 10x objective and 40 x and 60 x oil immersion objectives were used. Fluorophores were excited with an Argon, HeNe or Red Diode laser. Images were captured with Laserssharp 2000 software running on a Dell Poweredge 1400 computer.

M.2.9 Spindle quantification

The number of innervated spindles per muscle was counted under the fluorescent microscope described above.

Morphology of innervated spindles was examined in confocal images. These were viewed using ImageJ and Adobe Photoshop from the Biorad source (.pic) files using the appropriate plug-ins. For measurements, the measuring tool provided by ImageJ was used. All data were entered into Microsoft excel 2001 worksheets for further

analysis. For Figures, Adobe Photoshop was used to reconstruct muscles or spindles that required multiple confocal or fluorescent images. Adobe Photoshop was used for image enhancement and files were exported as uncompressed tiffs files or compressed jpg files.

The following data were collected; the number of intrafusal muscle fibres, the number of Ia, II and γ -motor axons innervating the intrafusal muscle fibres, the length of the primary ending (and the other endings, but this data was not used) and number of spirals in the primary endings.

Ia and II axons were differentiated by width of the axon and position and morphology of the ending on the muscle spindle. Figure M.4 indicates the measurements made.

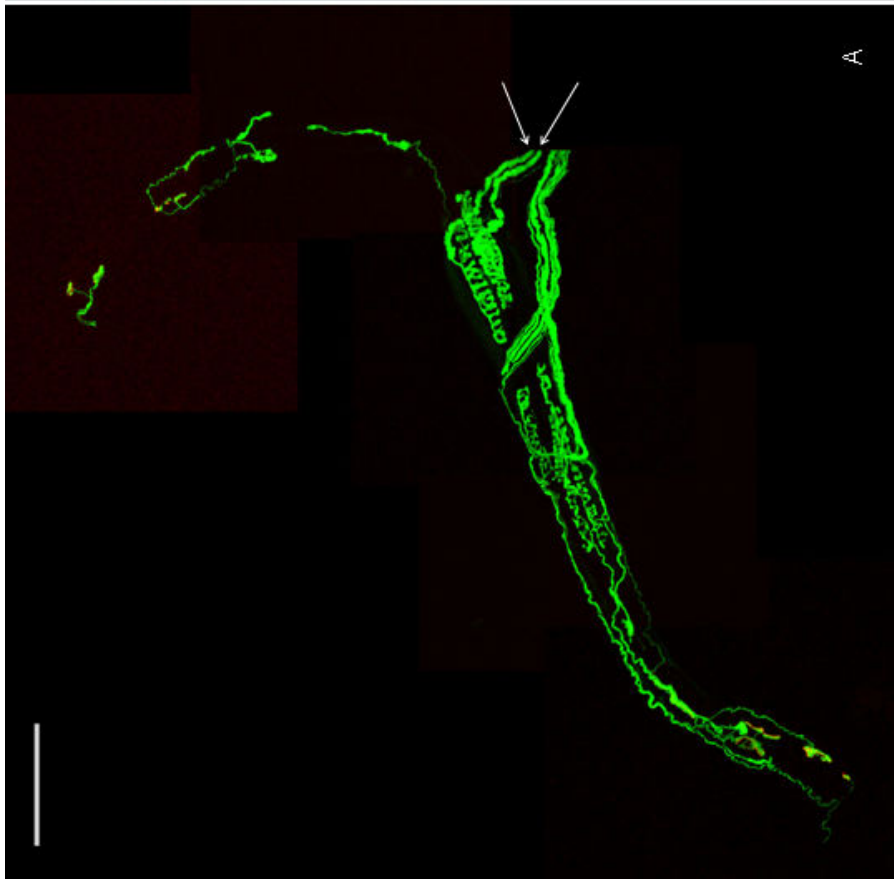
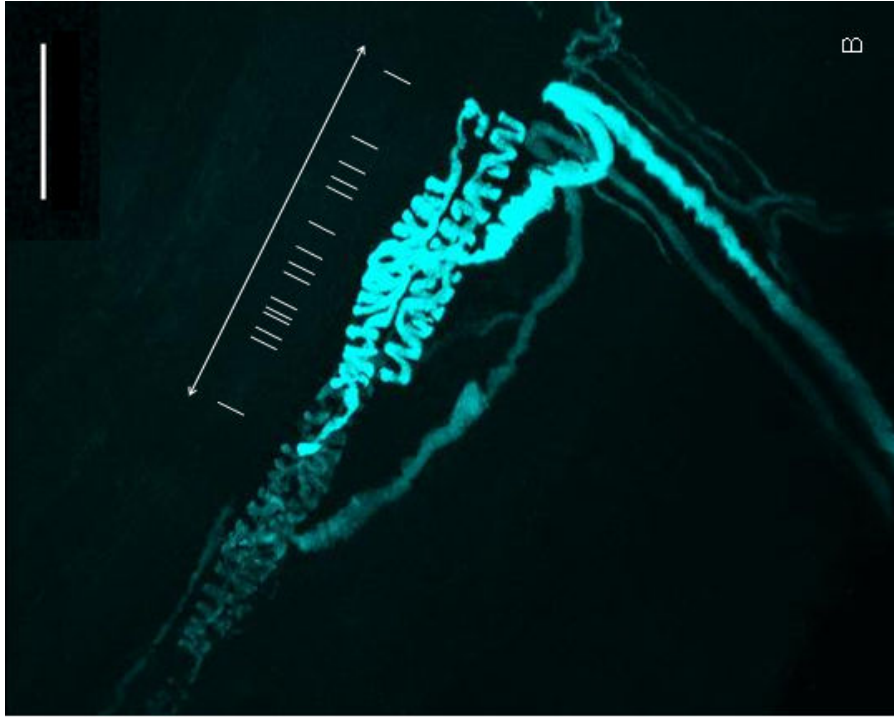


Figure M.4 Quantification of spindles

A) In order to count the number of I, II and γ -axons I used confocal microscopy of teased YFP16 muscle spindles, like the one shown here. Figure M.3 used arrows to indicate the primary, secondary and γ -endings. The I, II and γ -axons are the axons supplying each of these terminals, respectively.

The identity of axons was judged according their diameter, and position and morphology of the ending on the muscle spindle. In the example shown the primary, secondary and γ -endings are easy to recognise and although higher magnification would be necessary to discriminate the number of II and γ -axons, it is possible to see that 2 axons supply the primary ending. I have indicated these with two white arrows.

B) In order to measure the length of the primary ending and to count the number of spirals (or transverse bands) I used confocal images of spindles situated within a lumbrical muscle preparations, as shown here. The distance measured to be the length of the primary ending, was taken along the length of the intrafusal muscle fibre, starting and finishing at the furthest boundaries of the annulospiral. This is illustrated with a double headed arrow in the figure. The parallel lines indicate the 'top' of each annulospiral counted, 15 in total.

M.3 Western blotting

Unless otherwise stated, chemicals and reagents were obtained from Sigma Aldrich Company Limited.

M.3.1 Protein extraction

After sacrifice, spinal cords, between 6 and 8 DRG and dorsal and ventral roots were removed as quickly as possible, dipped in liquid nitrogen and put into eppendorfs on dry ice. During this process the mouse heads sat in dry ice. Once the other tissue was in dry ice the brains were removed either treated similarly (ie: frozen in liquid nitrogen and then transferred to dry ice) or homogenised directly into 1ml RIPA buffer (10mM HEPES, 150mM NaCl, 1mM EDTA, 1.1% SDS, 1% Triton X-100, 1% sodium deoxycholate with 1% EDTA-free, Roche protease inhibitor) on ice, then homogenised with a fine bore pipette, centrifuged at 10000g and 4°C and the supernatant collected and frozen in the -80° freezer until use.

Brains were removed last as they gave a strong Wld^S signal at the appropriate molecular weight using a variety of protocols, whereas spinal cord and dorsal root ganglia samples often produced degradation bands. Data collected using two different protocols were used for analysis of Wld^S expression in cerebellum.

Samples frozen in liquid nitrogen were subsequently homogenised in RIPA buffer each using a small glass pestle and mortar. The volume of RIPA used was equivalent to the weight of the sample. These were centrifuged and the supernatant collected.

Samples were then mixed with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and heated to between 80 and 100°C for 5 minutes.

M.3.2 SDS-PAGE

Gels for SDS-PAGE were purchased pre-cast from Novex, Invitrogen or prepared using stock solutions.

When gels were purchased they were Nupage 4-12% Bis-tris, 1.0mm 12 well gels used in conjunction with Tricine SDS running buffer.

Where gels were prepared, final concentrations were as follows: Resolving Gel, 375mM Tris-HCL, 10% Acrylamide, 0.1% SDS, 0.1% NNN'N'- tetramethyl ethylene diamine (TEMED), 0.01% Ammonium persulphate (APS); Stacking gel, 125 mM Tris-HCL, 4% Acrylamide, 0.1% SDS, 0.1 % TEMED, 0.01 % APS and they were used with Biorad 10x Tris-glycine reservoir buffer.

Samples were loaded along with a molecular weight marker, Precision Plus Protein marker (dual colour) (Bio-Rad) or SeeBlue pre-labelled standard (Invitrogen). The reservoir was filled with running buffer. Protein was then separated by electrophoresis at 150V for approximately one hour.

M.3.3 Transfer

Proteins were transferred to polyvinylidene difluoride (PVDF) membranes. This was done using a semi-dry method or a wet transfer as described.

Semi-dry

The membrane used for the semi dry blots was Millipore PVDF. The transfer was set at 13 V for 20 minutes using a semi-dry blotter (Bio-Rad) using a method adapted from Towbin et al (1979). PVDF was wetted briefly with methanol and washed with water prior to the transfer. The resolving gel, transfer membrane and Grade I filter papers (Whatman Laboratories), pre-soaked in transfer buffer made up from 0.192M Glycine, 25mM Tris and 20% Methanol, were assembled.

Wet transfer

Gels were placed onto Amersham Hybond-LFP PVDF membrane from GE Healthcare, and sandwiched between filter papers and sponges in an XCell II blot

module (Invitrogen), immersed in novex Tris-glycine transfer buffer and transferred overnight at 25 Volts.

M.3.4 Immunoblotting

Membrane was blocked for one hour in nonfat Marvel milk buffer (10% dried marvel milk in PBS-T PBS containing 0.2% Tween 20) to prevent non-specific binding of primary antibody. The membranes were then incubated with Wld^S18 antibody raised in rabbit (1/4000). Either Mouse anti- β -actin or mouse anti- β -tubulin monoclonal antibody was used as a loading control (1/4000) as stated. Following washes, the membranes were incubated with horse radish peroxidase (HRP) conjugated secondary antibodies. Signal of bound antibodies was developed by ECL chemiluminescence Western Blot detection reagent analysis system (GE Healthcare) according to the manufacturer's instructions. Chemiluminescence was detected using hyperfilm-ECL (GE Healthcare) and films were processed using a hyperprocessor (GE Healthcare).

M.4 Tissue Culture

M.4.1 Nerve explant culture

After sacrifice, mouse legs were immediately removed, skinned and placed in filtered oxygenated Ringer solution. Phrenic nerve was subsequently removed quickly and placed in a sterile dish containing culture medium (50% MEM-Earles + 25 mM HEPES <no L-glutamine>, 25% HBSS, 25% horse serum (heat inactivated), 1% 5000:5000 penicillin streptomycin solution and 0.5 ml 100x L-glutamine (200mM solution); All invitrogen) warmed to 37°C.

Sterile tweezers were used to place membrane filters (Millipore; Biopore; CM PICM ORG50) in culture dishes containing 1ml of warmed culture media. Isolated nerve segments were subsequently transferred on to the membrane filter using a sterile glass pipette. Up to 3 nerves were placed on one membrane filter in one dish of culture media.

Tibial and saphenous nerves were dissected from mouse legs and treated as described for the phrenic nerve above. In dissection of all nerve, non-neuronal tissue was removed as much as possible and length of the nerve was cut at approximately 1cm.

Dishes were incubated in a humid atmosphere at 37°C and 5% CO₂. Culture medium was replaced within half a day of culture and every 48 hours thereafter. Culture medium was warmed to 37°C before the replacement.

Visualisation of cultured nerves was undertaken periodically before the culture medium was due for refreshing. Dishes of cultured nerves were transported in a sterile, lidded dish to the alcohol swabbed platform of the fluorescence or confocal microscope and imaged as described in section M2.7. On returning to the tissue culture lab, culture media was exchanged before the dishes were returned to the incubator.

M.4.2 Spinal cord culture

Live spinal cord sectioning was performed by Carole Torsney in the Hugh Robson Building in George square. A 3 week old YFP-H *Wld^S* mouse was killed by decapitation and the spinal cord quickly removed. This was then stood upright in warm agar which was superglued to the cutting surface of a vibratome, and the chamber filled with oxygenated Ringer solution. The spinal cord was sliced at 300µm, 8 sections of spinal cord were retrieved and I placed these in oxygenated retinal culture media as described below. These were then brought back to 1 George Square (approximately a 5 minute walk) where they were transferred onto a biopore membrane on a dish containing 1ml of culture media as described for phrenic nerve.

M.4.3 Retinal culture

After sacrifice eyes were removed as quickly as possible and placed in filtered oxygenated Ringer solution. Retinas was removed from eyes under a dissecting microscope in a sterile Sylgard-lined dish. Retinas were then treated exactly as described above for phrenic nerve, the only difference being the composition of the culture media which was as above but with 8% Glucose and 2% B-27.

M.4.4 Retinal injury

Injury to the retinal explant was performed with a BD microlance or a Swann Motson razor blade.

Results Chapter 1: Does *Wld^S* protection differentiate between sensory endings and axons, as it does motor endings and axons?

1.1 Introduction

For decades the neuromuscular junction has been used as a model for studying form and function of synapses (Sanes and Lichtman, 2001). It is one of the easiest synapses to study as it is large and accessible to electrophysiological, morphological and molecular techniques. This has meant work on the neuromuscular junction has often guided research on other synapses, which has led to understanding of several general principles of synapse development, and neurotransmission. It seems likely that mechanisms of synaptic pathology might also be gleaned by appropriately designed studies of the neuromuscular junction.

Extensive work has been done in the Ribchester laboratory on the effect of *Wld^S* on degeneration of neuromuscular junctions at the terminals of motor axons (Ribchester et al, 1995; Mack et al, 2001; Gillingwater et al, 2002; Adalbert et al, 2005). These studies clearly show that neuromuscular junctions are not protected by *Wld^S* to the same extent as motor axons. Axons are robustly protected for up to a month after axotomy in *Wld^S* mice, irrespective of age or gene dose, however, in heterozygotes and in mice beyond 4 months of age, neuromuscular junctions cease to be protected (Gillingwater et al, 2002; Li Fan, 2007, PhD Thesis, University of Edinburgh). This has prompted the hypothesis that motor nerve terminals at neuromuscular junctions have their own mechanism for self-destruction (Gillingwater and Ribchester, 2001).

The finding that neuromuscular junctions are not protected by *Wld^S* in old and heterozygote animals implies, perhaps, that *Wld^S* does not protect any axonal ending in old or heterozygote animals. However in many ways the motor nerve terminal is a unique axonal ending. For example, it belongs to one of the largest neurones in the body, the motor neurone, which also projects further than most other neurones in the body (up to 5 cm in mice). The post-synaptic cell is non-neuronal and specific functional constraints have produced a number of adaptations, for example the

neuromuscular junction has very high fidelity transmission. ie: it is almost guaranteed that the post synaptic cell muscle fibre will respond and contract when the pre-synaptic axon fires.

This chapter addresses whether *Wld^S* protection discriminates between axons and endings in sensory neurones. I have focussed on the annulospiral sensory ending on the muscle spindle. This enables a direct comparison to the axonal terminals in muscle as the axons project similar distances and the environment of the endings is very similar.

Muscle spindles are sensory organs that signal changes in muscle length. The annulospiral endings of Ia fibres are morphologically distinctive and can be readily identified in muscles from *thy-1.2 XFP* mice which express fluorescent protein in sensory as well as motor neurones (Feng et al, 2000).

The possibility of a difference between sensory and motor axon protection in *Wld^S* mice has been reported previously (Brown et al, 1994). The degeneration of sensory axons in the sensory saphrenous nerve is slower than that in the phrenic nerve which contains mostly motor axons (Brown et al, 1994). In 1995, Fruttiger et al reported that they could see a distinct difference in the speed of Wallerian degeneration between muscle nerves and cutaneous nerves in 16 week old *Wld^S* mice. Five days post lesion almost all motor axons had degenerated but more than half of the sensory axons were still present (Fruttiger et al, 1995).

With the investigation of sensory ending degeneration in *Wld^S* mice being the overall aim, three issues were addressed in passing.

First, limited work has been undertaken on the mouse muscle spindle. The anatomy of the muscle spindle of the cat has been described in great detail (Banks et al, 1978; Banks et al, 1982; Barker and Scott, 1990). Descriptions of muscle spindles in the extra-ocular muscles of the cow (Blumer et al, 2003) and in various muscles of the rat and human have been published (Kim, 2007; Kararizou et al, 2005). However,

there appears to be a large variation in the anatomy of muscle spindles between species and possibly, within species from muscle to muscle (Edwards, 1975). Rosa P Edwards (1975) used electron microscopy to examine muscle spindles from the mouse biceps muscle in 3 month old mice. 7 muscle spindles were examined. Her primary aim was to study the capsule, as she was interested in the mode of entry of *Mycobacterium leprae* (that cause leprosy). For this reason, her observations of the sensory endings are concise.

Having examined the muscle spindle in unoperated mice as a control for those degenerating after axotomy, I was able to draw some conclusions about its morphology as visualised by fluorescent protein expression. In the following work, I used descriptive statistics to illustrate the basic components of a muscle spindle, as I have found them in the mouse lumbrical muscles.

Secondly, I asked whether spindle morphology changes with age. In humans, deterioration in gait, stability and both static and dynamic position sense occurs with aging (Madhavan and Shields, 2005). Functional and structural changes have been reported in the peripheral nerves of old subjects, for example: decreased nerve conduction velocity and demyelination. However, Kim (2007) suggests there may be age-related changes in the muscle spindles themselves. A physiological study has shown that dynamic and static length sensitivities of muscle spindle primary endings in response to ramp stretch were decreased in aged rats (Miwa et al, 1995). The possibility exists that this is due to morphological changes with age in the receptors on the muscle spindles.

Kim studied 121 teased spindles from rat muscle using light microscopy. These were divided into 3 groups depending on the age of the rat they had come from. He found no difference in the mean number of primary and secondary endings per spindle, but did find a difference in length and number of spirals (Kim, 2007).

This is important from the point of view of my *Wld^S* study, as any factor affecting spindle morphology would affect the quantification I undertook in order to examine

degeneration after axon transection. If spindle morphology changes with age, this must be taken into account when examining muscle spindles from animals in different age groups. For this reason, this question is addressed in mice as a control for my later experiments.

Thirdly, the degeneration of axons and their endings in *Wld^S* mice is complete by a month after axotomy, as opposed to within 48 hours in wild type mice. Is this a reflection of a) Wallerian degeneration proceeding at a slower pace, or b) A delayed start to Wallerian degeneration or c) an alternative form of axonal degeneration that occurs in the absence of Wallerian degeneration?

While quantifying the degeneration of Ia endings on muscle spindles it was possible to examine the pattern of degeneration of these spindles and compare this with the pattern of degeneration of wild type spindles. By comparing the pattern of degeneration in wild type and *Wld^S* mice it may be possible to discriminate between the options above. This adds information to the study of the mechanism of *Wld^S*-mediated interference with Wallerian degeneration.

1.2 Morphology of the muscle spindle

I used confocal microscopy of 65 muscle spindles in lumbrical muscles, and 15 teased muscle spindles to investigate the anatomy of the mouse muscle spindle and to test whether there was any change in this, due to aging.

I counted the number of intrafusal fibres that were innervated, and the number of axons innervating those fibres, classifying them as Ia, II sensory or γ -motor. I measured the length of the primary ending and counted the number of spirals, described elsewhere as ‘transverse terminal bands’, that the primary endings made on contact with the intrafusal muscle fibres. More detailed description of these measurements is included in the Methods section.

I divided the analysed spindles into two groups, based on the age of the mouse from which they were taken.

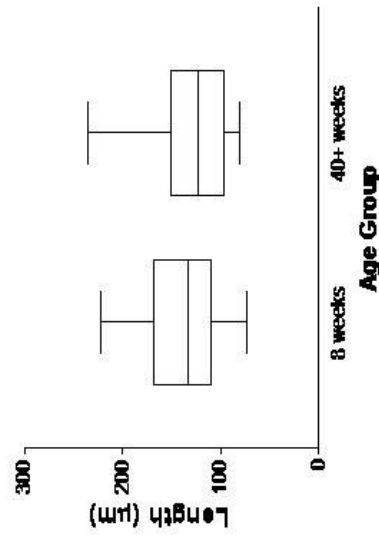
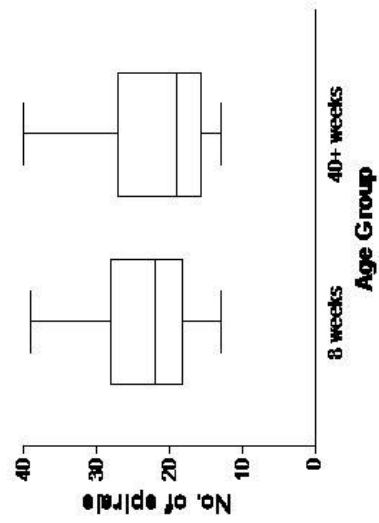
There was no significant difference between spindles from 8 week old mice and those from mice between 40 and 60 weeks of age when comparing the number of intrafusal muscle fibres innervated, or the number of Ia, II or γ -motor axons innervating the muscle spindle [Figure 1.1].

I also found no significant difference in the length or number of spirals of the muscle spindles in the two age categories. [Figure 1.1]

I conclude, therefore, that over the age range I studied in mice there are no overt morphological changes in the innervation of lumbrical muscle spindles with age.

Since there were no identifiable differences between the muscle spindles from older or younger mice, I used pooled the data in order to obtain a general description of the mouse lumbrical muscle spindle.

I found that there are between 2 and 5 intrafusal muscle fibres, innervated by either 1 or 2 Ia axons, with between 0 and 2 II axons. There were between 2 and 5 γ -motor axons supplying the poles with innervation [Figure 1.2]. The average length of the primary ending was $142.5 \pm 43.11 \mu\text{m}$, and the lengths are distributed with a Gaussian distribution (Kolmogorov-Smirnov Test). The average number of annulospirals was between 22 and 23, and the number of spirals was also distributed normally (Kolmogorov-Smirnov Test) [Figure 1.2].



| Unpaired t-tests | P Value |
|------------------|---------|
| Length | 0.6315 |
| No. of spirals | 0.7662 |

| Mann-Whitney tests | P value |
|---------------------------------|---------|
| No. of intrafusal muscle fibres | 0.6943 |
| No. of primary axons | 0.8665 |
| No. of secondary axons | 1.000 |
| No. of motor axons | 0.4634 |

Figure 1.1 No difference in spindle morphology with age

Neither the length, nor the number of spirals, was significantly different between spindles from 8 week old animals (n=36) and 40+ week animals (n=8). The number of intrafusal muscle fibres and the numbers of axons innervating the spindles were not significantly different between the age groups either (8 week olds, n=7; 40+ week old, n=8). The box-whisker plots shown indicate the median, interquartile range and outliers of the data set.

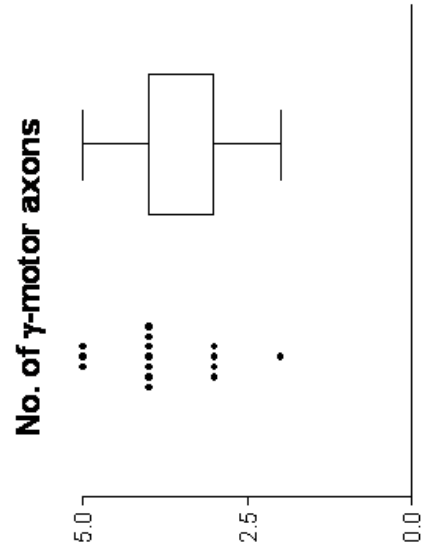
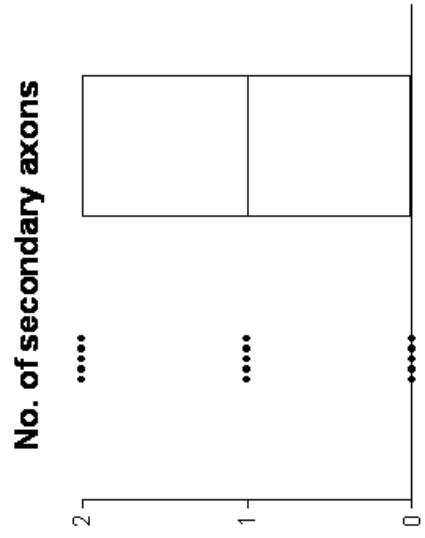
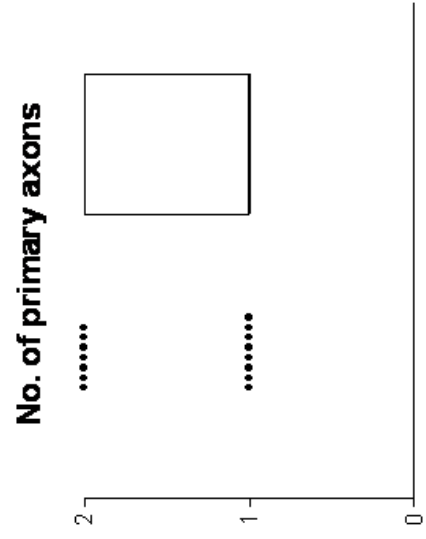
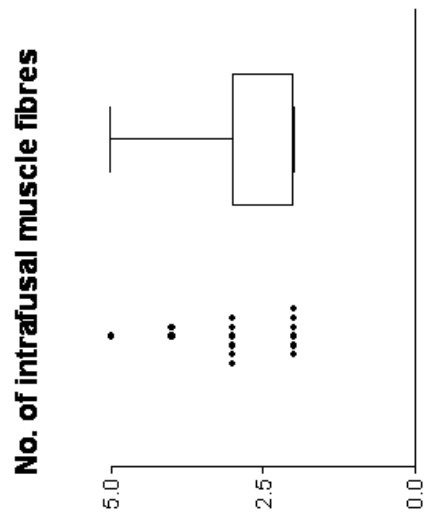


Figure 1.2 The components of a mouse lumbrical muscle spindle

Having examined 15 teased muscle spindles, the components of a mouse muscle spindle are presented here. Each dot represents a spindle. There are between 2 and 5, but most commonly 2 or 3, intrafusal muscle fibres; 1 or 2 primary axons in about equal proportions; up to 2 secondary axons and between 2 and 5 γ -axons. The box-whisker plots summarise the data by indicating the median, interquartile range and range of the data set.

1.3 Controls

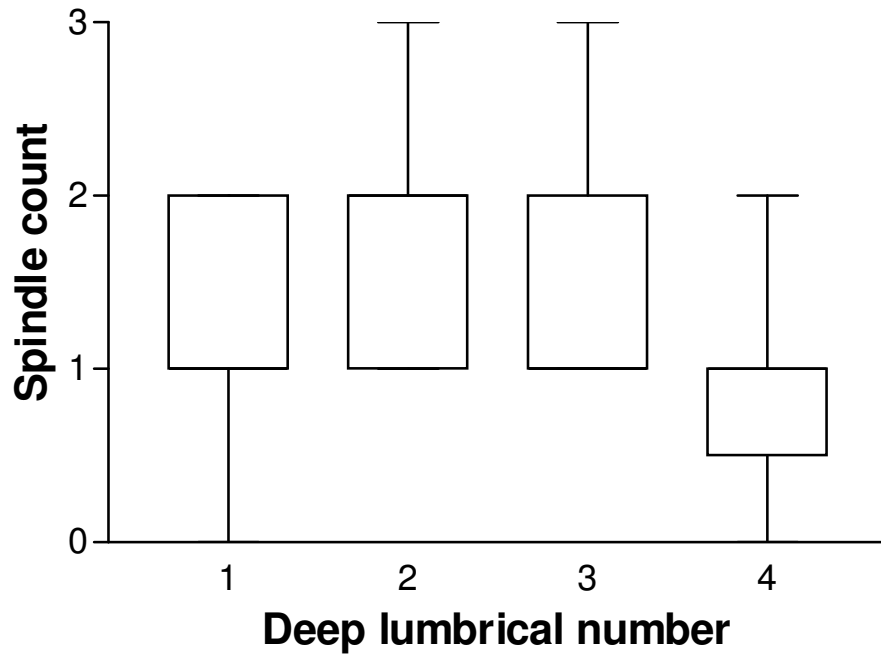
Figure 1.3 shows that each lumbrical muscle contains a different number of spindles. The fourth deep lumbrical has the fewest muscle spindles, which might be expected due to its slightly smaller size, and the second and third have the most. Interestingly, instances were found where the fourth or first deep lumbricals appeared to contain no muscle spindles at all. Throughout my analyses I counted the number of innervated spindles per foot as a measure that could indicate degeneration of Ia endings.

In order to be sure that the only factor affecting the number of innervated spindles per foot was degeneration due to axotomy, I tested for significant differences between the numbers of innervated spindles per foot in male/female, YFP16/CFP, left/right foot paired categories. These were all non-significant $p=0.7127$, 0.0942 , 0.7781 respectively (Mann Whitney test).

I also tested for any effect of *Wld^S* gene expression on the number of muscle spindles. There was no significant difference between *Wld^S* homozygotes, heterozygotes or wild type animals in the number of muscle spindles located in the lumbrical muscles, $p=0.8930$ (Kruskal-Wallis, non parametric analysis of variance test) [Figure 1.4].

Figure 1.3 The distribution of muscle spindles in the lumbrical muscle of the mouse foot

I examined 48 mouse feet. Due to a significant difference in the number of innervated spindles per lumbrical muscle, I used totals from whole feet in the rest of my analysis. The box-whisker plots indicate the median, interquartile range and range of the data set.



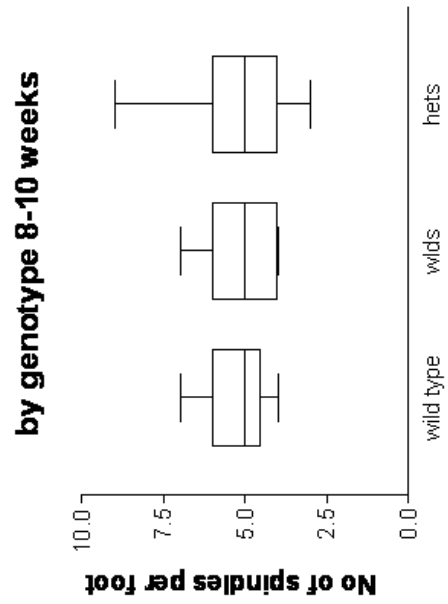
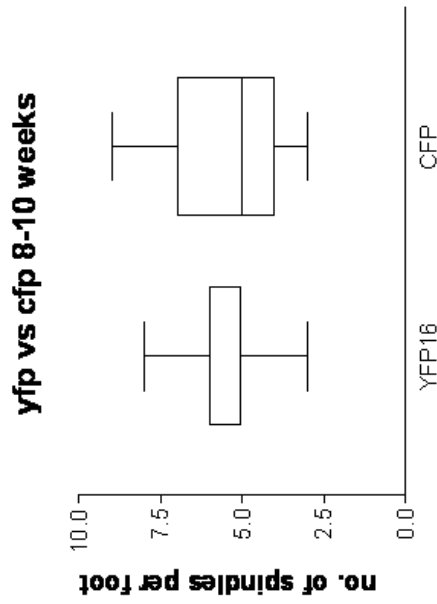
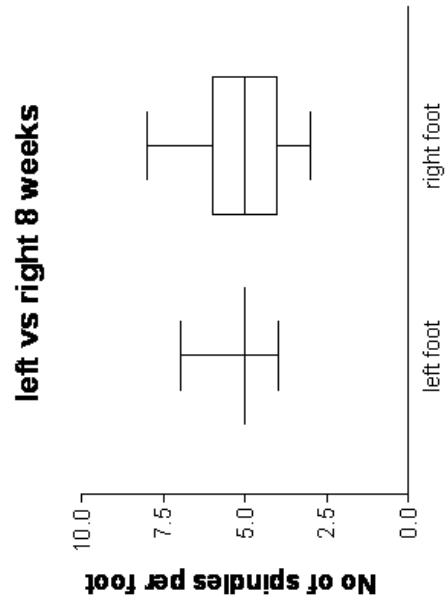
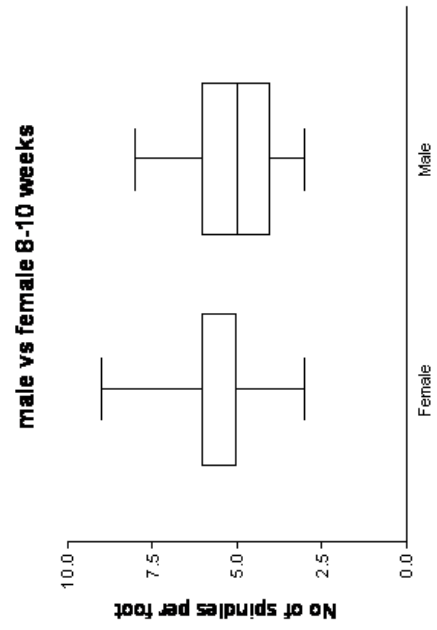


Figure 1.4 Controls

Box-whisker plots indicate the median, interquartiles and range of the data sets. Neither genotype, nor sex of the mouse affected the number of innervated muscle spindles per foot (male (n=37)/female (n=33) $p=0.7127$, YFP (n=41)/CFP (n=22) $p=0.0942$, *Wld^Shet* (n=23)/ *Wld^Shom* (n=21)/ WT (n=21) $p= 0.8930$). There was no difference between the number of muscle spindles in the left (n=8) and right feet (n=35) ($p= 0.7781$)

1.4 *Wld^S* strongly protects Ia annulospiral endings from degeneration after axotomy

As expected, in 8 week old *Wld^S* mice, annulospiral endings are apparent in muscles 2 days after axotomy, when wild type muscles are completely devoid of innervation. Remarkably, annulospiral endings could be identified in dissected lumbricals up to 20 days after axotomy [Figure 1.5]. Therefore, muscle spindles in *Wld^S* mice remain innervated 10 times longer than they do in wild type mice after nerve injury [Figure 1.6].

The number of innervated muscle spindles per foot was not significantly different from control feet, up to and including 5 days post axotomy [Figure 1.6].

I also noted that sensory axons innervating the muscle spindle survive intact up to 20 days post axotomy, while the endings themselves do begin to degenerate, as can be observed from Figure 1.5.

The length of the Ia ending in innervated spindles remains equivalent to control spindles up to 5 days post axotomy [Figure 1.7]. However, the number of spirals decreases within the same time frame. This pattern of losing spirals before losing length is examined further in section 1.7.

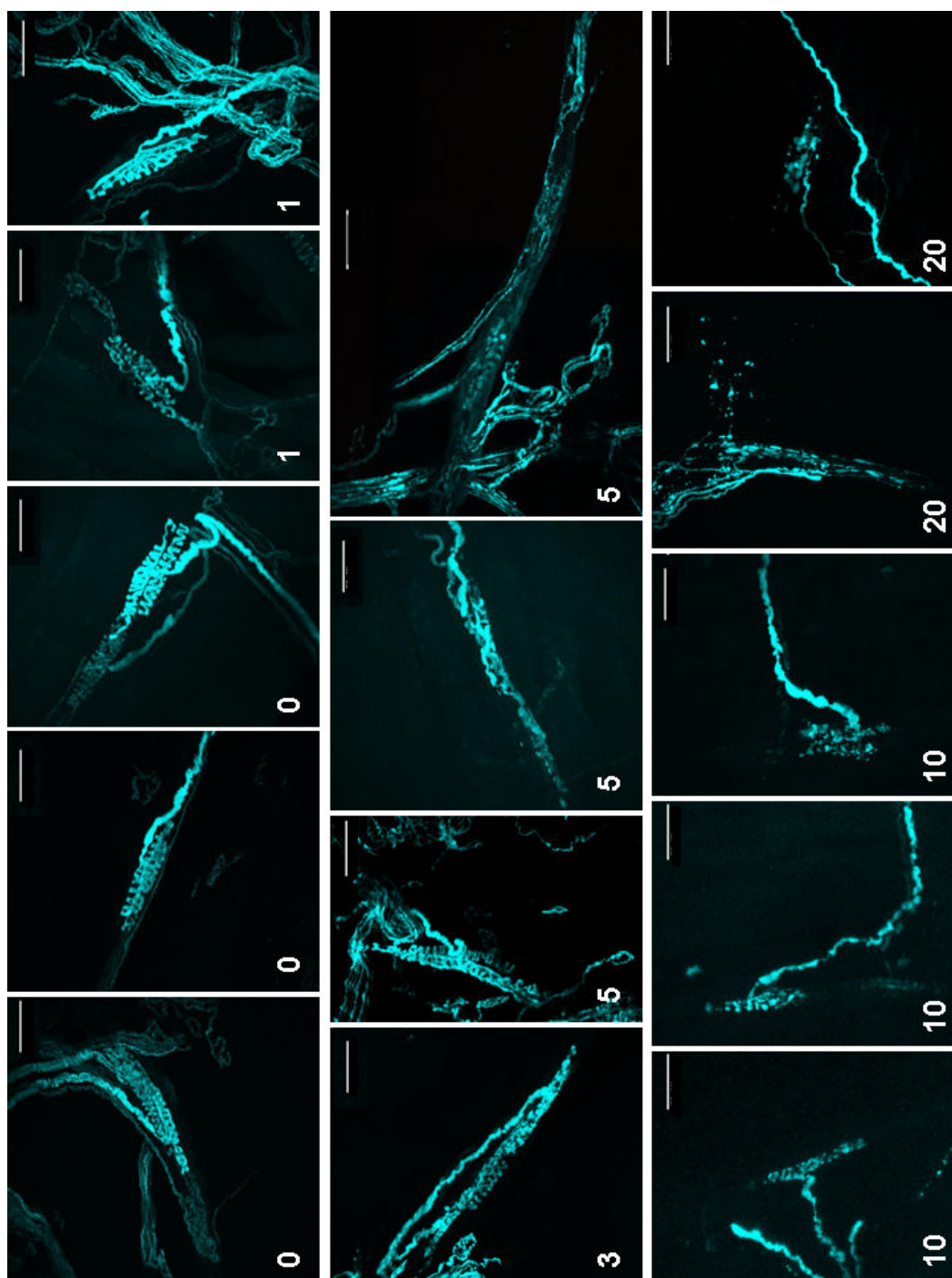


Figure 1.5 Confocal micrographs of preserved IA endings on muscle spindles in the muscles of *Wld^S* mice after axotomy

Confocal micrographs of innervated muscle spindles found in the muscles of 8 week old *Wld^S* mice. Marked on each panel is the number of days after axotomy. Each picture features one annulospiral ending.

This figure demonstrates that primary sensory endings are identifiable in the lumbrical muscles up to 20 days post axotomy. It shows the degeneration of IA endings with time post axotomy. It is also included in order that the reader can familiarise his/herself with the appearance of IA endings at various stages of degeneration as these are examined throughout this chapter.

In these images it can be observed that the axons innervating the sensory ending are always intact (when present). It can also be noted that the Ia Spindle ending itself degenerates over these 20 days, so that by 5 days post axotomy, although the primary ending retains the length of an unoperated spindle the annulospiral features begin to be lost. From 5-20 days length of the ending also decreases. These observations are explored further in later figures. Scale bars= 50µm.

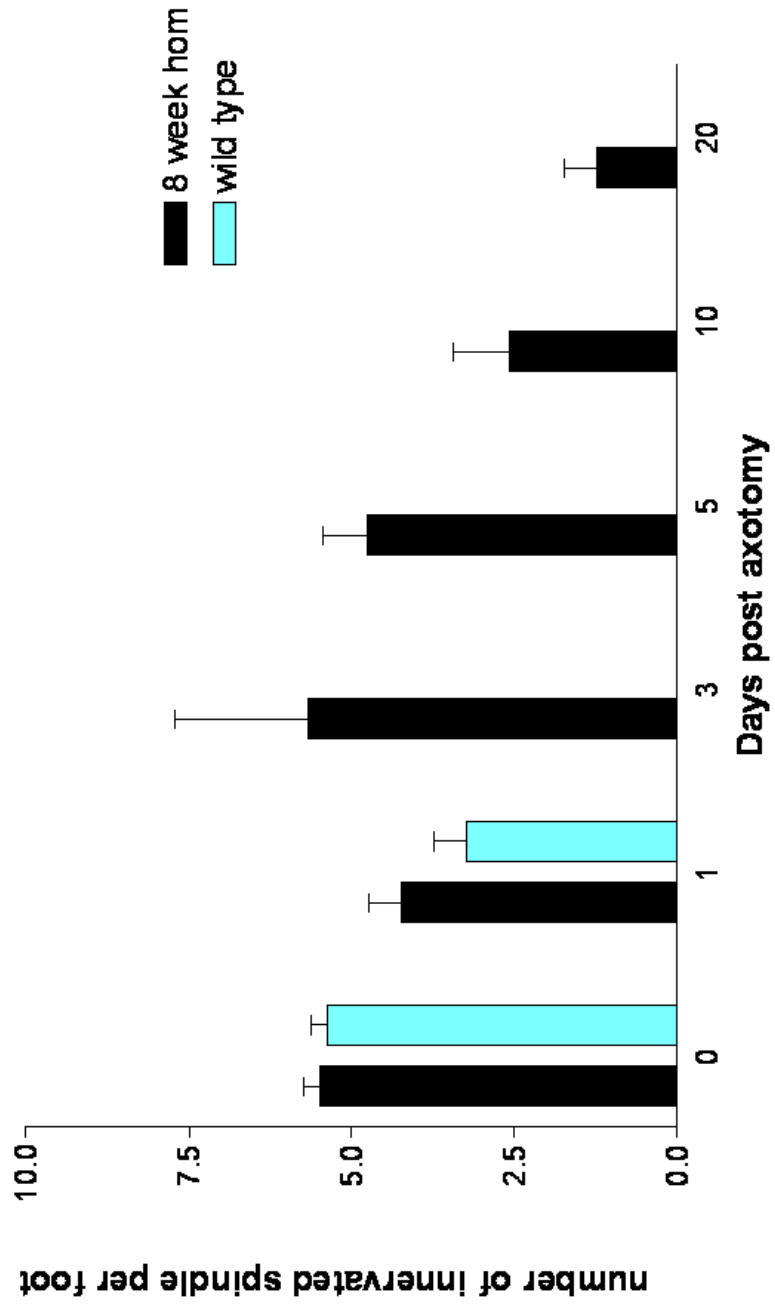


Figure 1.6 Persistence of innervated muscle spindles in the lumbricals of *Wld^S* mice after axotomy

The bar graph shows the number of innervated spindles counted per foot at the given number of days after axotomy in WT and *Wld^S* mice. Annulospiral endings could be identified up to 20 days post axotomy in 8 week old *Wld^S* mice, in contrast no annulospiral endings could be identified in wild type muscle 2 days after axotomy. This suggests *Wld^S* preserves Ia sensory endings for about 10 times longer than they would survive in wild type animals.

The number of innervated spindles per foot was not different in *Wld^S* mice in unoperated animals and up to and including 5 days post axotomy. However 10 days post axotomy and 20 days post axotomy there were significantly fewer innervated spindles per foot than in unoperated animals, $p < 0.01$ and $p < 0.001$ respectively (Tukey's multiple comparison test).

For 8 week old *Wld^S* animals, $n = 25, 4, 2, 8, 7, 8$ for 0, 1, 3, 5, 10 and 20 days post axotomy respectively. For wild type animals $n = 21, 4, 4, 4, 3, 1$ for 0, 1, 3, 5, 10, 20 days post axotomy respectively.

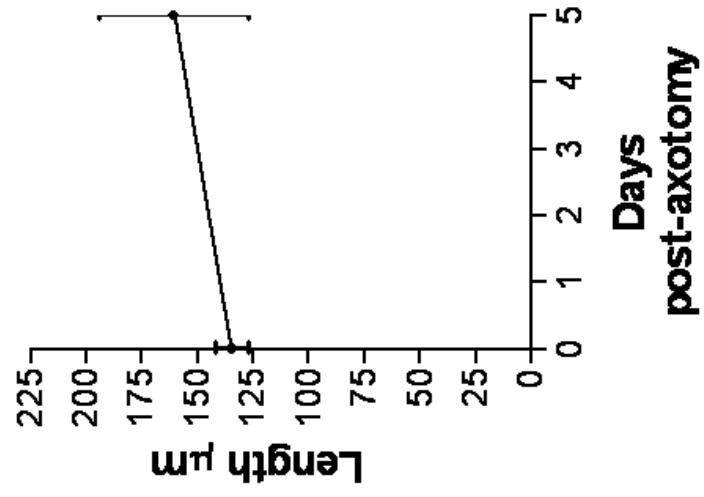
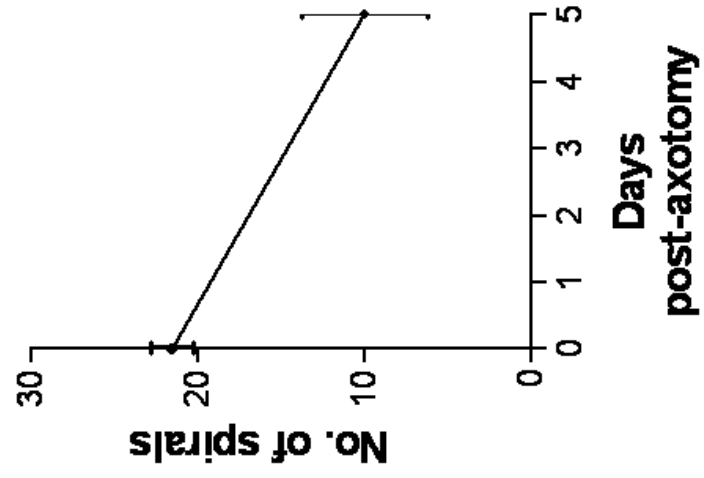


Figure 1.7 Quantifying degeneration of Ia annulospiral endings in *Wld^S* mice

There is no significant difference between the length of the primary ending in *Wld^S* mice up to 5 days post axotomy, and in unoperated animals. However, during the same time frame the number of spirals decreases.

N= 29 for unoperated feet, n=7 for feet 5 days post axotomy.

1.5 *Wld^S* protects Ia endings better than motor endings

In heterozygous *Wld^S* mice, and in mice as they age, motor endings lose the protection afforded by the gene. In order to investigate whether sensory endings have a similar phenotype, I examined innervation to the muscle spindle in old and heterozygote mice.

Surprisingly, Ia Sensory endings persist up to 20 days in heterozygous *Wld^S* mice and also in mice at 16 weeks and older [Figure 1.8]. This is in contrast to the motor endings, which are not protected in heterozygote [Figure 1.9] or old homozygous *Wld^S* mice [Figure 1.10].

There is also evidence that sensory endings in young homozygote *Wld^S* mice are protected to a greater extent than motor endings, as they persisted in muscles which had lost all motor innervation [Figure 1.11].

Figure 1.12 shows the percentage of innervated neuromuscular junctions in the lumbrical muscles, 5 days post axotomy, divided into groups dependent on mouse age and genotype. In order to compare this information about the motor innervation to information on sensory innervation, I converted the data on sensory innervation to percentages also. I did this using the number of innervated spindles in unoperated muscles as the denominator.

The oldest mouse I was able to investigate was 88 weeks old and 5 days after axotomy there were 4 innervated spindles one of which is pictured in Figure 1.13.

These results demonstrate that muscle spindle endings have stronger protection from Wallerian degeneration post axotomy than motor endings do.

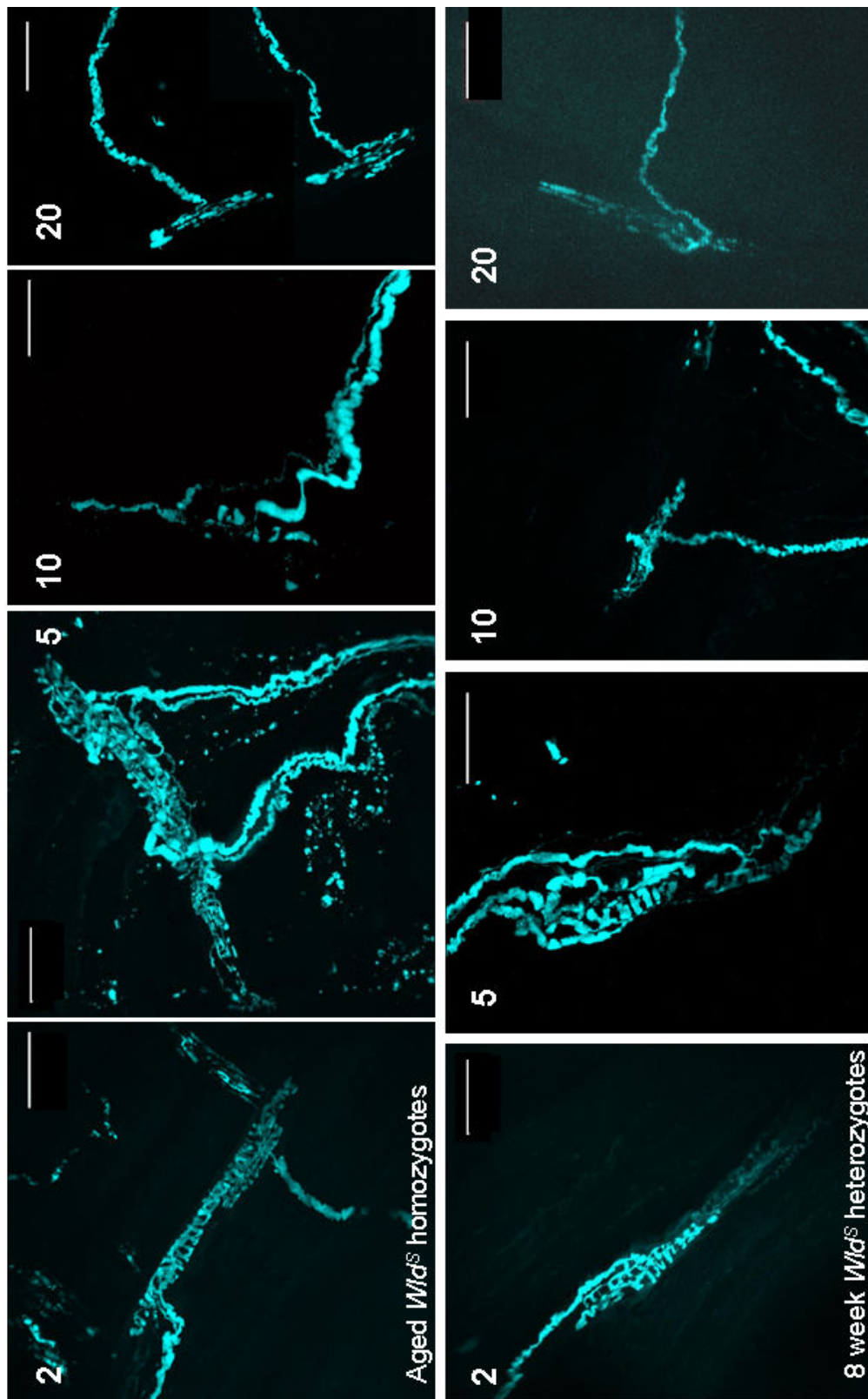
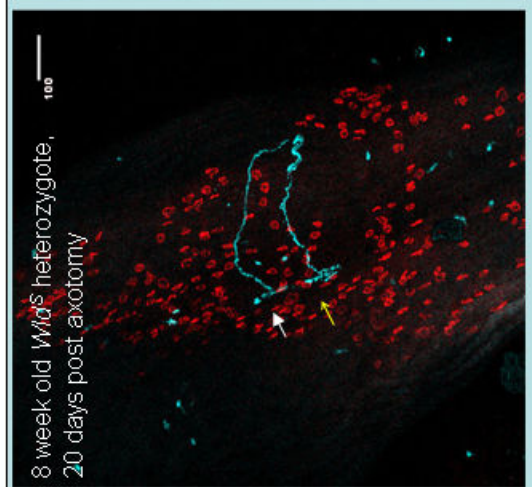
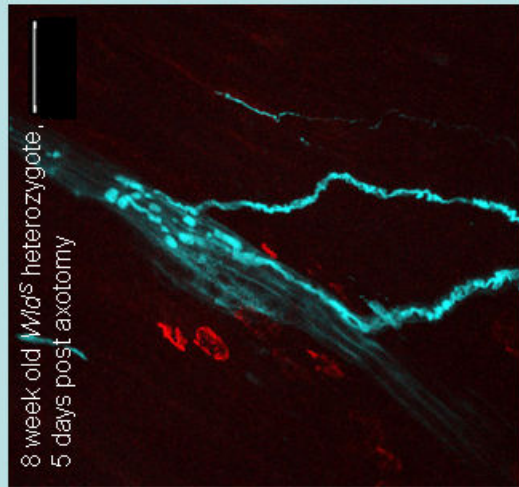
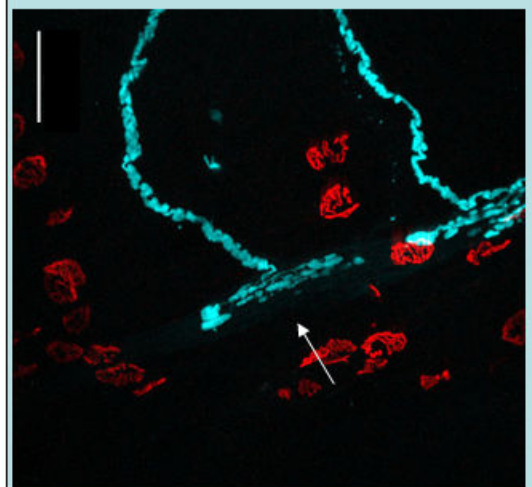
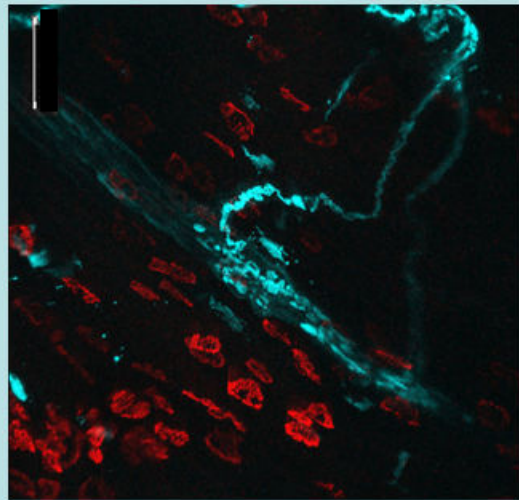
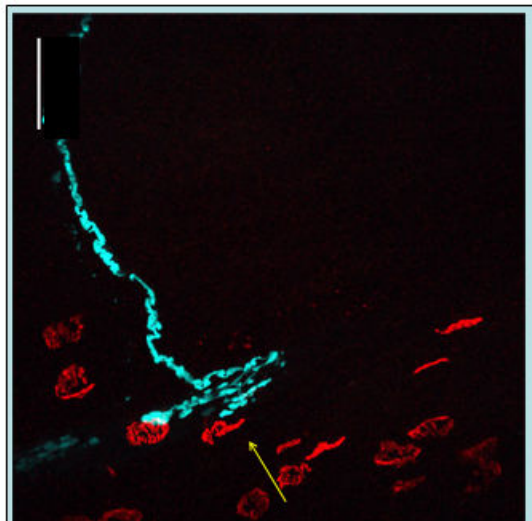
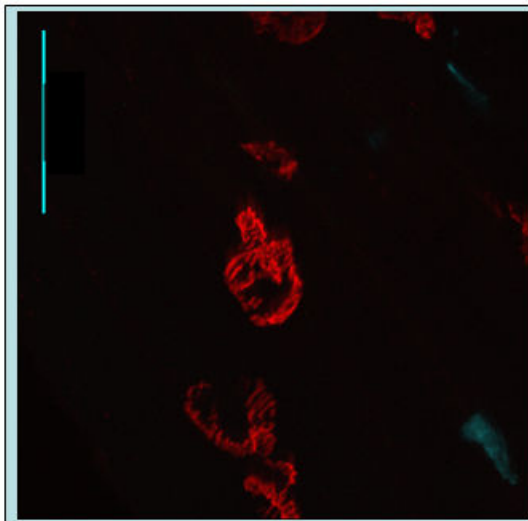


Figure 1.8 Protection of Ia annulospiral endings in heterozygous and aged *Wld^S* mice

Confocal images of preserved IA annulospiral endings on muscle spindles in lumbrical muscle preparations. Marked on each panel are the number of days after axotomy. Top row, examples from aged *Wld^S* mice (32-58 weeks of age), Bottom row, examples from heterozygous *Wld^S* mice.

As in Figure 1.5, this demonstrates that preserved annulospiral endings can be identified in muscles up to 20 days post axotomy. The degeneration of these spindles can be seen over time post axotomy. Features such as intact sensory axons supplying the endings, and degeneration of the annulospiral morphology occurring before reduction of the length of the primary ending can again be noted (but are the subject of later figures).

Scale bars= 50µm



8 week old *Wld^s* heterozygote,
5 days post axotomy

8 week old *Wld^s* heterozygote,
20 days post axotomy

Figure 1.9 Ia annulospiral endings are protected to a greater extent than motor endings in heterozygous *Wld^S* mice.

Confocal micrographs of lumbrical muscle from heterozygous *Wld^S* mice. Top row) 5 days post axotomy. Bottom row) 20 days post axotomy. Innervated muscle spindles and denervated neuromuscular junctions visualised with TRITC conjugated α -BTX are shown. Arrows indicate annulospiral endings which are shown in more than one panel. Scale bars= 50 μ m except where indicated to be 100 μ m.

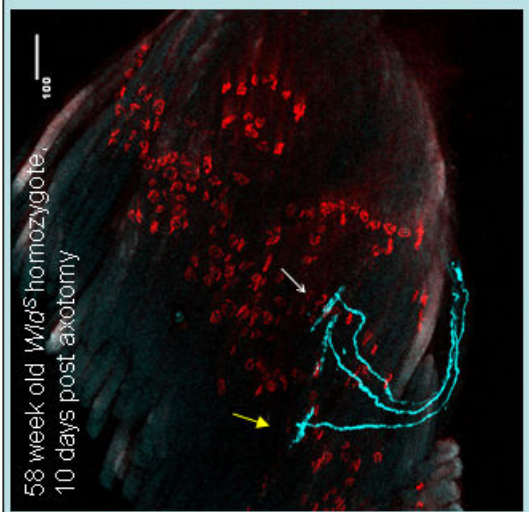
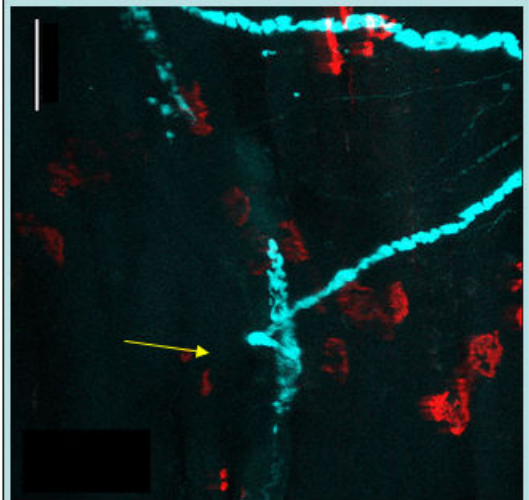
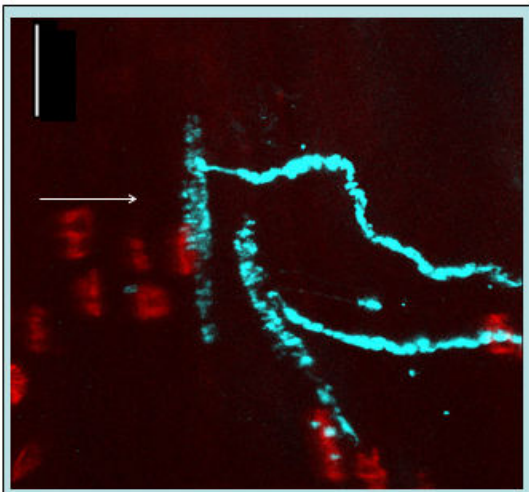
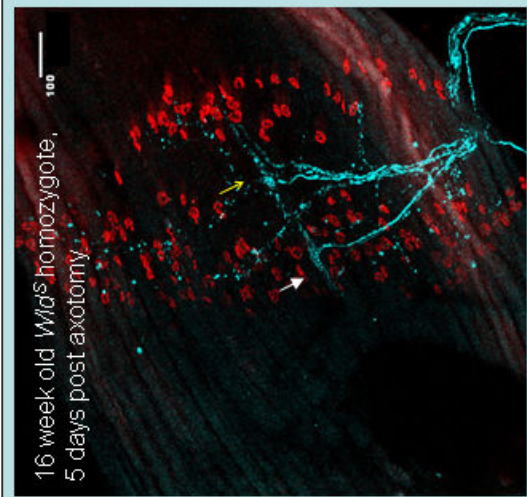
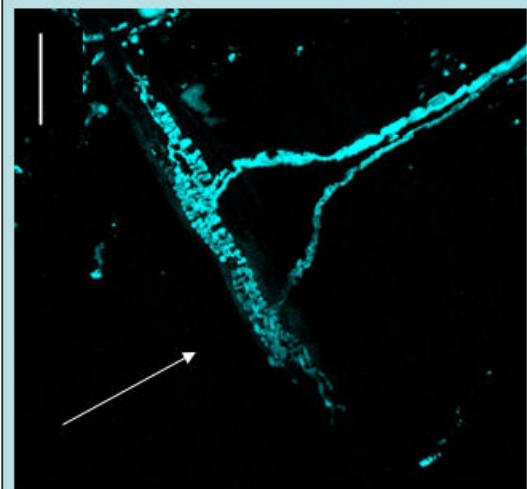
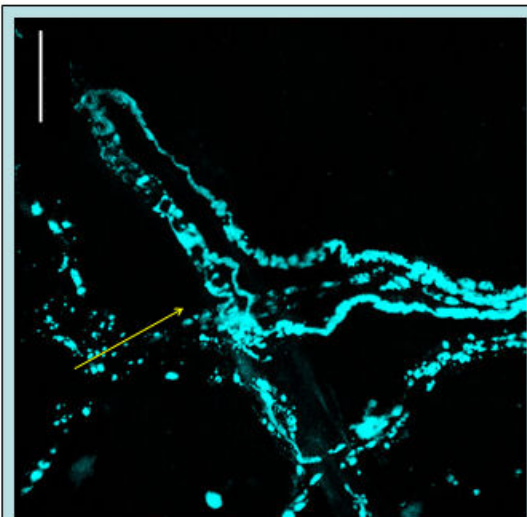
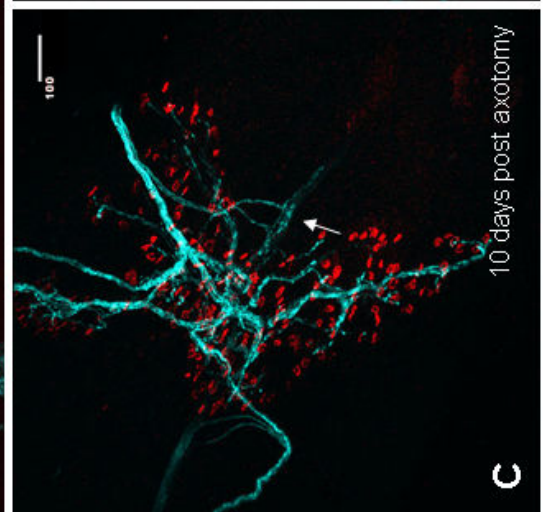
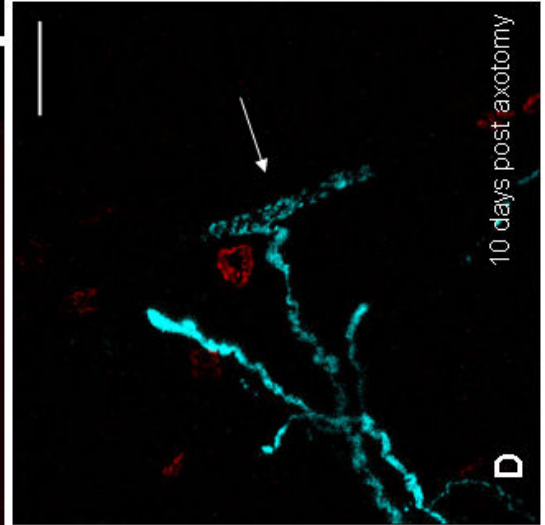
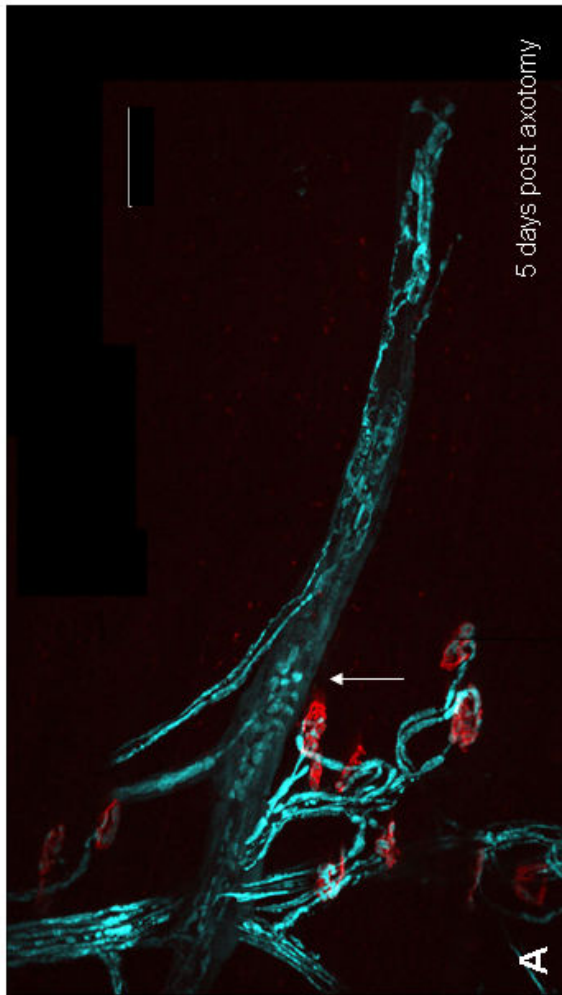
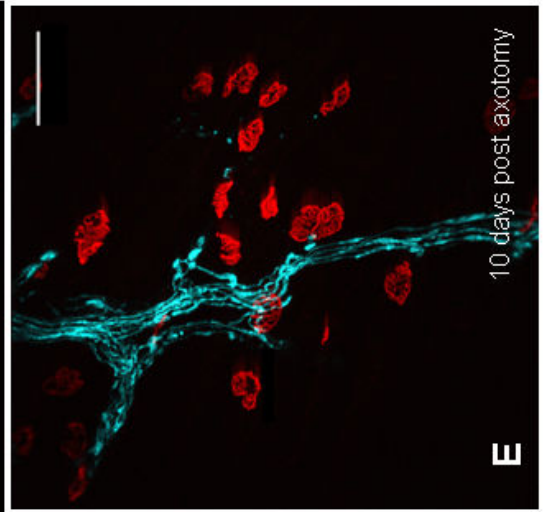
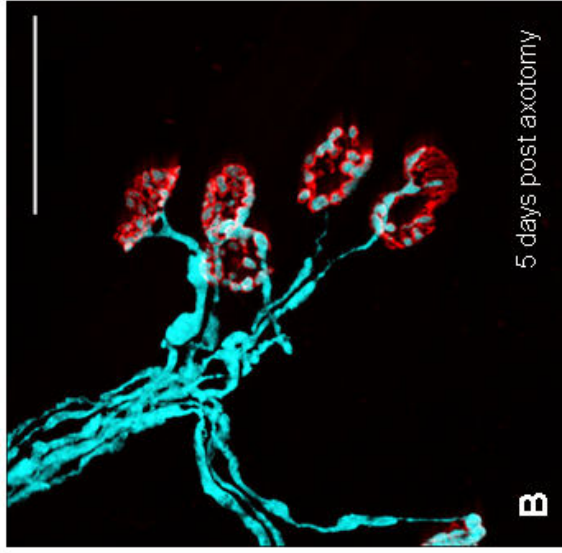


Figure 1.10 la annulospiral endings are protected to a greater extent than motor endings in aged *Wld^S* mice

Confocal micrographs of lumbrical muscles showing innervated muscle spindles and denervated neuromuscular junctions visualized with TRITC conjugated α -BTX. This figure gives examples of the relative protection of the sensory endings in comparison to the neuromuscular endings in mice older than 8 weeks. Top row, 16 week old *Wld^S* mouse 5 days post axotomy, bottom row, 58 week old *Wld^S* mouse 10 days post axotomy. Arrows indicate annulospiral endings which are shown in more than one panel. Scale bars= 50 μ m except where indicated to be 100 μ m.



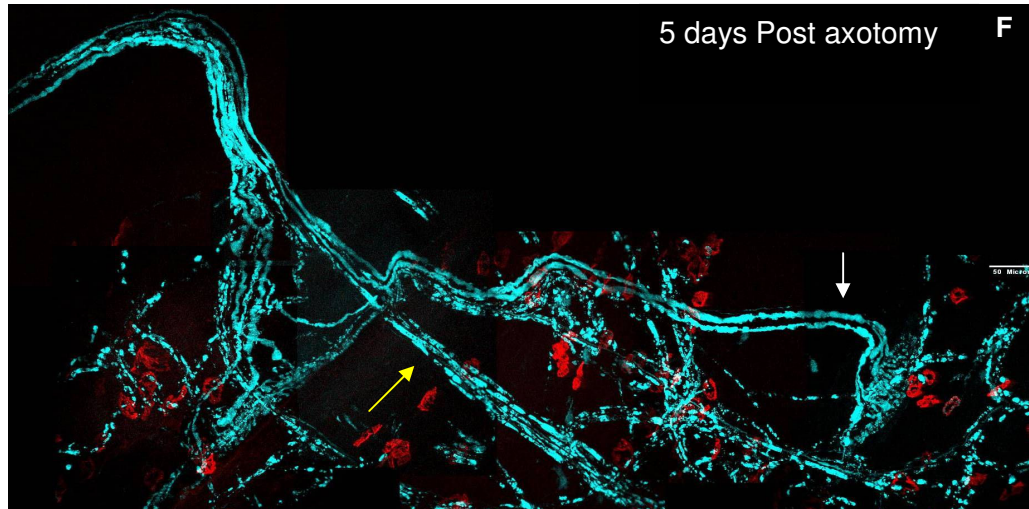


Figure 1.11 Ia annulospiral endings may be protected to a greater extent than motor endings in young *Wld^S* mice

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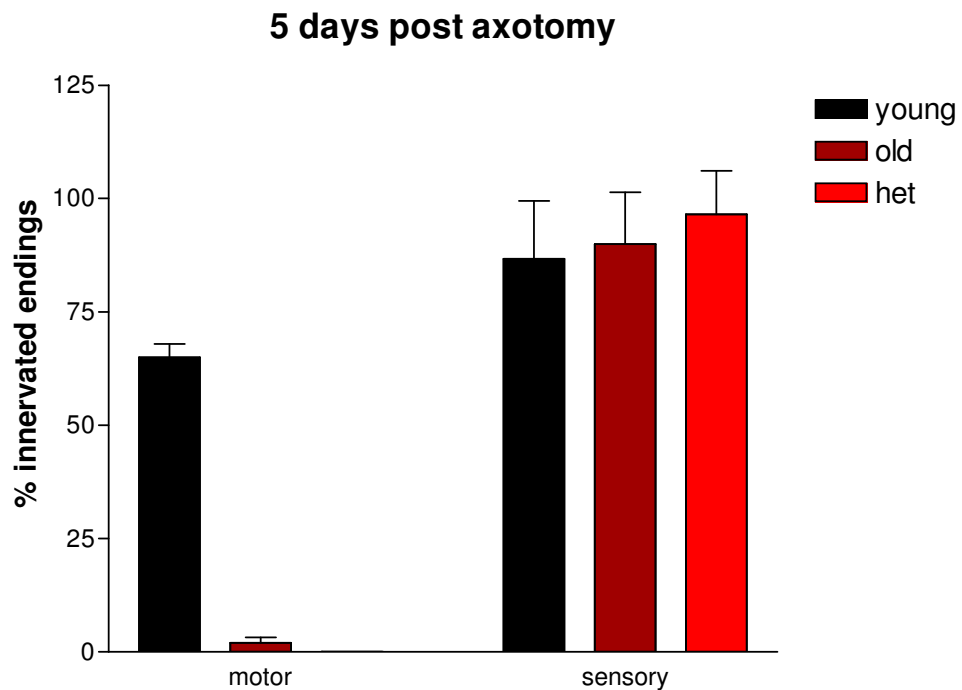
Confocal micrographs showing sensory and motor endings in 8 week old *Wld^S* mice. A and B) 5 days post axotomy, C, D and E) 10 days post axotomy.

Although at 5 days both sensory and motor endings show some protection from degeneration, at 10 days post axotomy, the preservation of the sensory endings can be seen to be greater than the preservation of the motor endings. Sensory endings indicated with white arrows. (Motor endings can be recognised due to staining of the post synaptic receptors with TRITC-conjugated α -BTX)

F) Reconstruction of part of a lumbrical muscle from an 8 week old *Wld^S* mouse, 5 days post axotomy. This shows intact axons (white arrow) leading to sensory endings and degenerating axons (yellow arrow) leading to motor endings. Scale bars= 50 μ m except where indicated to be 100 μ m

Figure 1.12 la annulospiral endings are protected to a greater extent than motor endings in old and heterozygote *Wld^S* mice.

A graph that shows the percentage of innervated motor endplates 5 days post axotomy in young (n= 8), old (n= 10) and heterozygote (n= 12) *Wld^S* mice. In contrast the number of innervated sensory endings, expressed as a percentage of the number of innervated sensory endings in unoperated mice, is higher in all three groups, but particularly in old and heterozygote *Wld^S* mice



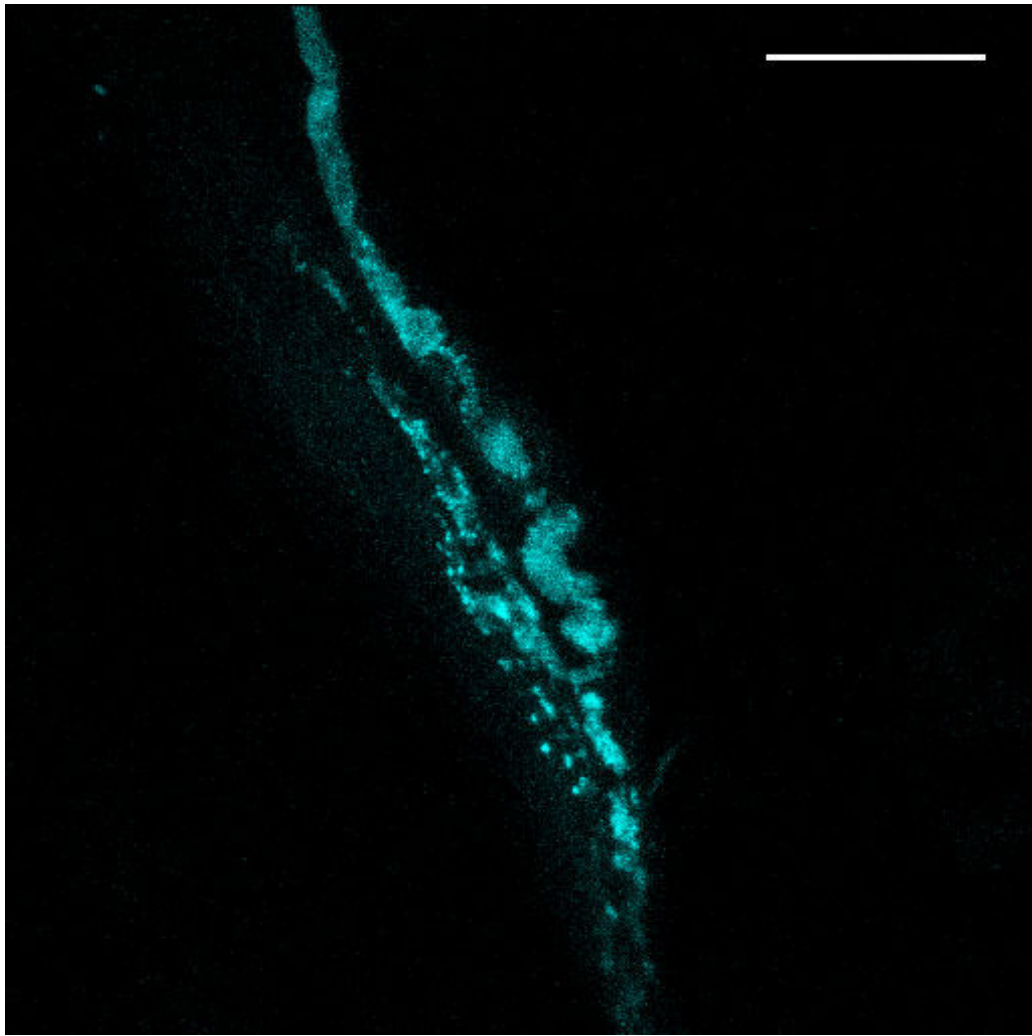


Figure 1.13 Preserved muscle spindles in the muscles of 88 week old *Wid^S* mice.

I was able to examine the four lumbrical muscles of one foot from an 88 week old mouse, 5 days post axotomy and found four innervated muscle spindles and no motor innervation. One of the spindles is shown here. Scale bar= 50 μ m.

1.6 Strong protection of sensory endings is independent of age or *Wld^S* gene copy number

It is clear from the measurements on lumbrical muscles of old and heterozygote mice, that *Wld^S* protects sensory endings better than motor endings. However, it is not obvious whether the protection of the Ia endings is dependent on the age of the animal or the number of copies of the gene.

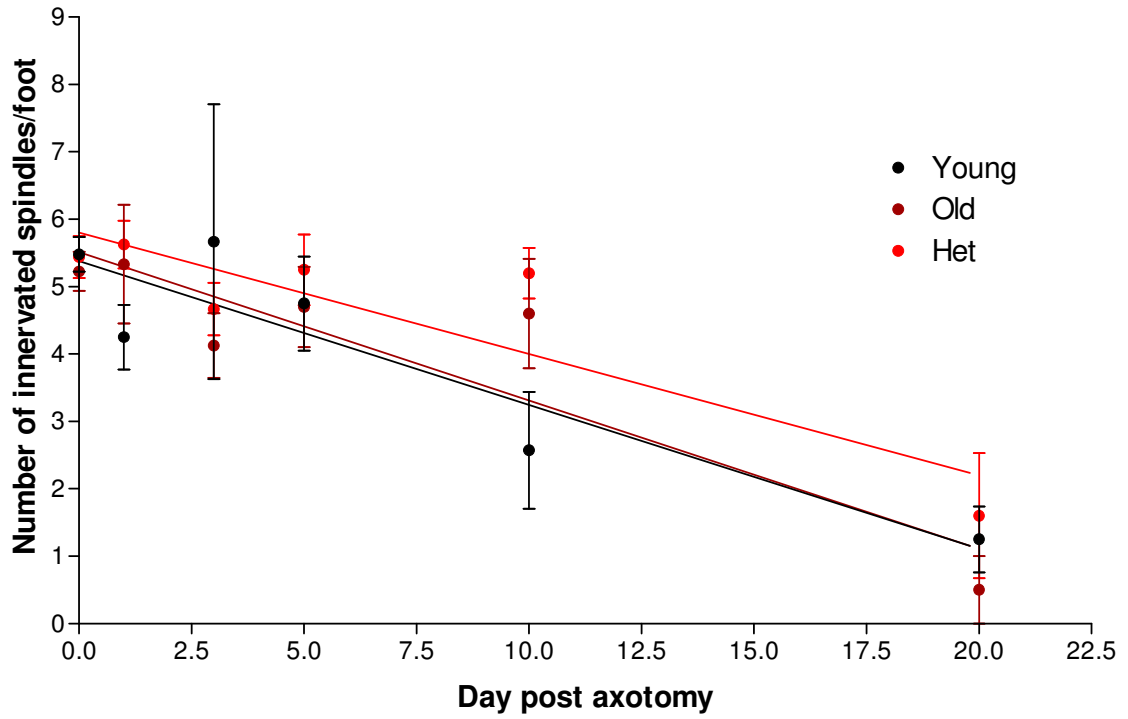
Figure 1.8 shows preserved spindles from old and heterozygote animals 2, 5, 10 and 20 days post axotomy. These spindles look similar to the Ia endings shown in young homozygotes [Figure 1.5].

Figure 1.14 shows a comparison between the numbers of innervated muscle spindles at each time point post axotomy. An analysis of co-variance suggests that there is no significant difference between the rate of degeneration of muscle spindles in older or heterozygote mice and young homozygotes, $p= 0.8175$.

Although there is no significant difference, the group that appeared superficially to lose protection of Ia endings first is the young homozygotes. Figure 1.14 shows that at 10 days post axotomy the number of innervated spindles in young homozygotes appears lower than the innervated spindles from heterozygote and old mice. However, this impression is likely to be an artefact due to the relative ease of counting spindles in lumbrical muscles where all the motor innervation has disappeared (such as in old and heterozygote *Wld^S* mice) in comparison to a lumbrical muscle with degenerating motor endings and axons that mask the degenerating Ia endings (as in young homozygote *Wld^S* mice).

The surviving Ia endings appeared to degenerate at similar rates in old and heterozygote mice. There is no effect of group on the change in length or the change in number of spirals (Two-way ANOVA) [Figure 1.15]. Again, the observation can

be made that the length of the Ia ending does not change in the first 5 days post axotomy, while the number of spirals decreases.



| n numbers | 8-12 week old <i>Wld^S/Wld^S</i> | 32-58 week old <i>Wld^S/Wld^S</i> | 8 week old <i>Wld^S/WT</i> |
|-------------------------|---|--|---|
| 1 day post axotomy | 4 | 3 | 8 |
| 3 days post axotomy | 3 | 8 | 9 |
| 5 days post axotomy | 8 | 10 | 12 |
| 10 days post axotomy | 7 | 6 | 6 |
| 20 days post axotomy | 8 | 2 | 5 |

Figure 1.14 There is no significant difference between the protection of Ia annulospiral endings in old, young or heterozygote *Wld^S* mice

The graph shows the number of innervated spindles per foot at the given number of days post axotomy for each of the three groups of mice, young homozygotes (aged 8-12 weeks), old homozygotes (aged 32-58 weeks) and 8 week old heterozygote *Wld^S* mice. The table shows the n numbers for each group at each time point.

Linear regression was used to plot the rates of decline for each group. The rates of decline in the numbers of innervated sensory endings were not significantly different between heterozygote, old and young *Wld^S* mice $p = 0.8175$ (ANCOVA).

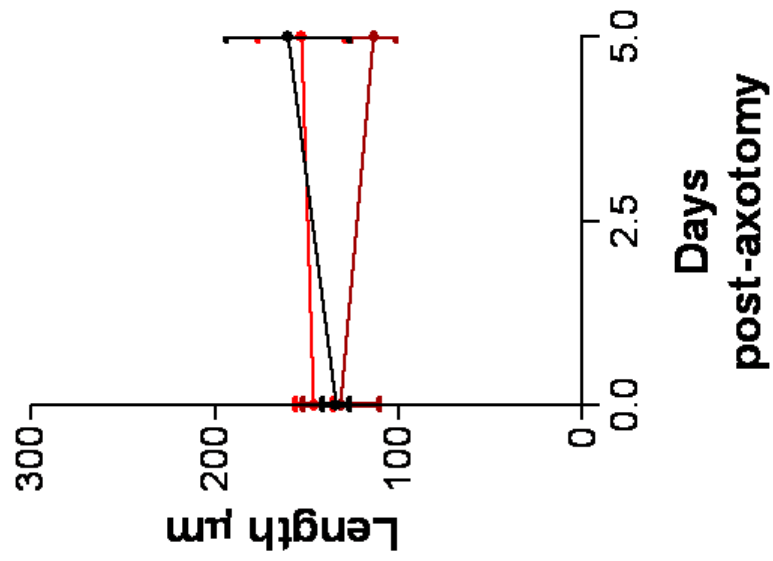
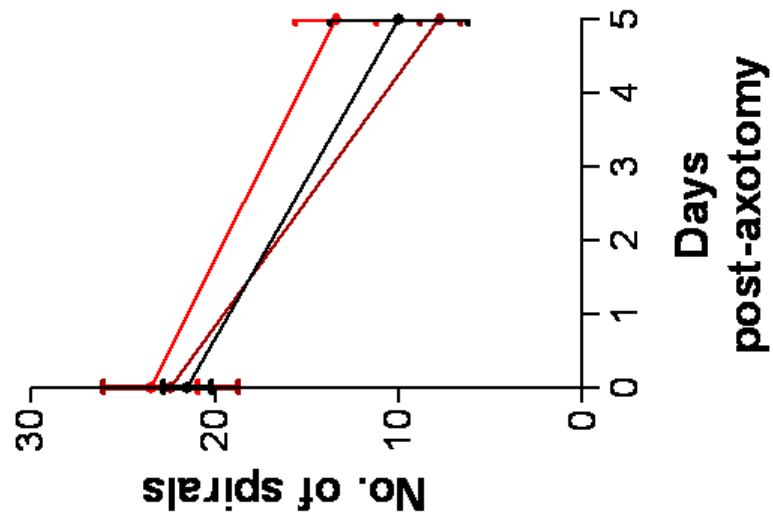


Figure 1.15 No significant difference between groups in the degeneration of annulospiral endings

Graphs show the number of spirals and length of the primary ending in unoperated mice and mice 5 days post axotomy. There was no significant difference in the change in length ($p= 0.2177$) or no. of spirals ($p=0.5998$) between young, old and heterozygote Wld^S mice (Two-way ANOVA). As seen in young homozygotes, the degenerating spindles in all groups of Wld^S mice lost spirals before they lost length.

For unoperated feet $n= 29, 7, 8$ and at 5 days post axotomy $n= 7, 13, 10$; for 8-12 week Wld^S mice, 30+ week old Wld^S mice and 8-12 week old heterozygote Wld^S mice respectively.

1.7 *Wld^S* protects axons more robustly than endings

The previous data suggest that sensory endings are preserved irrespective of age and gene dose, which means they have more robust protection in old and heterozygous animals than neuromuscular junctions. However, they still appear to degenerate ahead of their parent axons. Figure 1.16 shows muscles containing Ia annulospiral endings that have nearly completely degenerated. However, the axons appear intact.

In order to examine which compartment degenerates first, I examined 105 spindles and classified them as to whether they were fragmented or intact, and connected or disconnected (ie: whether the axons were unbroken). Figure 1.17 demonstrates that *Wld^S* Ia afferents lose their endings, before their axons begin to degenerate.

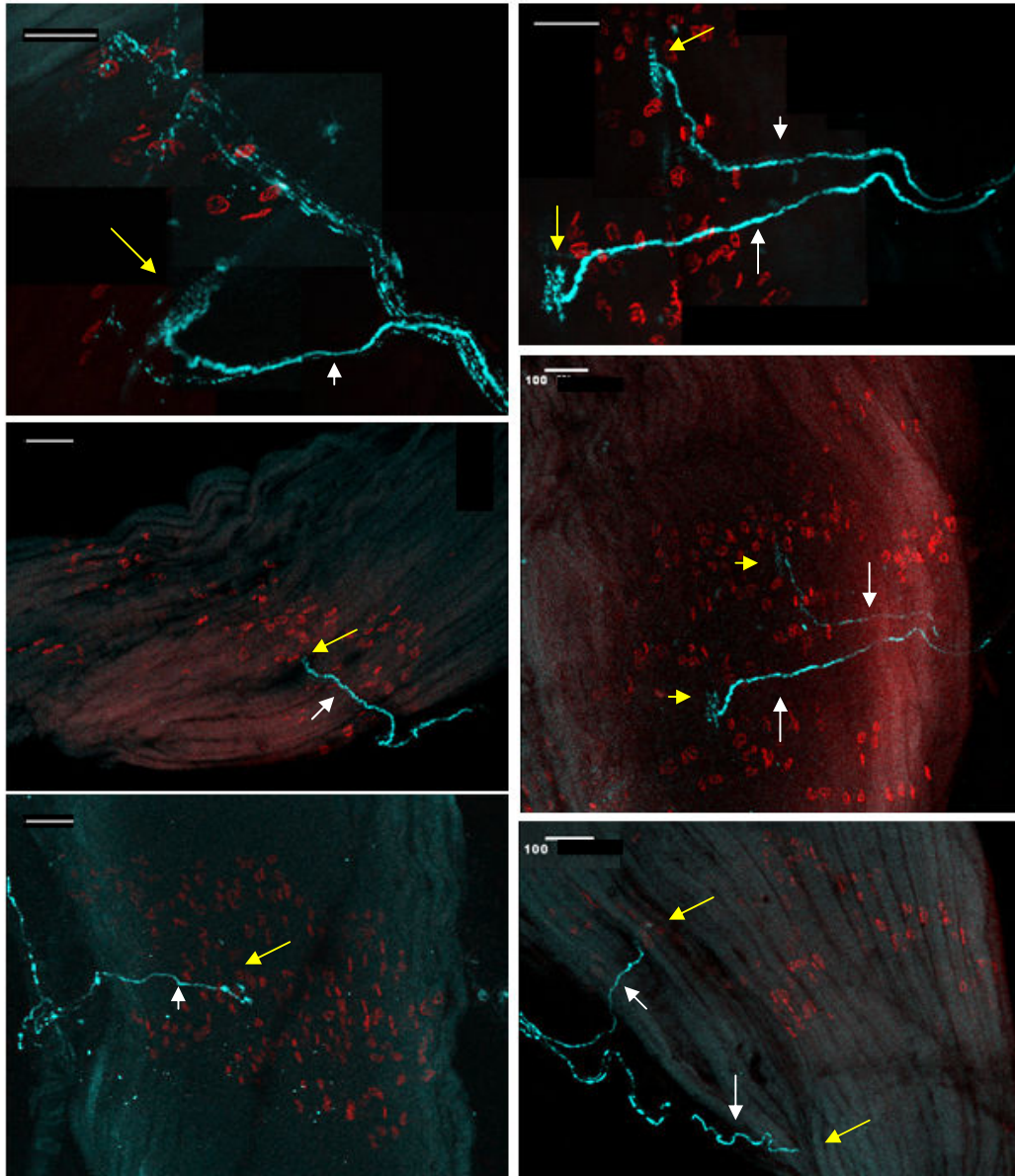


Figure 1.16 Sensory axons are better protected than sensory endings in *Wld^S* mice.

Confocal microscope pictures of lumbrical muscle preparations from *Wld^S* mice 5-20 days after axotomy. These images show Ia annulospiral endings at various stages of degeneration (yellow arrows), but with strongly protected unbroken parent axons innervating them (white arrows). Neuromuscular endplates are also visible due to incubation with TRITC-conjugated α -BTX. All scale bars=100 μ m.

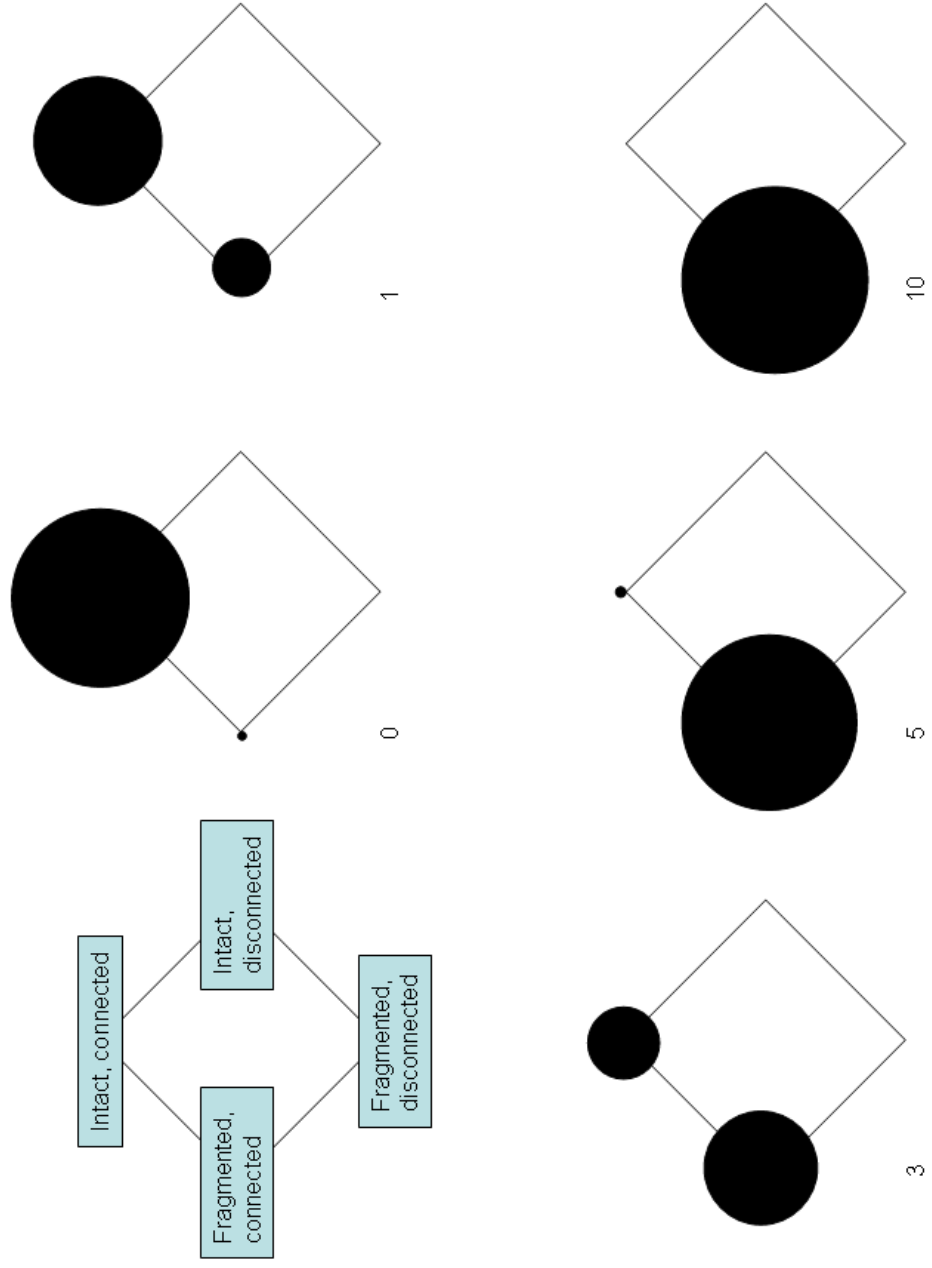


Figure 1.17 *Wld^S* sensory endings degenerate before their attached axons

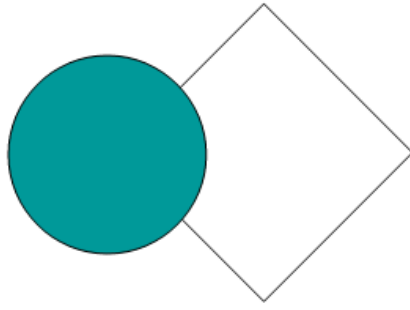
Figure to show percentage of spindles in different categories at given number of days after axotomy. Categories are designed to discriminate between annulospiral endings that are degenerating before or after their parent axon. Diameter of the circle at each vertex of the kite represents the percentage of spindles that fell within that category. 105 spindles were examined from more than 30 mice. No spindle at any time point, remained intact while its parent axon degenerated (ie: no annulospiral ending examined was intact but disconnected). In addition no spindle at any time point degenerated alongside its parent axon (ie: no annulospiral ending was degenerated and disconnected). Instead all spindles examined were either intact and connected ie: neither the annulospiral ending nor the axon showed signs of degeneration, or fragmented and connected ie: the annulospiral ending was degenerating but the axon remained unbroken. This suggest Ia annulospiral endings spindles degenerate before their parent axon in *Wld^S* mice. The progress of the degeneration can also be seen in this figure, with the proportion of intact connected Ia afferents decreasing over time post-axotomy.

N= 12, 4, 6, 11, 11, 4 for 0, 1, 3, 5, 10 and 20 days post axotomy respectively.

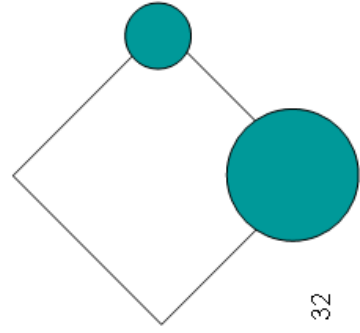
1.8 Pattern of degeneration

In order to examine whether wild type Ia afferents degenerated with a similar pattern to *Wld^S* Ia afferents, I classified them in a similar manner to the *Wld^S* Ia afferents in 1.6. In wild type mice there were more instances where an intact or near intact Ia ending was connected to an axon that contained breaks [Figure 1.18]. Examples of these are pictured in Figure 1.19. This suggests that Ia annulospiral endings in wild type mice degenerate alongside their axon after axotomy in wild type animals. This is qualitatively different to the pattern of degeneration in *Wld^S* mice where the endings degenerate first.

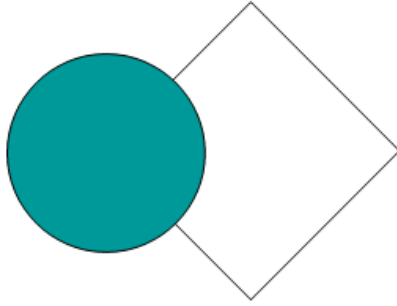
Between 1 and 2 days post axotomy the number of innervated spindles in wild type muscle decreased [Figure 1.6], however the number of spirals and length of the remaining endings remained high [Figure 1.20]. This quantifies an observation I had noted; most wild type spindles that were innervated had well preserved morphology, unlike the *Wld^S* Ia endings which lost spirals as they degenerated over time. Wild type endings appear to remain intact after axotomy and must abruptly degenerate within a few hours. Figure 1.21 is a diagram to illustrate the differences in the pattern of degeneration between wild type and *Wld^S* Ia endings.



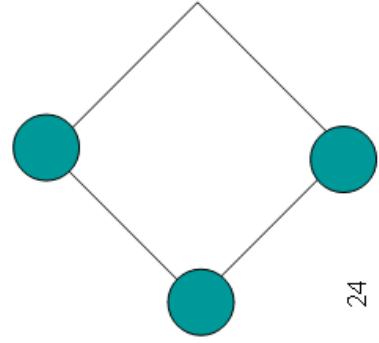
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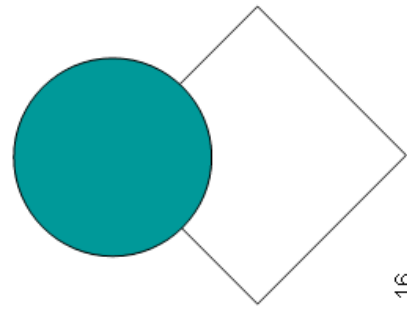
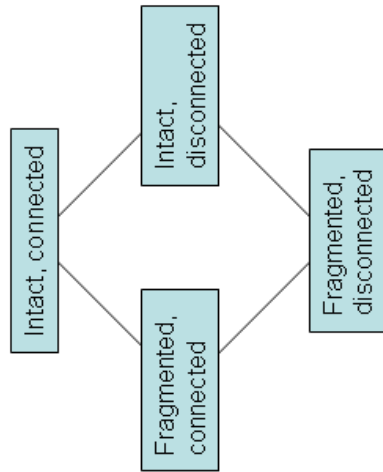
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16

Figure 1.18 WT sensory endings degenerate alongside their attached axons

Figure to show percentage of spindles in different categories at given number of days after axotomy. Diameter of the circle at each vertex of the kite represents the percentage of spindles that fell within that category. Unlike degenerating *Wld^S* spindles, spindles in lumbrical muscles from wild type animals, appear to degenerate alongside their parent axons giving rise to a number of spindles falling into both the intact, disconnected category and the fragmented, disconnected category.

N= 3, 4, 6, 6, 3 for 0, 0.3, 0.6, 1 and 1.3 days post axotomy respectively.

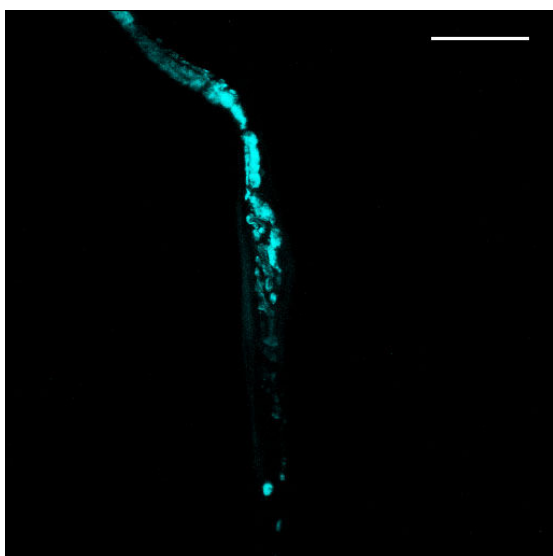
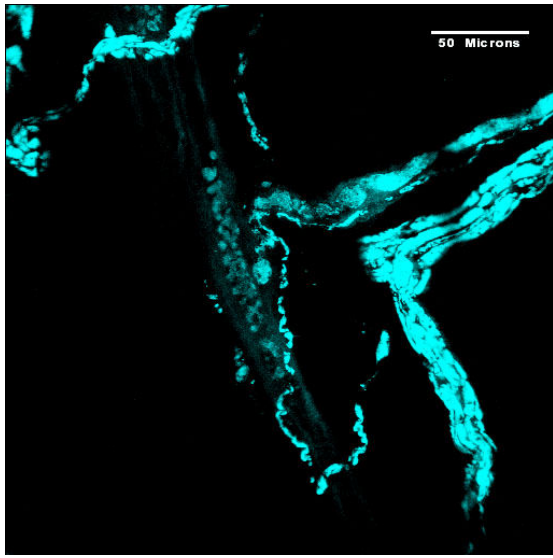
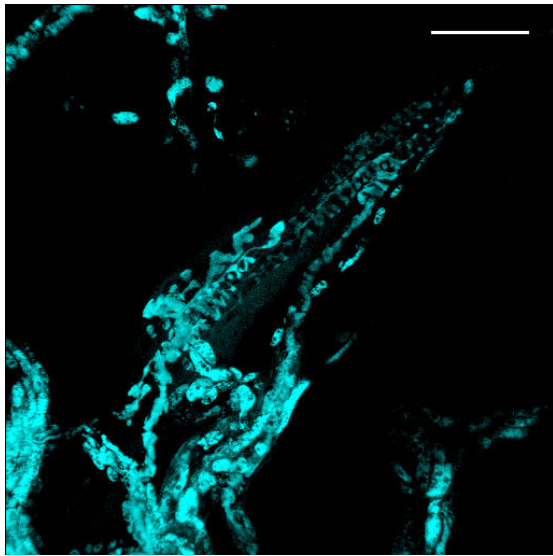


Figure 1.19 WT sensory endings degenerate alongside their attached axons

Confocal microscopy shows spindles in wild type animals during their degeneration 1-2 days after axotomy. Top image shows a spindle with intact morphology whose parent axon is broken. Remaining images show degenerating spindles attached to broken axons. Scale bars= 50 μ m.

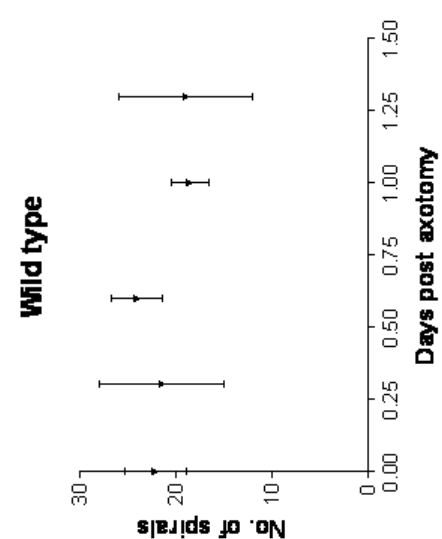
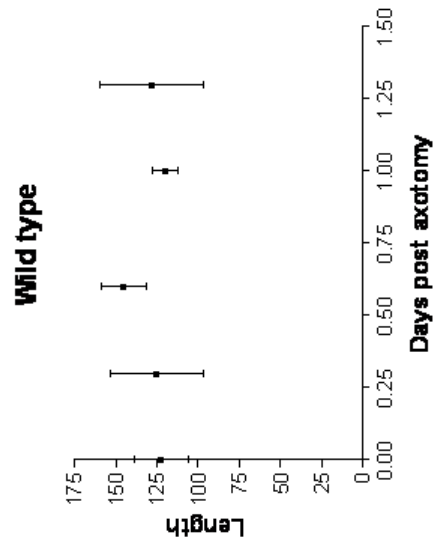
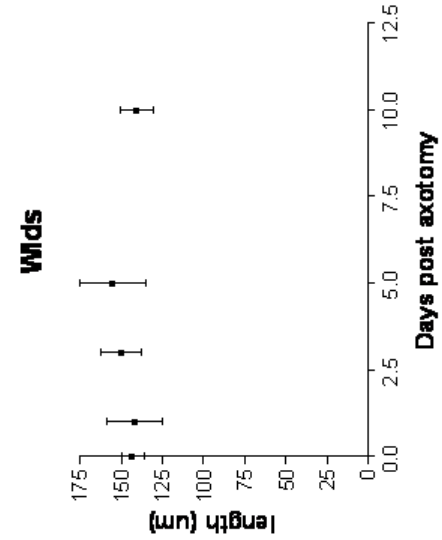
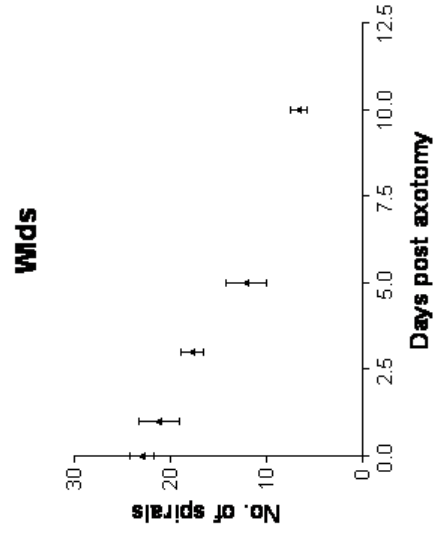


Figure 1.20 Different pattern of degeneration in WT and *Wld^S* spindles after axotomy

Graphs that show the number of spirals, or length of the primary endings, at given amount of time post axotomy. While *Wld^S* spindles lose spirals before they lose length after axotomy, which indicates that they do not retain normal morphology over time after disconnection, innervated wild type spindles retain their morphology with both length and number of spirals remaining high for innervated spindles in axotomised muscles. This suggests they must degenerate rapidly once degeneration is initiated.

N= 6, 4, 10, 10, 3 for spindles from WT mice, 0, 0.3, 0.6, 1, 1.3 days post axotomy; and n= 31, 13, 16, 16, 20 for *Wld^S* mice 0, 1, 3, 5, 10 days post axotomy

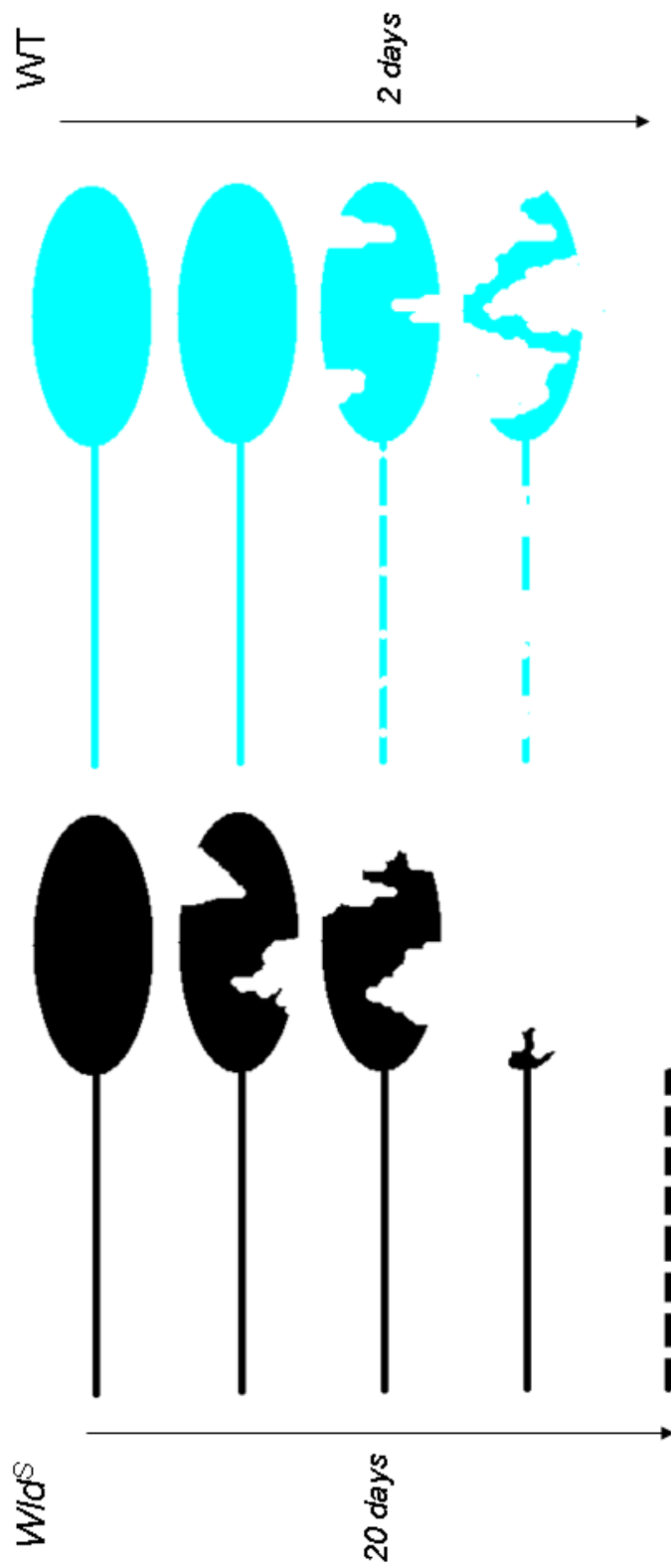


Figure 1.21 Diagram to show pattern of degeneration of *Wld^S* and WT Ia sensory endings

Three to 5 days after axotomy, *Wld^S* endings begin to show morphological degeneration while maintaining their length. Between 5 and 10 days after axotomy the ending loses length and morphology and between 10 to 20 days after axotomy, the ending has completely degenerated. Breaks only begin to appear in the parent axon once degeneration of the ending is complete.

WT endings maintain their morphology for up to 32 hours, but rapidly degenerate alongside the parent axon.

1.9 Discussion

In this chapter I have described the innervation of the lumbrical muscle spindle in the mouse and the degeneration of Ia afferent endings after axotomy in wild type and *Wld^S* mice.

I characterised the innervation of the mouse muscle spindle and found that there were no changes with age over the age-range studied.

I present evidence that suggests that slow Wallerian degeneration of sensory terminals, occurs with a qualitatively different pattern from Wallerian degeneration. I have found that wild type Ia endings degenerate rapidly after a latent phase in which they maintain their normal morphology. In *Wld^S* animals, however, the degeneration is gradual, over a longer time period and involves loss of spirals from the primary ending occurring before loss of length.

I demonstrated that Ia afferent endings are protected by *Wld^S* to a much greater extent than neuromuscular junctions are, both in terms of length of survival after axotomy and also in terms of robust survival in older animals and in heterozygotes.

I will review these findings and their implications and then discuss the contribution of this work to the compartmental hypothesis of neurodegeneration.

1.9.1 Morphology of the mouse lumbrical muscle spindle

There is very little literature on the anatomy of the mouse muscle spindle. Having studied numerous mouse muscle spindles as a control for my degeneration experiments I was able to use descriptive statistics to outline the components of the mouse muscle spindle in the lumbrical muscle.

Edwards (1975) previously reported that the number of intrafusal muscle fibres in the mouse biceps muscle was 2, 3 and 4. When four were present two were nuclear bag

and two nuclear chain. I have found the range to be between 2 and 5, but this minor discrepancy may simply be due to either the different muscles studied or the difference in sample size as there was only one muscle spindle with 5 intrafusal muscle fibres out of the 15 that I examined and Edwards only studied 7 spindles in total.

I found the average length of the primary ending to be 142.5 +/- 43.11 compared Kim's finding in rats of 167.1 +/- 32.8 (Kim, 2007). This probably reflects their smaller body size.

I also tested for any structural changes in the mouse muscle spindle that occurred during aging, but found no difference in any of the parameters measured between spindles from mice aged 8 weeks and those between 40 and 60 weeks of age.

This was done as a control for my degeneration experiments, however, a change in spindle morphology has also been suggested to be a factor in the decline of postural stability with age (Kim, 2007), so to some extent, the result is interesting in its own right. Addition of a third, more elderly group of mice could perhaps elucidate any changes that may occur later in life.

1.9.2 The pattern of Ia terminal degeneration

My findings that Ia sensory terminals degenerate with a qualitatively different pattern in *Wld^S* mice than in wild type mice supports the conclusion made by Beirowski et al (2005) that *Wld^S* mutant mice undergo a fundamentally distinct process rather than simply following the same molecular pathway in slow motion.

Beirowski et al (2005) studied degeneration of sciatic and tibial nerves from YFP-H mice. They found that delayed degeneration in individual *Wld^S* axons is directional with an exclusively anterograde pattern after either axon transection or crush injury. This pattern of degeneration was qualitatively different from what was observed in the nerve of wild-type mice. Wild type nerves degenerated with asynchronous, bidirectional fragmentation.

Indeed, even before 2005, differences in slow Wallerian degeneration and Wallerian degeneration in wild type mice had been noted. Gillingwater et al (2003) report that different ultrastructural events occur during degeneration of neuromuscular junctions in *Wld^S* mice and in wild type mice.

In contrast the ultrastructural events in the degeneration of synapses in the brains of *Wld^S* mice after cortical ablation were delayed but indistinguishable from the degeneration in wild type mice (Gillingwater et al, 2006b).

My results support the hypothesis that, rather than Wallerian degeneration proceeding at a slower pace, or a delayed start to Wallerian degeneration, Wallerian degeneration is abolished altogether and an alternative degeneration occurs in its absence. Electron microscopy of degenerating sensory endings in wild type and *Wld^S* mice could throw further light on this possibility.

Beirowski et al (2005) suggest that this alternative degeneration may be passive degeneration due to disconnection from the cell body as Wallerian degeneration was once thought to be. Gillingwater et al suggest that there may be similarities between the degeneration of motor nerve terminals in *Wld^S* mice and the degeneration that occurs during development or in some diseases suggesting *Wld^S* mouse could be used as a model for these situations.

1.9.3 Stronger protection of Ia afferent endings than motor endings in the *Wld^S* mouse

The result that sensory endings are protected by *Wld^S* to a greater extent than motor endings has implications for the use of *Wld^S* in neurodegenerative therapies.

Wld^S mice have been crossed with various mouse models of motor neurone disease. In these crosses, *Wld^S* has had variable success in improving the disease signs and lifespan of the animals. One factor that may affect how successful *Wld^S* is, in the treatment of these diseases, is that the protection of neuromuscular junctions is

reduced with age. Indeed the most promising study has come from the study of the progressive motoneuropathy mouse (*pmn* mouse) which develops disease very early in life. Ferri et al, crossed the *pmn* mouse with *Wld^S* mice and found the *Wld^S* gene product attenuates symptoms, extends life span, prevents axon degeneration, rescues motor neurone number and size, and delays retrograde transport deficits (Ferri et al, 2003). However, the SOD1-G93A mouse, an extensively used model of familial ALS that develops disease later in life, demonstrated no benefits from a cross with the *Wld^S* mouse at time points beyond 80 days. There was no significant difference in innervation of neuromuscular junctions, and the mice had no phenotypic improvements as measured by rotorod (Fischer et al, 2005).

The age-related decline in *Wld^S*-mediated protection of neuromuscular junctions may be a huge barrier to clinical application of *Wld^S* research in motor neurone disease and up until now the assumption has been made that any axonal ending would be similarly unprotected from degeneration in aged *Wld^S* mice. This renders *Wld^S* protection far less imminently useful, because age is a common risk factor to most neurodegenerative diseases. However recent studies suggest that other mutations can modify and enhance the strength of synaptic protection in *Wld^S* mice. These include point mutations in the *Wld^S* gene itself (B. Beirowski, M.P. Coleman, R. R. Ribchester et al, submitted) and random ENU-induced mutations at as-yet uncharacterised loci (F. Wong, G. Blanco and R. R. Ribchester, in preparation). This may be cause for optimism that the downstream signalling mechanism responsible for *Wld^S* protection might ultimately be translated into new treatments that could benefit humans with these neurodegenerative diseases. However, as things currently stand and given the result that sensory endings are protected to a far greater extent than motor endings and with no effect of age, perhaps neurodegenerative diseases or neuropathies that primarily effect sensory neurones stand to benefit more from therapies based on a mechanism of *Wld^S* protection.

Sensory axons degenerate in response to chemotherapeutic drugs used in cancer eg: Paclitaxel (Lipton et al, 1989) and cisplatin (Krarup-Hansen et al, 2007). The neurotoxic side effects of these drugs limit doses and their therapeutic use (Lipton et

al, 1989). Sensory axon degeneration is also a complication in patients with diabetes (Muller et al, 2008), HIV (Keswani et al, 2006) or diseases such as chronic cholestasis, abetalipoproteinaemia, celiac disease or cystic fibrosis which lead to vitamin E deficiency (Aslam et al, 2004). There are also rare inherited or sporadic genetic sensory neuropathies.

Wang et al (2002b) demonstrated that young homozygote *Wld^S* animals were less susceptible to the side effect of sensory axon degeneration in response to paclitaxel treatment using behavioral, physiological, and pathological measures. It would be a very interesting experiment to repeat this in heterozygotes and in older animals. Success could mean that those researching *Wld^S* for translational and clinical gains might switch attention from ALS to sensory disorders.

Another important clinical implication is for other neurodegenerative diseases. Axonal endings in the brain have not been tested in mice over 8 weeks due to the presupposition that, like the neuromuscular junction, axonal endings in the brains of older *Wld^S* mice would degenerate rapidly. Perhaps the CNS experiments are now worth repeating in aged animals in order to assess whether synapses in the brain retain protection when neuromuscular junctions have lost it. This would demonstrate whether therapy based on *Wld^S* is worth pursuing for the common neurodegenerative diseases such as PD and AD.

There are also scientific implications of the finding that different neuronal cells may have different degrees of axon and axon terminal protection due to *Wld^S* expression. Investigators work on the mechanism for *Wld^S* action in many different classes of neurones in vivo and in culture. Results may not be repeatable or comparable if one group are testing molecules on cultured DRG neurones, another group are working with cultured SCG neurones and a third group test in vivo by quantifying neuromuscular innervation after axotomy.

1.9.4 The compartmental theory of neurodegeneration

The compartmental theory of neurodegeneration suggests that the separate compartments of the neurone which are morphologically and functionally distinct also have separate mechanisms of degeneration.

If the same molecular mechanism was involved in the destruction of the axon and axon terminal, then it would be expected that any genetic intervention would have an equivalent effect on the destruction of both these compartments. However, in the case of *Wld^S*, aged or heterozygote *Wld^S* mice have robustly protected axons but the neuromuscular terminals proceed to degenerate on the same time scale as in wild type animals.

The finding that there is strong protection of sensory endings in homozygote *Wld^S* mice independent of age, and that there is also equivalent strong protection in heterozygous *Wld^S* animals, suggest it may not be possible to generalise from the study of neuromuscular junctions to make assumptions about all axonal endings. This observation may therefore suggest that the compartmental theory of neurodegeneration, where the soma, axon and axon terminal are considered as separate compartments can only be specifically applied to motor neurones.

However, despite the robust protection of sensory endings, I believe other evidence presented in this chapter supports more general application of the compartmental theory of neurodegeneration.

Though the protection of sensory endings was more robust than that of motor nerve terminals, like motor nerve terminals, the axonal endings of sensory neurones were the first compartment to degenerate in *Wld^S* animals. Intramuscular sensory axons remained unbroken up to 20 days post axotomy, at a time point when all Ia endings had substantially degenerated. This suggests a retrograde pattern of degeneration occurring in the lumbrical muscle.

Beirowski et al, 2005 reported that all degeneration in the injured nerves of *Wld^S* animals occurs anterogradely and proposed to rename slow Wallerian degeneration

as “slow anterograde axon decay”. The authors suggest two hypotheses to explain the anterograde degeneration that they observe. They note that the degeneration rate occurs at about the same speed as slow axonal transport. They suggest that a net anterograde gradient of cytoskeletal proteins could underlie the anterograde gradient of axonal atrophy as structural proteins are gradually depleted at the proximal end of the axon stump. The second explanation they propose is that a proximal-distal temperature gradient could be the cause of the difference in degeneration rate along the axon, as a decrease in temperature has been shown to delay degeneration in *Wld^S* axons after injury (Tsao et al, 1999a).

If, in *Wld^S* animals, the axonal terminals were degenerating with the same mechanism as the rest of the axon, it would be expected that they would be last to degenerate. Instead I have observed that sensory terminals degenerate first. Given the observation that the axon degenerates anterogradely, there is a sharp contrast between the distal axon which lasts longest and the terminal which degenerates first.

Taken together, the data from my own observations of sensory degeneration in *Wld^S* and the data from observations of axonal and neuromuscular terminal degeneration it appears that there is likely to be a separate axonal terminal compartment which undergoes its own molecularly distinct form of degeneration in *Wld^S* mice and therefore possibly in wild type mice under certain conditions. Whether or not there is a common mechanism which is responsible for both sensory and motor axon terminals is beyond the scope of the data presented here, but a question that would be interesting to tackle.

Another question that arises is to find out where the first degenerative events happen after axon transection. To what extent is the degeneration in *Wld^S* animals bidirectional, given that there appears to be retrograde degeneration in the muscle and anterograde degeneration in the nerve?

1.9.5 What causes the sensory-motor difference?

The difference in protection of sensory and motor axons and endings may affect clinical and scientific work in the future. However, the difference itself is also curious. Why is it that sensory axons and endings are protected to a greater extent than motor axons and endings?

This question will be the subject of my following chapter.

2 Investigating the difference between sensory and motor protection in *Wld^S* mice

2.1 Introduction

In Chapter 1, I found that protection of sensory axonal endings was independent of age and gene copy number in *Wld^S* mice. In contrast, I confirmed that the protection of α -motor endings declines with age and there is no protection in heterozygote *Wld^S* animals. This means *Wld^S* protection of sensory endings is better than that of α -motor endings in both homozygous animals over 16 weeks of age, and in those animals which are heterozygous for *Wld^S*.

The present chapter examines three hypotheses which might explain this aspect of the *Wld^S* phenotype. These are protein expression levels, differences in the branching patterns of the axons and differences in activity experiences by sensory and motor axonal stumps. The results essentially rule out all three alternatives.

2.2 Protein expression

Given the similarity in phenotype for aged *Wld^S* and heterozygous *Wld^S* mice, I asked if decreased protein expression could be a common cause. Protein expression has been studied before using Western Blot. No differences were found in the amount of *Wld^S* protein in whole brain homogenate from mice between 2 and 12 months of age (Gillingwater et al, 2002). However, given the results of my first chapter, it seems possible that the age-effect is not specific to all the neurones in the brain. I investigated cerebellum, spinal cord and dorsal root ganglia to see whether there was a decline in *Wld^S* protein expression in these tissues, in older mice. The spinal cord samples were particularly important as these contain the cell bodies of motor neurones which we know to have an age-dependent phenotype.

While running Western blots to detect *Wld^S* protein changes with age in the spinal cord, DRG and cerebellum, I also ran sciatic nerve, and dorsal and ventral roots and

detected Wld^S protein in these tissues. This result followed personal communication from Dr M. P. Coleman, whose group now has preliminary evidence that Wld^S works directly through a mechanism in the axon. Other recently presented data demonstrated that Wld^S is not required to be localised to the nucleus in order to cause the phenotype (Sasaki et al, 2006). Perhaps a difference in sensory and motor axons causes the difference in the level of Wld^S protection. For these reasons it also seemed appropriate to re-examine immunostaining for Wld^S in nerve.

2.2.1 Wld^S protein expression is unchanged in the nervous system of aged Wld^S mice

I collected cerebella from wild type mice, young and old homozygote Wld^S mice and heterozygote Wld^S mice, extracted the protein and ran these samples for Western Blot. I detected no discernable difference in the amount of Wld^S protein extracted from the cerebellum of old and young Wld^S mice [Figure 2.1A]. In contrast, there is a striking difference in the amount of Wld^S extracted from heterozygote brains.

I collected spinal cord samples from wild type mice and from young and old Wld^S mice. I found no apparent differences between the amount of Wld^S protein extracted from the spinal cords of young and old Wld^S mice [Figure 2.1B].

I ran dorsal root ganglia samples from wild type mice and from young and old Wld^S mice. Again, I was unable to detect a difference in the amount of Wld^S protein extracted from old mice in comparison to that extracted from young mice [Figure 2.1C].

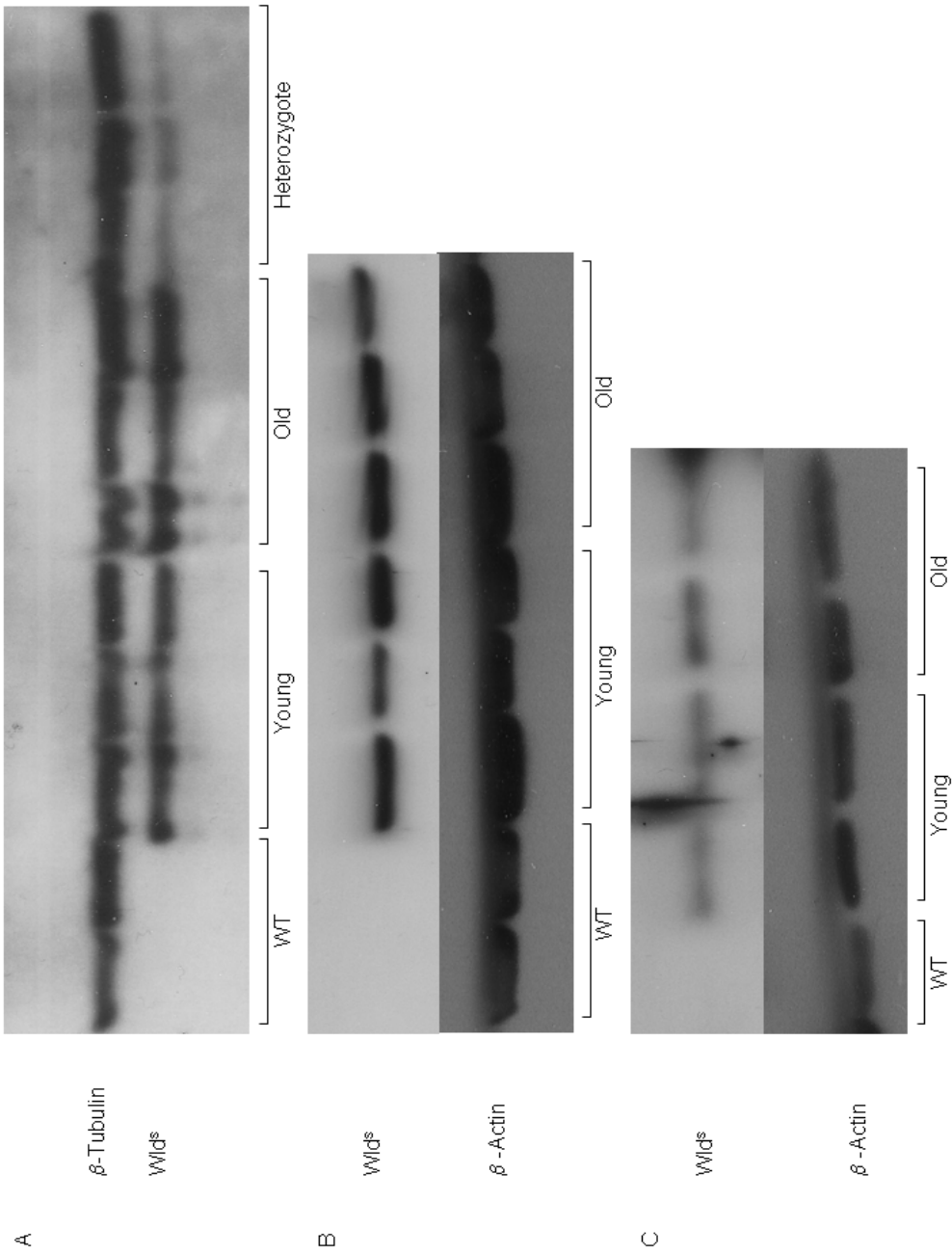


Figure 2.1 No detectable difference in *Wld^S* protein expression in cerebellum, spinal cord or dorsal root ganglia with age

Western blots demonstrating no discernable difference in *Wld^S* protein levels in the nervous system of young and old *Wld^S* mice. A) Cerebella from WT, Young, old and heterozygous *Wld^S* mice. B) Spinal cord from WT, young and old *Wld^S* mice. C) DRG from WT, young and old *Wld^S* mice.

2.2.2 Wld^S protein is readily detected in nerve preparations by Western blot

While Western blotting, I also ran samples from sciatic nerve, dorsal and ventral roots. Although there were some unspecific bands and noise, probably due to the long exposure time necessary to detect the small amounts of protein extracted, I detected Wld^S in all of these [Figure 2.2].

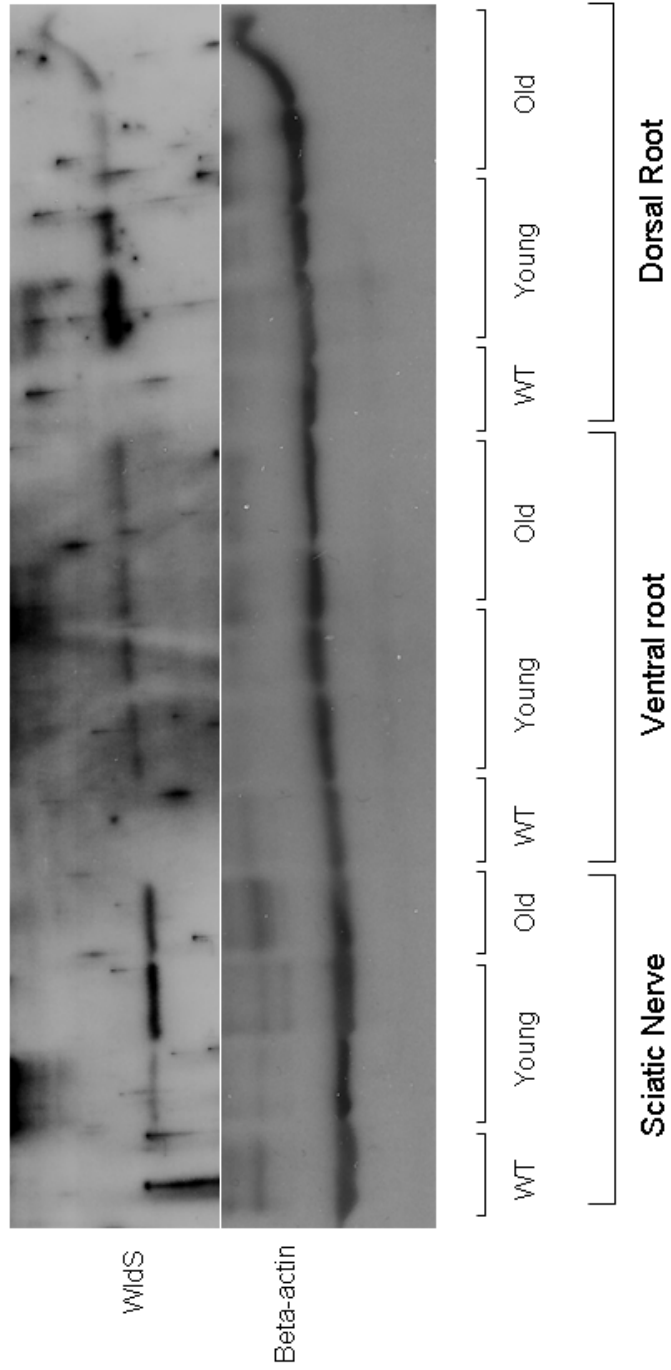


Figure 2.2 Wld^S protein detected in sciatic nerve, ventral and dorsal roots.

Western blot of sciatic nerve, ventral and dorsal root preparations from WT and *Wld^S* mice. These nerve preparations from *Wld^S* mice demonstrate immunoreactivity to Wld^S.

2.2.3 Wld^S protein is not readily identified by immunohistochemistry in axons or their endings

Given that Wld^S is present in the Western blot of nerve and roots, and given more evidence (M.P. Coleman and colleagues, unpublished observations) that Wld^S is present in axons; and given evidence that axonal Wld^S may have an effect (Sasaki et al, 2006), I considered the possibility that there might be a difference in Wld^S protein expression in the axons and endings of sensory in comparison to motor axons and endings. I examined tibial, phrenic and saphenous nerve as well as the motor and sensory endings in lumbrical muscles using DRG from *Wld^S* animals as a positive control. I was unable to detect immunoreactivity to Wld^S in any nerve examined [Figure 2.3].

In order to thoroughly investigate axonal localisation of Wld^S, I repeated the immunoprotocol on nerves using an antigen retrieval method based on Namimatsu et al (2005). The idea behind this is that fixation in paraformaldehyde cross links proteins which then mask the antigen of interest. Again I was unable to detect immunoreactivity to Wld^S in either axon or axonal terminals in muscle. I was also unable to detect the fluorescence of the thy-1.2 YFP in these nerves or muscles after this treatment (data not shown).

While I was unable to detect Wld^S immunoreactivity in nerves or nerve endings in lumbrical muscles, I did see specific Wld^S staining localised to the cytoplasm of the DRG cell somas, which I was using as controls [Figure 2.3].

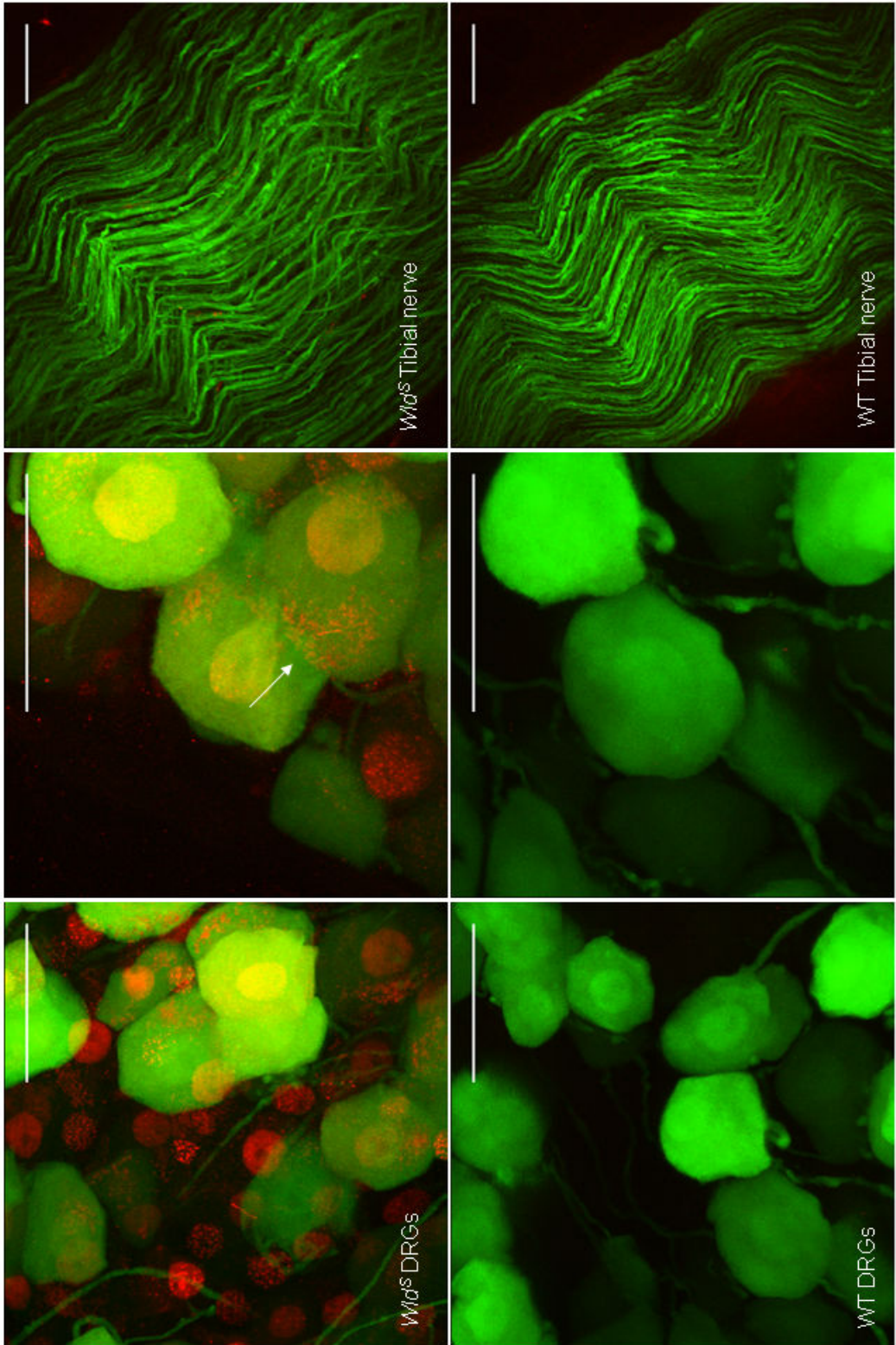


Figure 2.3 No sign of *Wld^S* immunoreactivity in nerve from *Wld^S* mice.

Confocal microscopy showing *Wld^S* immunoreactivity in the nuclei of DRGs from *Wld^S* mice and none in the DRGs from WT mice. There is no *Wld^S* detectable in sciatic, tibial, phrenic or saphenous nerve from *Wld^S* mouse. Tibial nerve is shown; from a *Wld^S* mouse above and WT below. Interestingly there appears to be immunoreactivity to *Wld^S* in the cytoplasm of the *Wld^S* DRG neurones, indicated by a white arrow, as well as in the nuclei. Scale bars= 50µm.

2.3 Axonal branching

Next, I asked whether the difference in protection might be associated with different degrees of axon branching. The axon of an α -motor neurone branches many times to innervate each endplate of a motor unit, in mouse lumbrical muscles the motor unit is between 35 and 80 muscle fibres with a mean of about 54. In contrast, a sensory neurone travels unbranched all the way to its target.

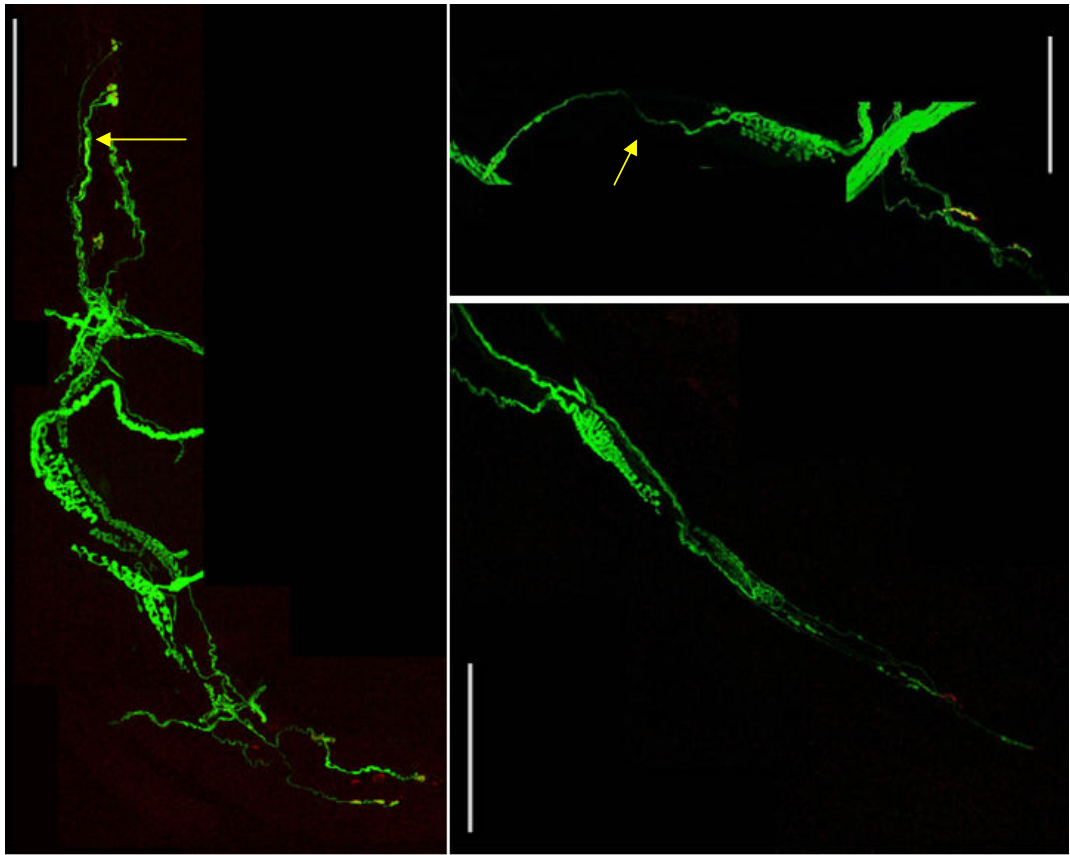
It is plausible that axonal branching is somehow causal to the weakening of *Wld^S* protection, because although motor axons in the nerve are preserved in old and heterozygote mice, the intramuscular axons are not (Li Fan, PhD Thesis, University of Edinburgh). Given that *Wld^S* protection decreases at the point of intramuscular branching, there could be something about this branching that reduces the effectiveness of *Wld^S* protection. For instance, if there was a fixed amount of a neuroprotective molecule in the axon, its concentration would be diluted by distribution down several axon branches, but sustained in single axons with no branches, like Ia afferents.

In order to investigate whether branching has an effect on *Wld^S* protection, I examined γ -motor units. These are motor neurones which innervate the poles of the muscle spindle. They have a small motor unit size of about 2 endplates per neurone, therefore they branch about 25 times less than α -motor neurones but at least twice as many times as sensory motor neurones. If the rate of axon degeneration were influenced by branching I would expect their terminals would degenerate at a rate intermediate to α -motor and Ia degeneration. I would expect the rate of γ -motor ending degeneration to more closely resemble the rate of Ia ending degeneration than that of α -motor endplate degeneration.

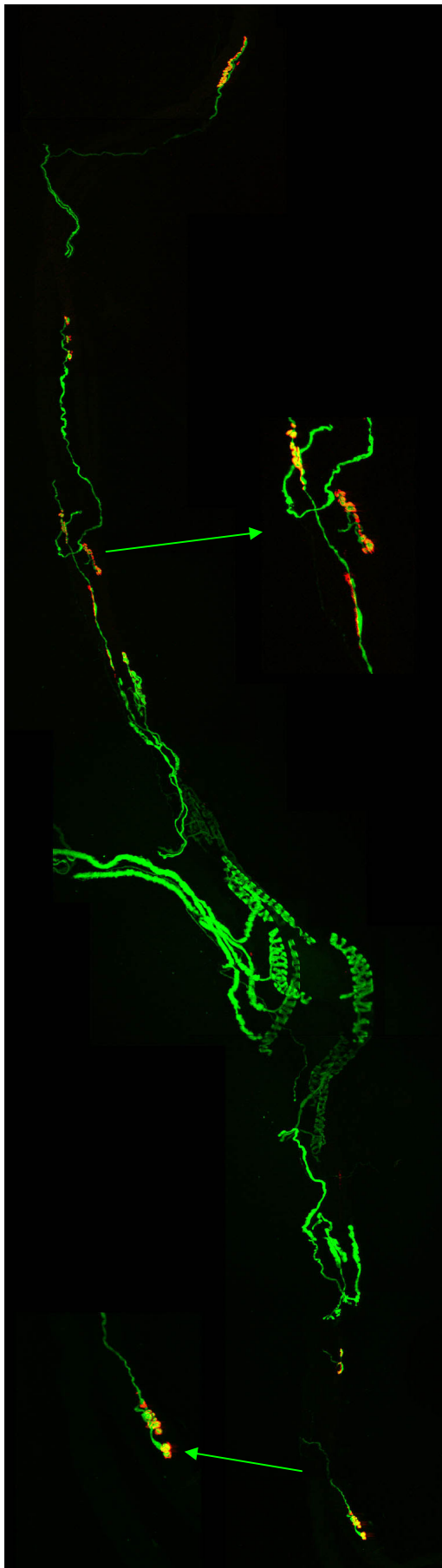
2.3.1 γ -motor units are non-FP in CFP animals, FP in YFP16 animals

Teased spindles from unoperated CFP mice revealed no fluorescent motor units, in contrast to teased spindles from YFP16 mice in which innervation to the γ -endplates

was visible in every spindle teased [Figure 2.4]. This contrasts with the data on this CFP line published by the creators, who state that all motor neurones are labelled in the CFP mouse (Feng et al, 2000). This evidently does not extend to γ -motor neurones.



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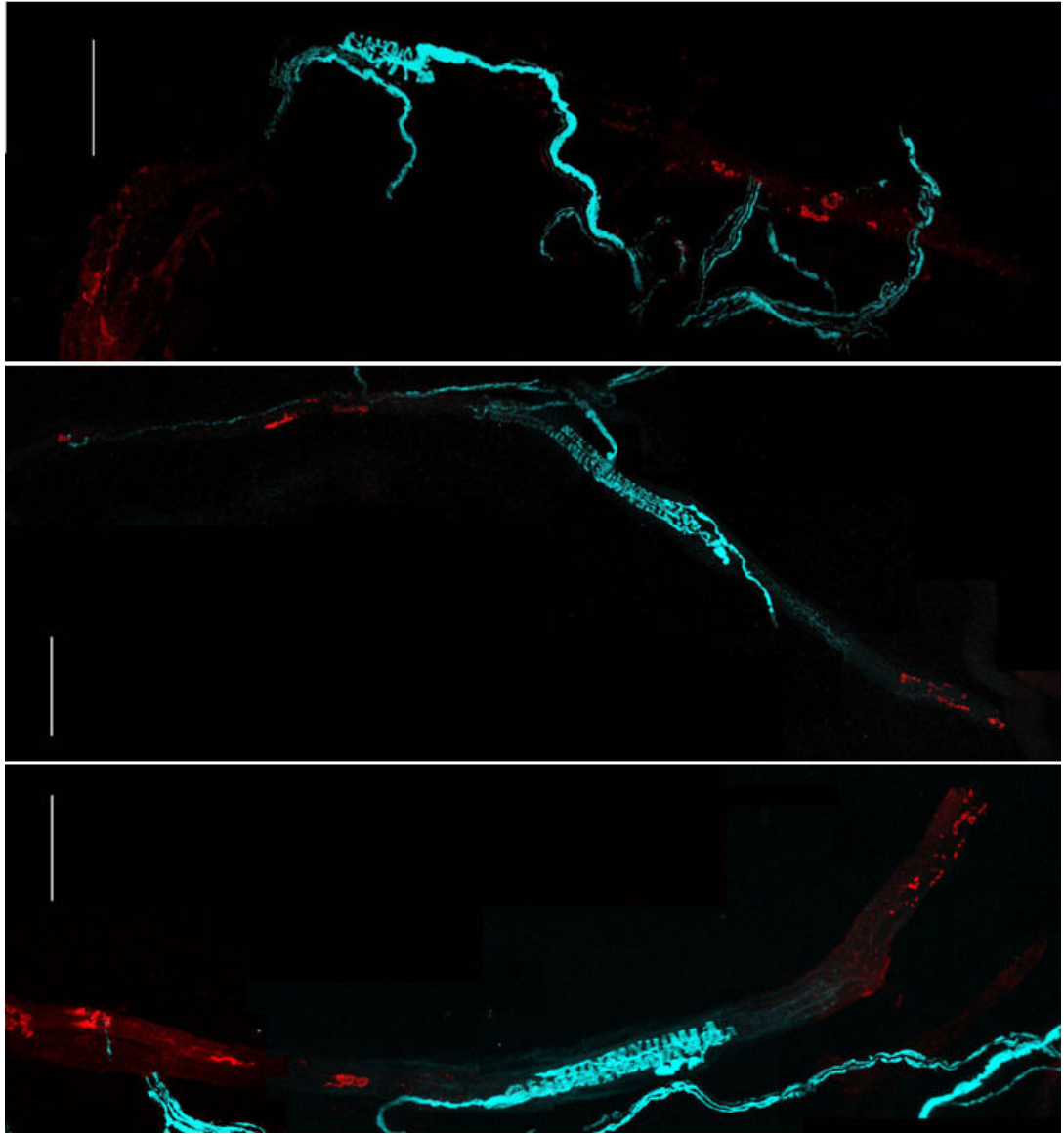


Figure 2.4 γ -motor neurones are fluorescent in YFP16 mice, not fluorescent in CFP mice

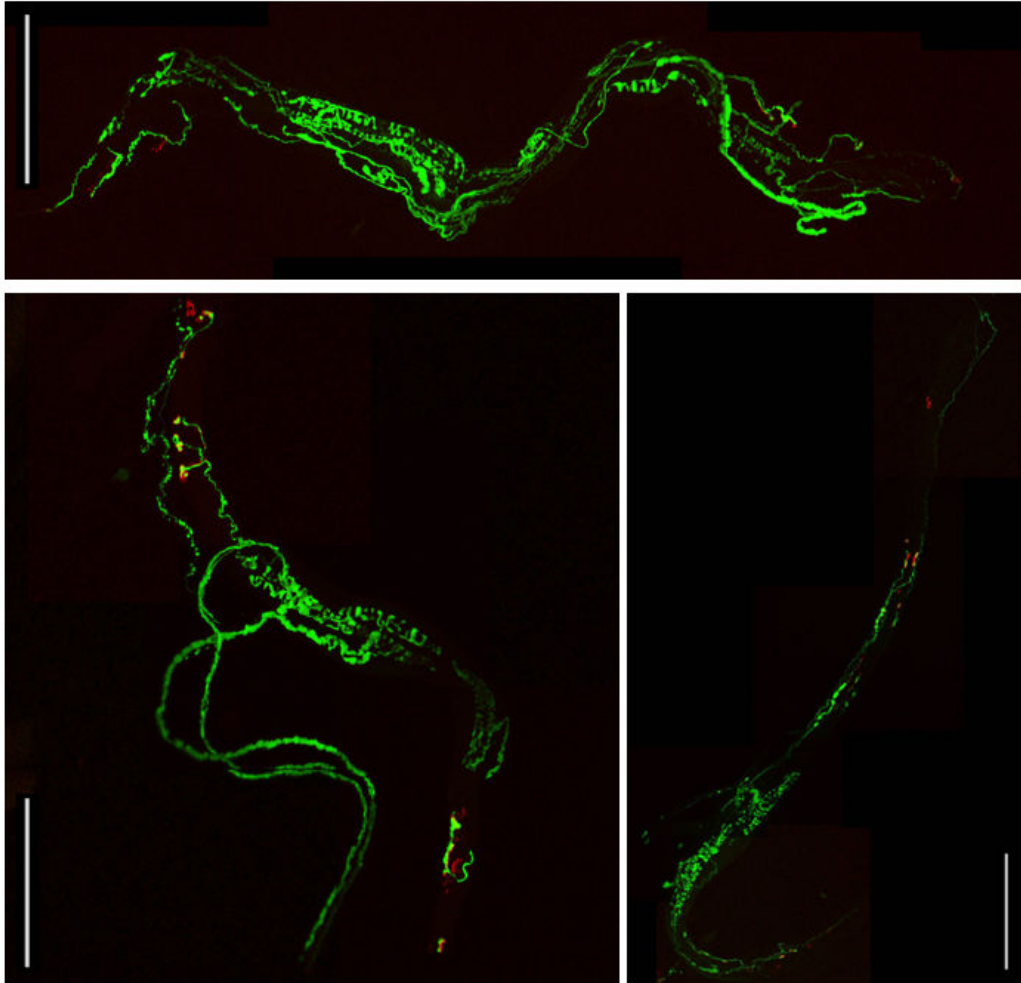
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Confocal microscopy of teased spindles from YFP16 and CFP mice show intramuscular γ -motor gamma motor axons and γ -motor nerve terminals on the intrafusal muscle fibres which are fluorescent in YFP16 (yellow arrows indicate a few of these) but not in CFP mice. The post-synaptic receptors on the γ -motor endplates are shown in red, due to incubation with TRITC conjugated α -BTX. Scale bars= 100 μ m

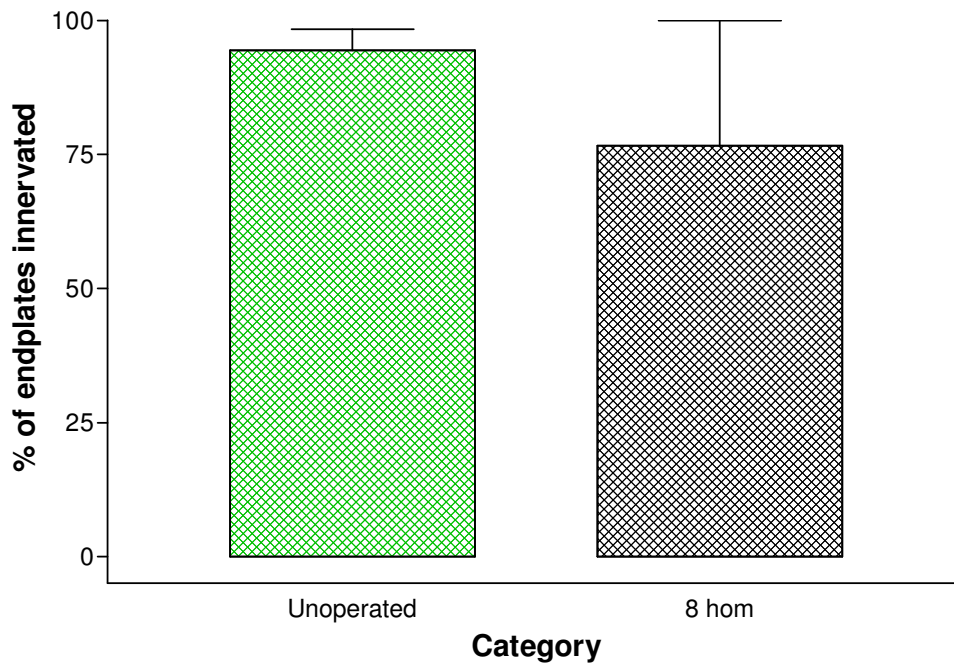
Green arrows indicate the gamma endplates shown in scale with the teased muscle spindle at higher magnification.

2.3.2 *Wld^S* protects γ -motor endings

Seven teased spindles from 8 week old YFP16 *Wld^S* homozygotes, sacrificed 5 days post axotomy show protection of the γ -innervation of the intrafusal muscle fibres as was expected [Figure 2.5]. The percentage of innervated γ -endplates on muscle spindles from *Wld^S* mice 5 days post axotomy is not significantly different to the percentage of innervated γ -endplates in teased spindles from unoperated mice ($p=0.4051$ Mann Whitney U test) [Figure 2.5].



A



B

Figure 2.5 γ -motor endings are preserved in young homozygote Wld^S mice

A) Confocal microscopy of teased muscle spindles from Wld^S mice 5 days post axotomy showing innervated γ -motor endplates. Scale bars= 100 μ m. B) Graph to show the percentage of innervated endplates from teased muscle preparations of unoperated (n=9) and 8 week old homozygote Wld^S mice (n= 7). These are not significantly different (p=0.4051).

2.3.3 Heterozygous *Wld^S* mice show no protection of γ -motor endings

Teased spindles from *Wld^S* heterozygotes showed no γ -innervation at all at either 3 (n=7), or at 5 days post axotomy (n= 6) [Figure 2.6].

This fast rate of degeneration is equivalent to the rate of degeneration of α -motor endings in heterozygous *Wld^S* mice.

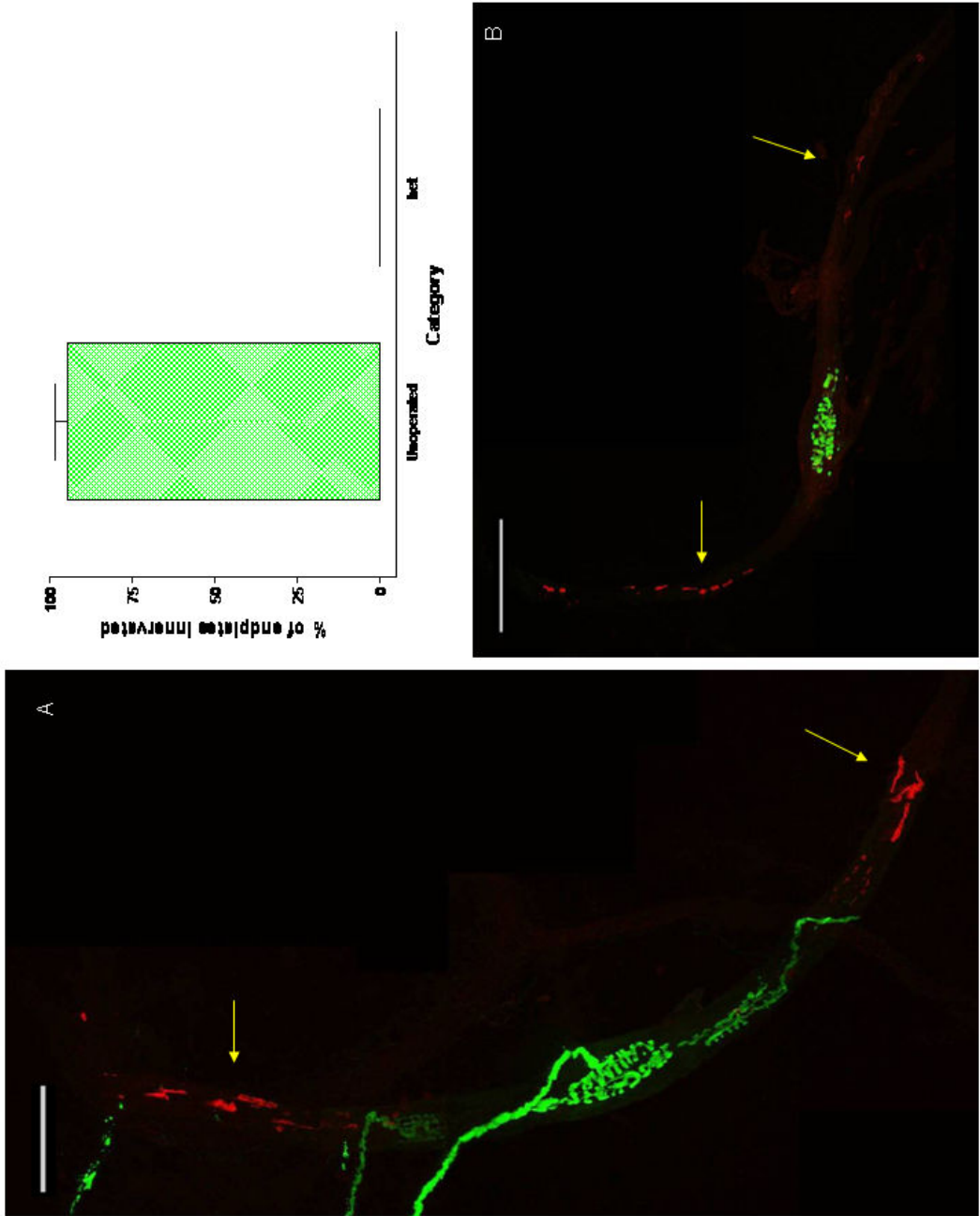


Figure 2.6 γ -motor endings are not protected by *Wld^S* in heterozygote *Wld^S* mice

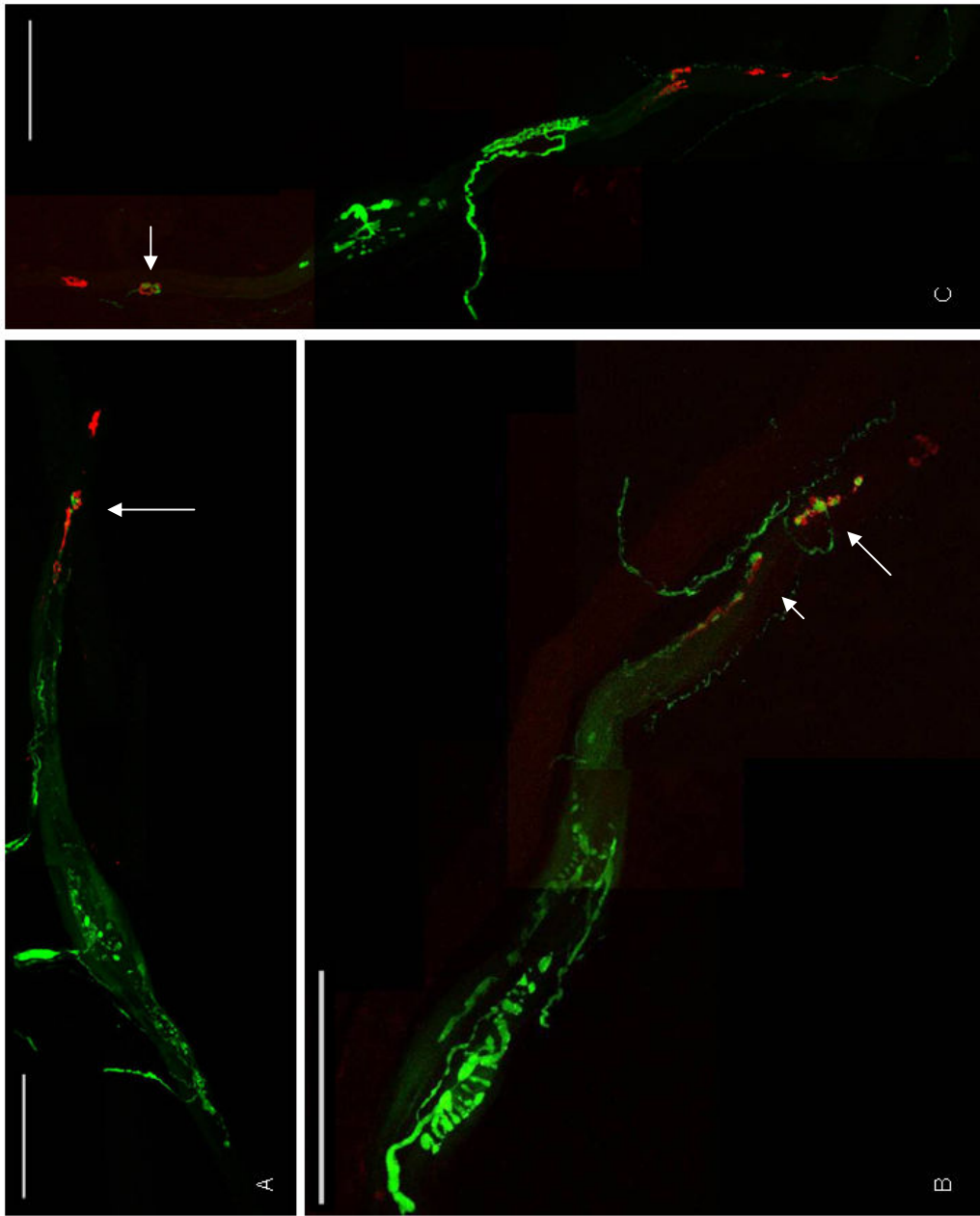
Confocal microscopy of teased muscle spindles A) 3 days post axotomy, B) 5 days post axotomy showing completely denervated γ -motor nerve terminals indicated with yellow arrows. Scale bars= 100 μ m. Graph to show the percentage of innervated γ -motor nerve terminals in teased muscle spindle preparations from unoperated (n=9) and heterozygote mice 5 days post axotomy (n=6).

2.3.4 Age effects *Wld^S* protection of γ -motor endings. However, γ -motor units are better protected than α -motor units in 16-42 week old animals

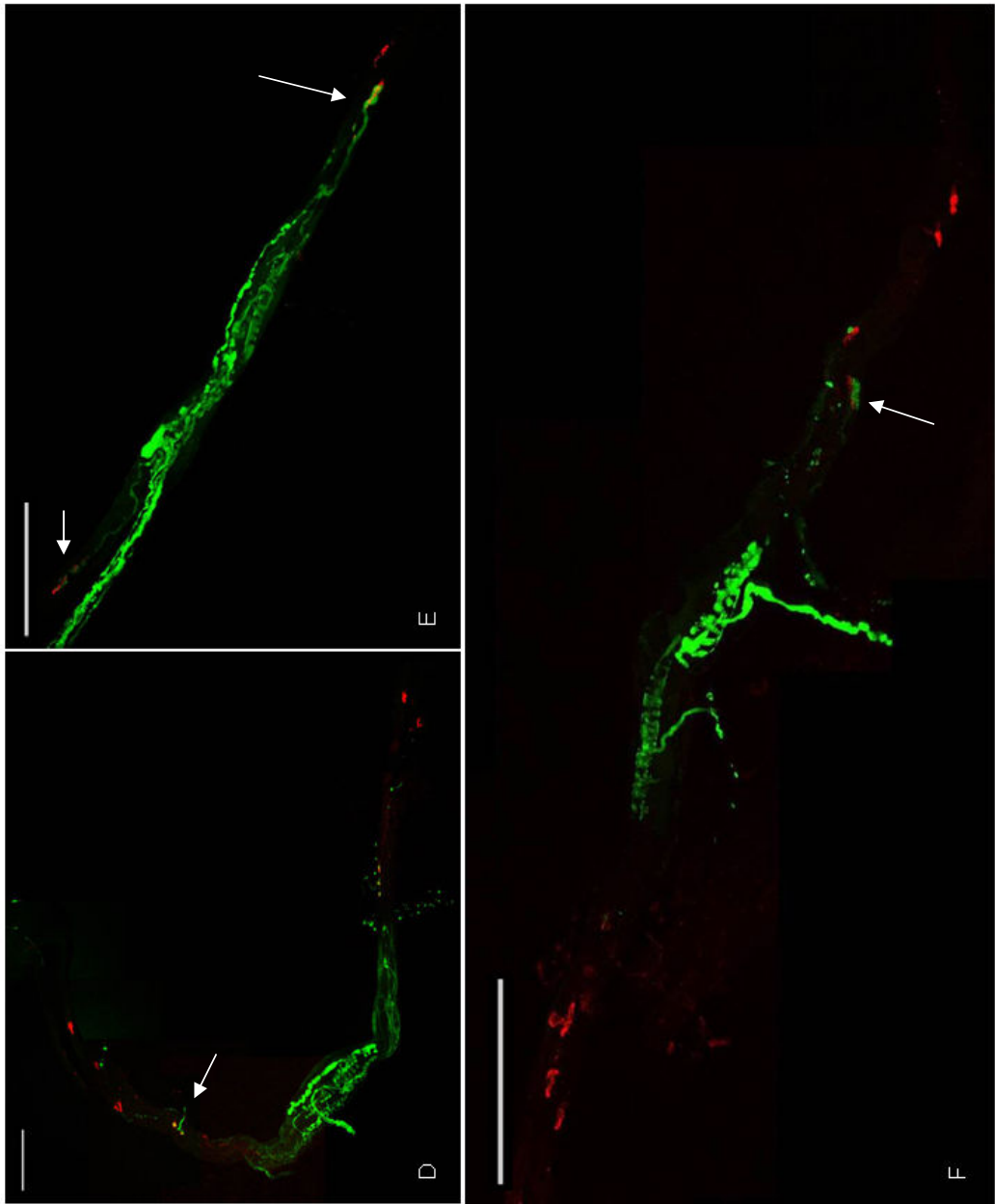
15 teased spindles from mice up to 42 weeks of age, including seven from mice aged 42 weeks, showed γ -innervation 5 days post axotomy. Three out of 6 teased spindles from mice aged 42 weeks showed some γ -innervation at 10 days post axotomy [Figure 2.7]. The percentage innervation of the γ -endplates was not significantly different between teased spindles from unoperated mice and teased spindles from *Wld^S* mice 5 days post axotomy, in the 8 week, 16 week or 32 week old age groups (Dunn's Multiple Comparison test) [Figure 2.7].

However, there is a significant difference between the percentage innervation of γ -endplates in 42 week old *Wld^S* animals, 5 days post axotomy, and from unoperated spindles ($p < 0.05$ Dunn's Multiple Comparison test) and teased spindles from mice aged 58 weeks showed no γ -innervation at 3 (n=7) or 5 (n=7) days post axotomy [Figure 2.7].

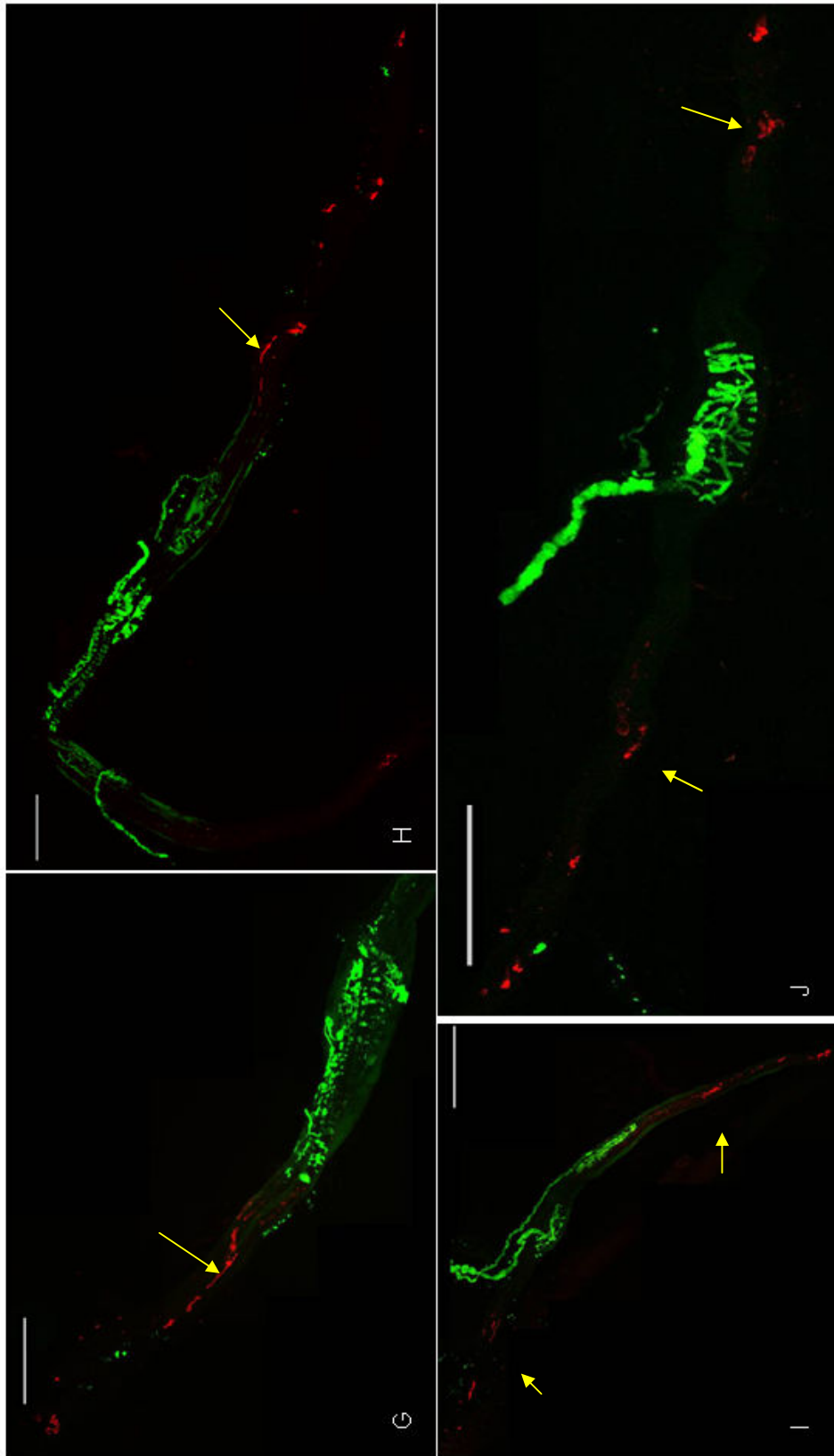
Taken together, these data suggest that γ -innervation is preserved by *Wld^S* at ages when α -motor innervation is no longer robust. However, the *Wld^S* protection observed in these mice was not equivalent to the protection of sensory endings, which were robustly protected regardless of age. Here, there is an effect of age on the quality of *Wld^S* protection and no protection found at all in mice at 58 weeks. This means in mice aged 58 weeks that the protection of γ -motor endings is equivalent to the protection of α -motor endings.



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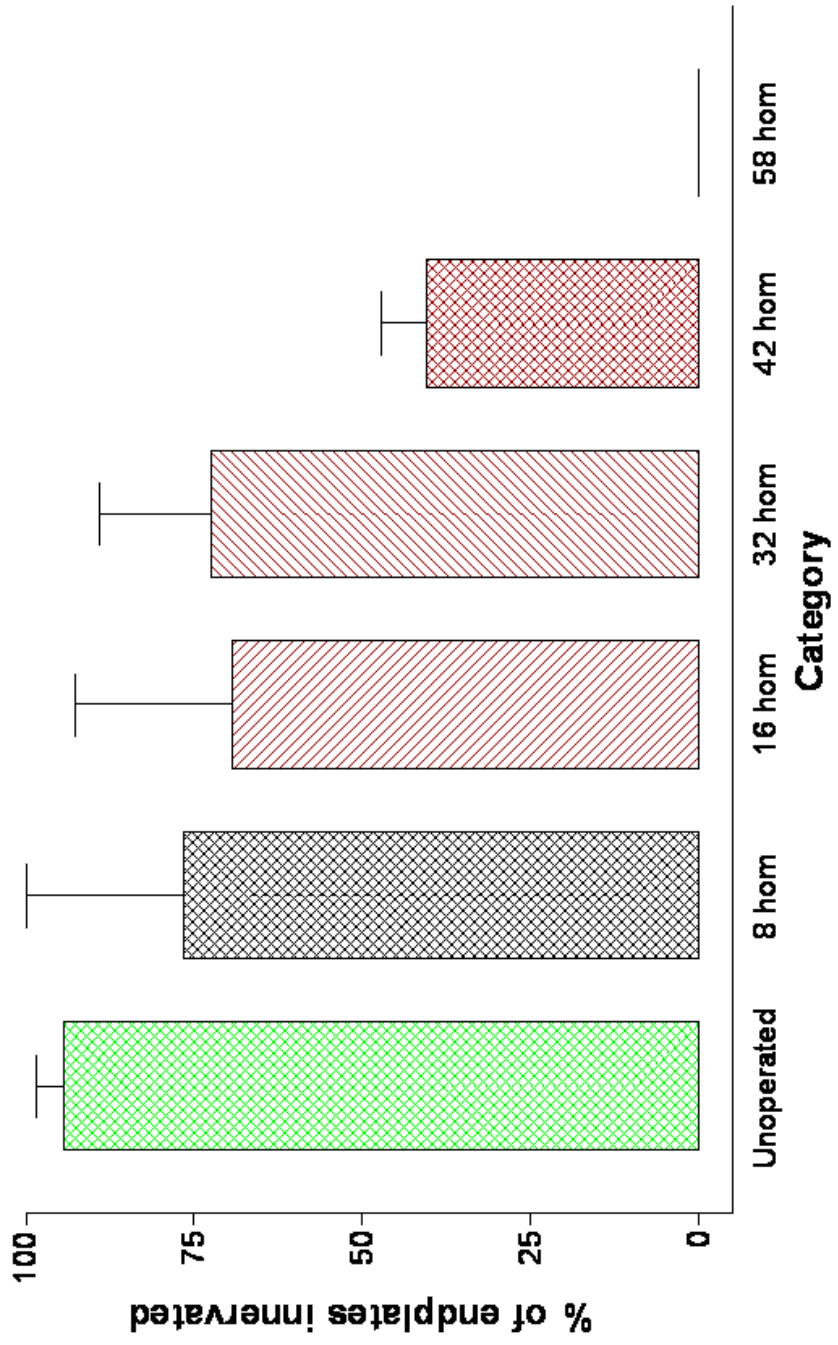


Figure 2.7 The protection of γ -motor endings depends on age in homozygote *Wld^S* mice

Confocal microscopy of teased muscle spindles. This figure extends over the 4 preceding pages.

A-C spindles from 32 and 42 week old animals, 5 days post axotomy. All of these still show some motor innervation of γ -motor endplates indicated by white arrows. D-F Spindles from 42 week old animals 10 days post axotomy with γ -motor innervation indicated by white arrows. G-J spindles from 58 week old *Wld^S* mice 5 days post axotomy showing no γ -motor innervation, empty γ -motor endplates visualised with TRITC conjugated α -BTX are indicated with yellow arrows. Scale bars= 100 μ m.

Graph shows the percentage of γ -motor endings innervated 5 days after axotomy in various mouse age-groups. This declines with age. However there is no significant difference between the values in unoperated mice (n=9) and operated mice up to 32 weeks of age. N numbers = 8 week old (7), 16 week old (4), 32 week old (4), 42 week old (7), 58 week old (7).

In conclusion, it appears that there may be an effect of branching on the protection of axon terminals in *Wld^S* mice as in these experiments, γ -motor neurone terminals appear to be more robustly protected than α -motor neurone terminals. However, it is unlikely that branching alone is responsible for the improved protection of sensory endings as compared to α -motor endings in *Wld^S* mice as the protection of γ -motor endings declines with age and in 58 week old animals and heterozygotes the protection of these γ -motor terminals is equivalent to α -motor terminals while the protection of Ia annulospiral endings remains high.

2.4 Activity

The final factor I have investigated in this chapter is activity. Axons are not spontaneously active so a motor axon stump, having been disconnected from its inputs, most probably does not generate action potentials, although they are able to conduct them (Tsao et al, 1994). However, a sensory stump is still attached to its input; the sensory receptor, and could possibly still be generating potentials in that receptor during passive muscle stretching. These action potentials could propagate up the axon to the transection point.

Neuronal activity is hypothesised to be protective in other systems. During synapse elimination in development more active inputs to a neuromuscular junction out compete inactive inputs (Ribchester and Taxt, 1983). One hypothesis is that the activity within the neurone promotes survival of its inputs to the synapse (Personius and Balice-Gordon, 2002). Increased mental activity decreases risk of neurodegeneration in the CNS (Valenzuela et al, 2007, 2008; Ickes et al, 2000) and exercise has suggested therapeutically for motor neurone disease sufferers (Kirkinetzos, 2003). Although there are many factors that might mediate these benefits, it is possible that activity in the neurones is at least partially responsible for the neuroprotection. This could be mediated by activity-regulated genes such as *c-fos* which seems to be transcribed in response to synaptic activity in a neurone and

neurotrophin, BDNF, also appears to be upregulated by neural activity (Flavell and Greenberg, 2008). Perhaps residual action potentials, in sensory axon stumps, cause a cascade of events that give the axon a better chance of survival.

I attempted but was unable to obtain electrophysiological recordings from axotomised sensory endings to verify that disconnected sensory axons were functional so I used indirect methods to ascertain this.

Ia sensory afferents terminating on muscle spindles contain 50nm clear vesicles which resemble synaptic vesicles in their morphology, association with synaptic vesicle proteins and FM1-43 uptake (Bewick et al, 2004). Bewick et al (2004) demonstrated that these vesicles under-go recycling in an activity and Ca²⁺ dependent manner. I used FM1-43 to examine whether the preserved spindles in *Wld^S* animals after axotomy retained any normal activity.

Next, in order to test the effect of activity on the survival of disconnected axons, I examined primary explant cultures of nerve. The nerves used to answer this question were the phrenic nerve, which supplies the diaphragm and is more than 90% motor axons and the saphenous which is 100% sensory axons. With no axonal endings of any kind in the primary culture, both nerves ought to have the same amount of activity. If activity were responsible for the differences in degeneration of sensory and motor nerve seen in vivo (Brown et al, 1994) then these cultured sensory and motor nerve explants should have degenerated at equivalent rates.

2.4.1 Vesicle recycling occurs in sensory endings on lumbrical muscle spindles

Following the protocol put together by Dr Guy Bewick (University of Aberdeen), I was able to demonstrate stretch-dependent labelling of sensory endings in mouse lumbrical muscle spindles using FM1-43fx [Figure 22.8].

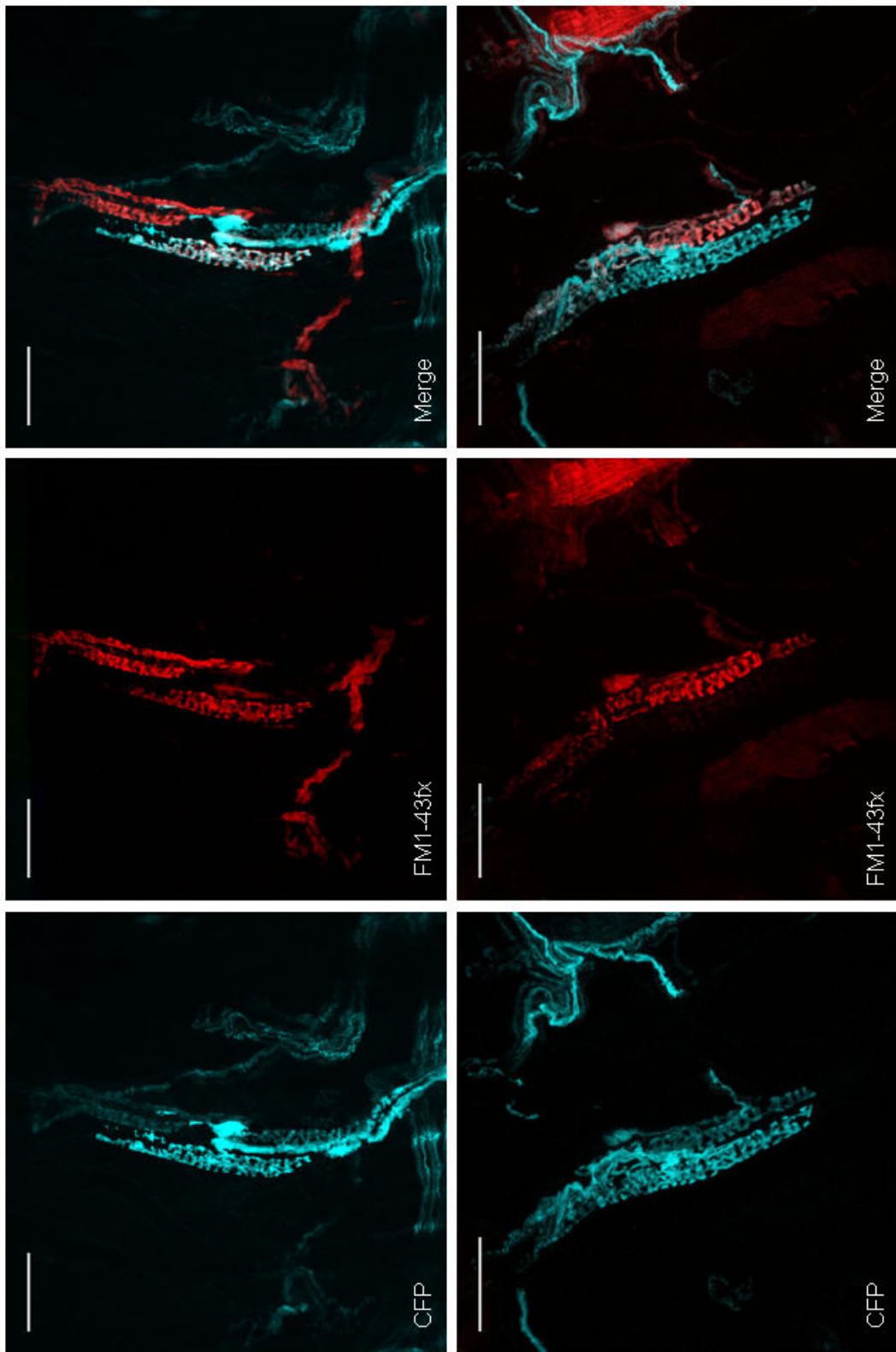


Figure 2.8 FM1-43 labels mouse lumbrical muscle spindles,

Two examples of muscle spindles, in the lumbricals of CFP mice, showing that FM1-43 has been taken up by the IA endings. Confocal microscopy of the CFP (left) showing the structure of the ending, and the fixable fm1-43 (middle) showing where the styryl dye has been taken up, merged in the final panel. All scale bars = 50 μ m.

2.4.2 Vesicle recycling occurs in preserved sensory endings in *Wld^S* mice

I used FM1-43fx to label sensory endings on muscle spindles in 8 week old, aged and heterozygous *Wld^S* mice, 5 days post axotomy. This demonstrated that the vesicles are able to recycle in the ending even 5 days after the axon has been transected and suggests that at least one of the cellular processes continues to occur and the preserved structure may still have some functionality [Figure 2.9].

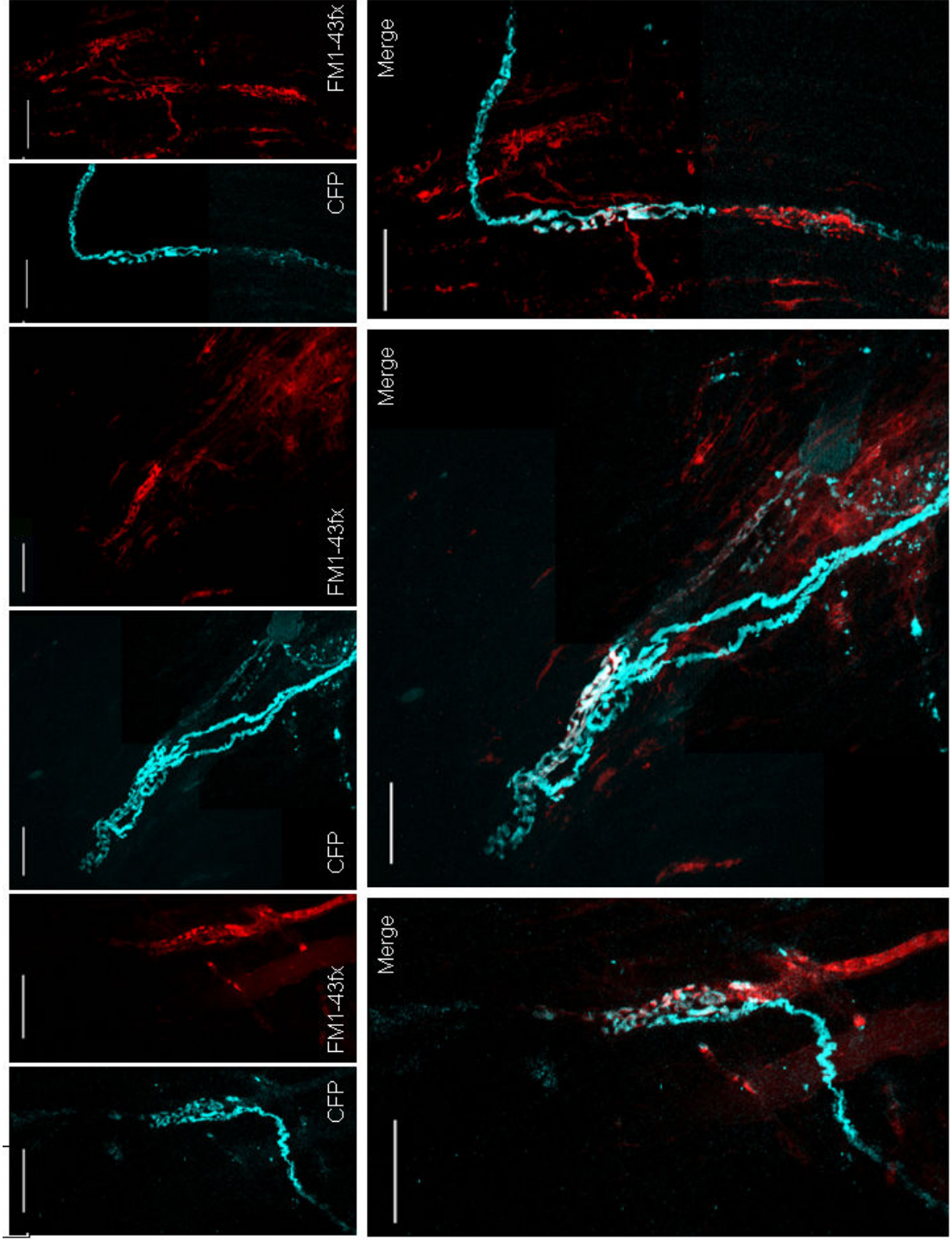
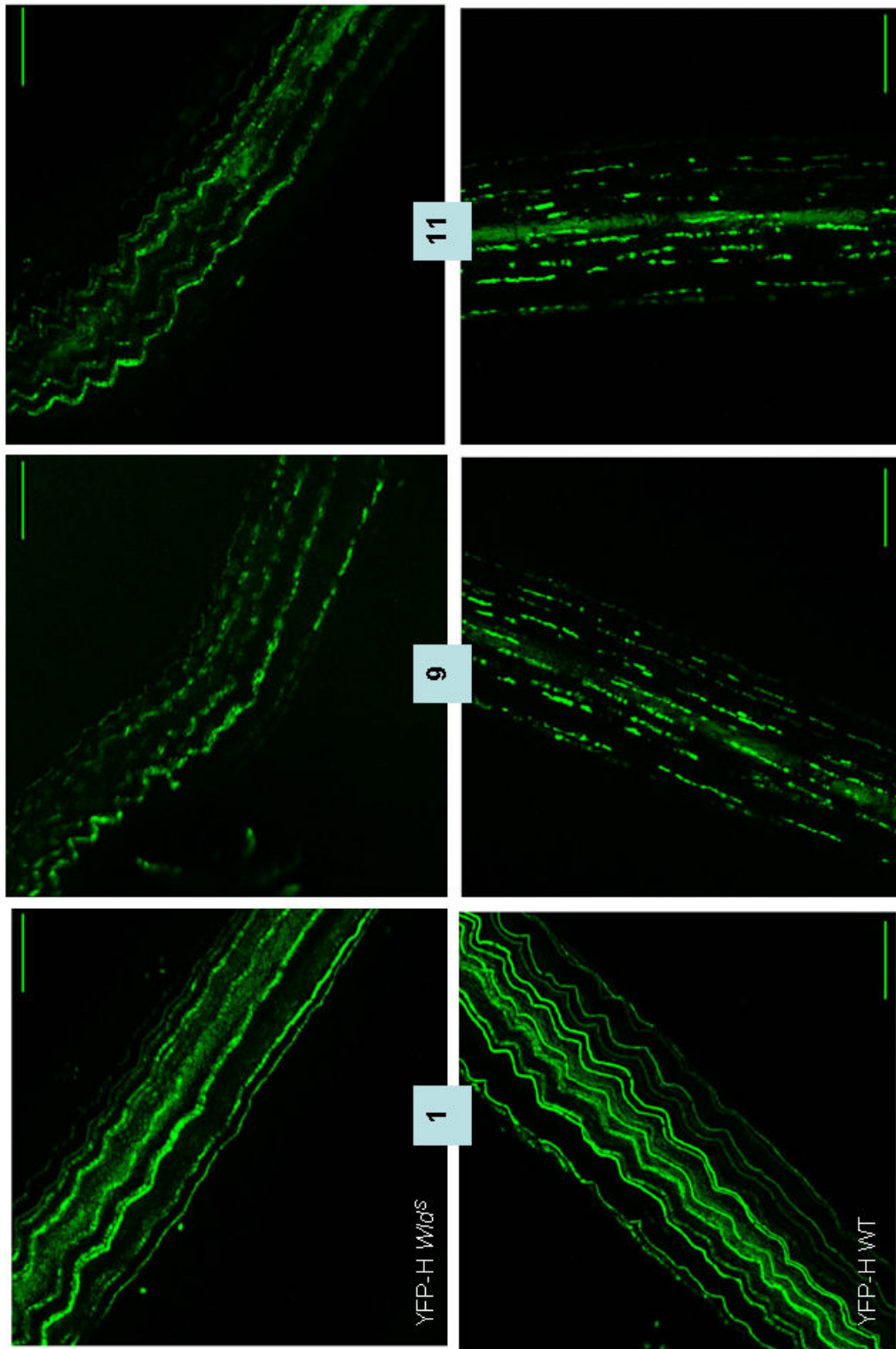


Figure 2.9 FM1-43 is taken up by sensory endings in *Wld^S* mice after axotomy.

Three examples of CFP *Wld^S* spindles, 5 days post axotomy. Confocal microscopy shows the CFP structure of the IA ending and the fixable FM1-43, which has been taken up by the annulospiral endings. Scale bars = 50 μ m.

2.4.3 *Wld^S* phenotype is reproduced in culture

Degeneration of tibial nerve from *Wld^S* mice is slower than that of wild type mice, in vitro [Figure 2.10]. Up to 11 days after the nerve was cultured (ie: 11 days post axotomy) *Wld^S* nerve the majority of axons appear to have long intact lengths and few breaks, while in WT nerve, axons were broken into short intact lengths four days after axotomy and these continued to be reduced. This result demonstrates that even after undergoing what is effectively a double axotomy, followed by tissue culture, *Wld^S* nerve contains more robust axons than wild type nerve.



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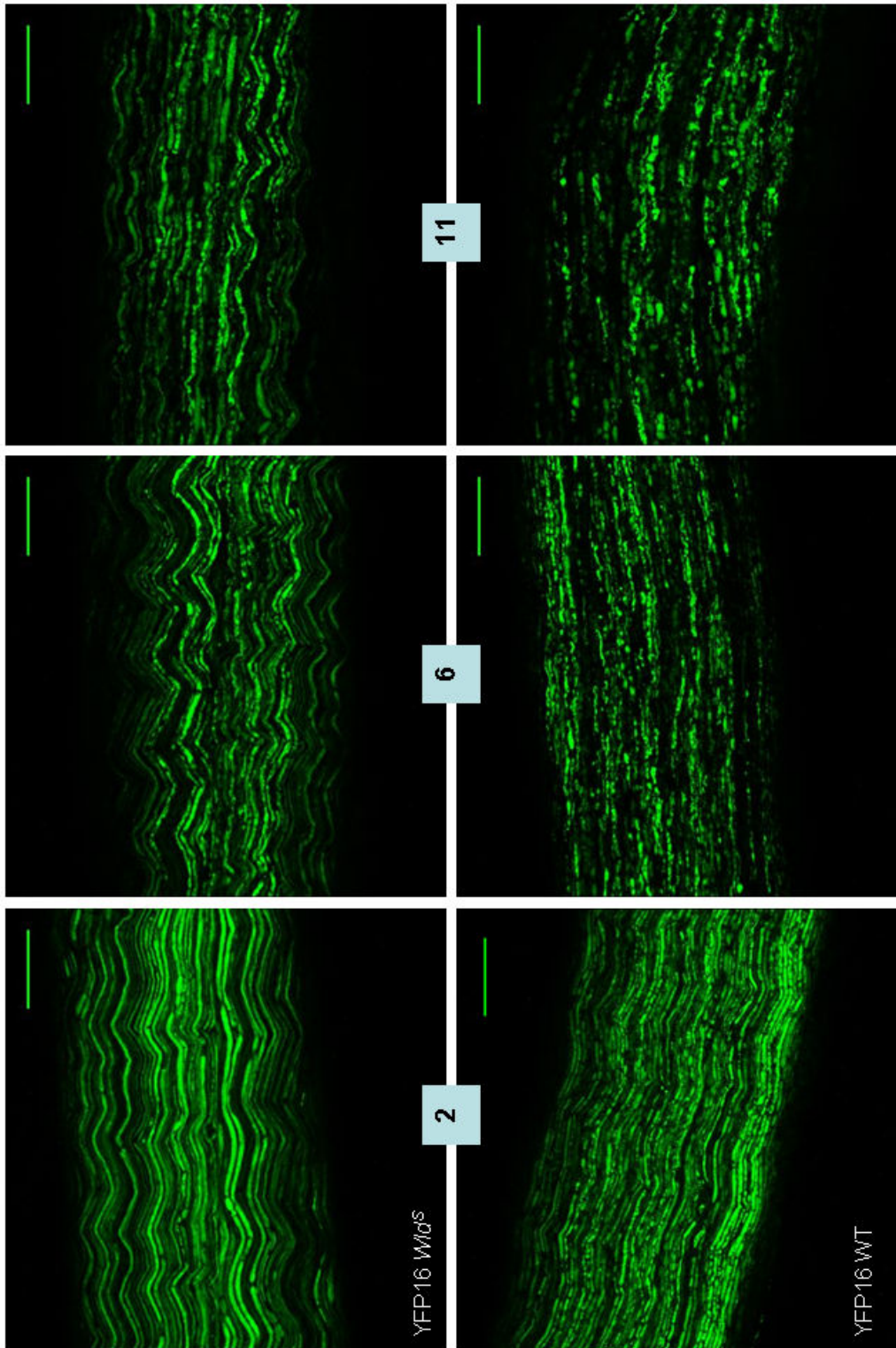


Figure 2.10 *Wid^S* axonal phenotype is reproducible in culture

This figure extends over the previous two pages.

Confocal micrographs of tibial nerve from mice expressing yellow fluorescent protein, after increasing duration of time in culture. A) YFP-H tibial nerve, top row from a *Wid^S* mouse, bottom row from a wild type mouse, at given days post axotomy (Left to right 1, 9, 11). B) YFP16 tibial nerve. Top row from a *Wid^S* mouse, Bottom row from a WT mouse, at given days post axotomy (Left to right 2, 6 and 11). The difference in the degradation of the nerve is observed from 3 days in culture. Scale bars = 100 μ m

2.4.4 There is differential *Wld^S* protection of the phrenic and saphenous nerve in culture

Having demonstrated that it is possible to discriminate WT and *Wld^S* nerves in culture, I examined phrenic and saphenous nerve from *Wld^S* mice in order to see if there was a difference in their rates of degeneration in vitro. Phrenic nerve cultured from old *Wld^S* mice degenerates faster than that of saphenous nerve from the same mice [Figure 2.11]. Axon breaks appeared in phrenic nerve within a week, and by 11 days axon fragments were considerably shorter, whereas saphenous nerve retained unbroken axons even at 11 days in culture.

There was no discernable difference in the rate of degeneration between phrenic and saphenous nerve from wild type animals [Figure 2.11].

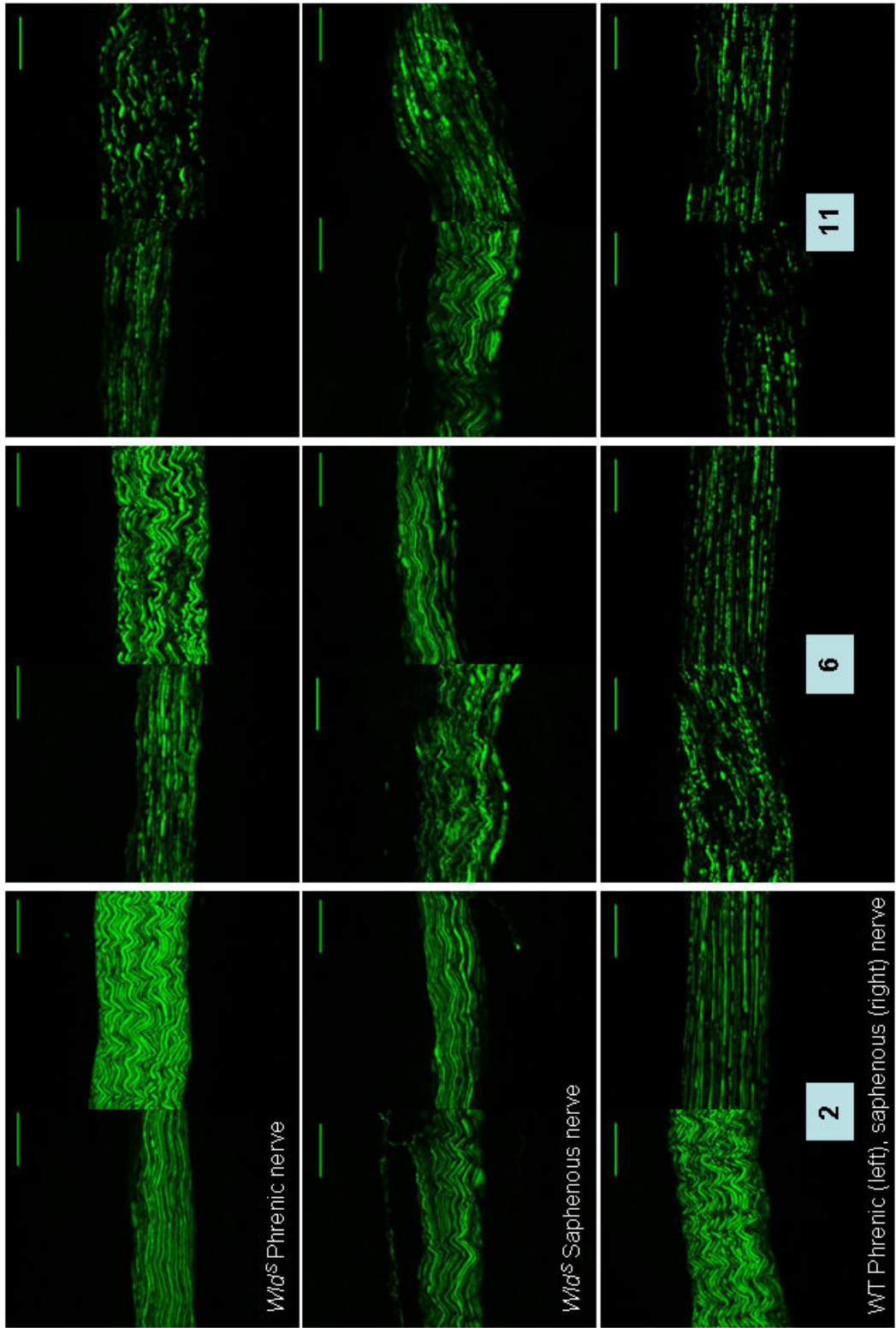


Figure 2.11: Motor axons degenerate faster than sensory axons in culture

Confocal micrographs of peripheral nerves at increasing durations of time in culture. Top row phrenic nerves from YFP16 *Wld^S* mice, middle row saphenous nerves from YFP16 *Wld^S* mice. There are two examples of nerve at each time point. Bottom row: Examples of phrenic and saphenous nerve from YFP16 WT mice. From left to right, 2, 6 and 11 days in culture.

Both examples of *Wld^S* phrenic nerve contain many more broken axons than the examples of *Wld^S* saphenous at both 6 and 11 days post axotomy. The degeneration of the two nerves is comparable in wild type animals.

Scale bars=100μm

2.5 Discussion

2.5.1 Protein expression

The first part of this chapter examined the hypothesis that the amount of Wld^S protein decreases with age, potentially providing the age phenotype and the heterozygous phenotype with a common cause. Due to the reports in the literature that Wld^S protein is neuronally located I chose to examine cerebellum, as a region on CNS in which Wld^S protein expression is high; spinal cord, where the motor neurone somas are located and dorsal root ganglia which house the cell bodies of sensory neurones. The amount of Wld^S protein in each nervous tissue examined remained high in older animals. There was also no evidence to suggest that the amount of protein fell selectively in the motor neurones, as samples of spinal cord where the motor cell bodies are located, showed similar levels of Wld^S protein in young and old animals. Taken together, there is no evidence of differential protein expression in old *Wld^S* animals in the nervous system.

During the final year of my studies, evidence has emerged that Wld^S protein may in fact be located in the axons of *Wld^S* animals at very low levels (M.P. Coleman, B. Beirowski, personal communication). There are also data that suggest Wld^S protein located in the axon may give rise to the protective phenotype (Sasaki et al, 2006; B. Beirowski et al, submitted). However, up until now there has been overwhelming evidence in the literature that Wld^S protein was all located in the nucleus and none in the cytoplasm (Mack et al, 2001; Fang et al, 2005; Gillingwater et al, 2006a).

I detected Wld^S protein in Western blots of sciatic nerve, ventral and dorsal roots. However, this not unequivocal evidence for Wld^S protein in axons as there are many cell types that make up nerve. I also examined Wld^S protein expression in the axon using immunocytochemistry. I was unable to detect any protein in axons from sciatic, tibial, phrenic or saphenous nerve, with this method. However, I was able to see some specific Wld^S staining in the cytoplasm of DRG neurones that I was examining as controls.

Given the signal from Western blots, and the accidental observation of cytoplasmic Wld^S in the cytoplasm of DRG neurones that was noticed in confocal images designed to examine the nuclear staining, it is puzzling that cytoplasmic localisation of the protein was not reported before.

Notwithstanding the above, I find no compelling evidence to suggest that a difference in the amount of Wld^S protein in axons is responsible for the differing motor and sensory phenotypes. However, investigation of cytoplasmic deposits of Wld^S in the soma of spinal motor neurones should be a priority, and perhaps improved techniques for detection of axonal proteins could allow re-examination of peripheral nerve.

Given that there is no evidence in favour of a decrease in the amount of Wld^S protein in the nuclei or axons of neurones as an animal ages, neither generally, nor specific to motor neurones, it seems unlikely that the heterozygote and aged Wld^S phenotype share a common cause.

2.5.2 Axon Branching

The second hypothesis examined in this chapter was that branching reduced the effect of Wld^S . This was tested by examining the protection of γ -motor units, which branch intermediately between α -motor and sensory units.

Here γ -motor endings demonstrated no protection in heterozygous Wld^S animals. There was an effect of age on the protection of γ -motor endings and Wld^S mice at 58 weeks of age exhibited no protection of the γ -motor endings. However, protection of γ -motor endings was excellent up until 32 weeks of age, and Wld^S mice at 42 weeks of age still demonstrated some protection of γ -motor endings at 3, 5 and 10 days post axotomy.

This suggests that there may be a mild effect of branching on the protection conferred by Wld^S on axonal endings. This could be investigated further with a comparison of Wld^S protection to endings in muscles with larger motor unit size

versus smaller motor unit size. Perhaps this effect on the *Wld^S* phenotype can tell us something about the mechanism of *Wld^S* protection.

If there was a protective factor produced by *Wld^S* in the nucleus that was transported down the axon by anterograde axonal transport, at axonal branch points the factor could get diluted down each arm of the axon. This could explain why no protection was seen even with smaller γ -motor unit sizes in the heterozygous *Wld^S* mouse. With less *Wld^S* in the heterozygous mouse there may be less of the protective factor to start with, this may protect a single axon adequately, but dilution even over a few branches may reduce the effectiveness.

The sensory endings appear to be disproportionately better protected than even the γ -motor endings, so branching cannot be the only explanation for the improved sensory phenotype.

2.5.3 Activity

The third part of this chapter addressed the possibility that activity underlies the increased protection of sensory endings. Experiments on cultured nerves where neither sensory nor motor axons receive any stimulus and should not experience any activity (or if they do, for some reason, it should at least be equivalent) showed that a difference in the protection of sensory and motor axons still exists. This is compelling evidence that an intrinsic difference rather than activity is the cause of more robust protection of *Wld^S* sensory axons.

2.5.4 Other explanations

The conclusion from this chapter is that the amount of protein in the nuclei of motor neurones does not change sufficiently with age to explain the loss of protection of the neuromuscular synapse. Activity and branching are also probably not the main causes of the sensory/motor difference.

Given that none of the hypotheses I have tested can completely explain the differences between the robust protection of sensory axons and endings in *Wld^S* mice

and the conditional protection of motor axons and endings which is weaker in aged or heterozygous *Wld^S* mice, what other explanations might there be?

The differences between sensory and motor neurones are vast. *Wld^S* protection is not the only feature to discriminate between them. The selective death of motor neurones in amyotrophic lateral sclerosis (ALS) has not yet been satisfactorily explained. In 10-20% of familial ALS, a mutant SOD1 has been demonstrated to be responsible for the disease. However this protein is expressed in neurones throughout the nervous system so why is it that motor neurones selectively degenerate in both humans and mice expressing the mutant SOD1?

This question is echoed throughout neurodegenerative disease research. In Parkinson's disease there is selective death of dopamine neurones, in Alzheimer's disease the hippocampus is primarily affected, in fronto-temporal dementia the frontal lobes degenerate first. In all of these neurodegenerative diseases, degeneration is not necessarily restricted to one population of neurones; however it is one particular population that is affected to the greatest extent. As with most questions in biology, the explanation for this is likely to be genetic, environmental or a mixture of the two.

Returning to the example of ALS, perhaps motor neurones have a particular genetic pre-disposition that interacts with the mutant SOD1 in such a way as to cause pathology unseen in other neurones in the nervous system. Raoul et al (2002) identified a novel Fas signalling pathway that requires transcriptional upregulation of neuronal NOS and no evidence for involvement of this pathway was found in cells other than motor neurones. Motor neurones from transgenic mice overexpressing SOD1 mutants displayed increased susceptibility to activation of this pathway. They were more sensitive to Fas- or NO-triggered cell death but not to trophic deprivation or excitotoxic stimulation.

Genetic death pathways restricted to specific neuronal classes could exist for precise control of developmental neuronal death and also underlie the selectivity of neuronal loss in neurodegenerative disease.

Genetic factors, for example as yet unknown death pathways unique to the motor axon terminals, may explain why they degenerate where sensory axon terminals remain intact after injury in older or heterozygote *Wld^S* mice. However it may be that motor axons experience a particular environment conducive to degeneration in this situation while sensory axons experience a supportive environment. Environmental factors could include a different compliment of neurotrophins, different activity, or different energy requirements.

Hoke et al, (2006) used RT-PCR to examine the expression levels of 11 growth factors and demonstrated that Schwann cells of cutaneous nerve and ventral root exhibit differing growth factor profiles at baseline and respond differently to denervation.

Expression of vascular endothelial growth factor-1 (VEGF-1), insulin-like growth factor-1 (IGF-1), and pleiotrophin (PTN) was much higher in intact ventral root than in intact cutaneous nerve. In contrast, baseline expression of BDNF, neurotrophin-3 (NT-3), hepatocyte growth factor (HGF), and GDNF was higher in cutaneous nerve than in ventral root. NGF, the prototypical sensory growth factor, was expressed similarly in both nerve types, as were IGF-2, fibroblast growth factor-2 (FGF-2), and ciliary neurotrophic factor (CNTF) (Hoke et al, 2006).

Between 5 and 30 days after denervation of the femoral cutaneous nerve and the L4 and L5 ventral roots, five of the growth factors, NGF, BDNF, IGF-1, HGF, and VEGF were significantly upregulated in denervated cutaneous nerve but minimally if at all in denervated ventral root. When viewed in light of baseline expression levels, the predominance of BDNF and HGF in sensory nerve is accentuated further and that of NGF is unchanged; the relative levels of VEGF and IGF-1 are lower (Hoke et al, 2006).

The differing baseline state of neurotrophins experienced by the axons and the differing responses to injury could underlie the differing susceptibilities to degeneration in genetic disease, or after environmental insult, and differing responses to protection from that degeneration.

Age is a risk factor in all neurodegenerative diseases. Therefore, it seems likely that something about the neural constitution changes as they mature. In the *Wld^S* mouse similar genetic and environmental factors that change or develop with age, may be responsible for the loss of the protective phenotype that appears to be specific for old motor nerve terminals.

Discovering what causes motor neurones in aged and heterozygous *Wld^S* mice to be susceptible to degeneration even when sensory neurones demonstrate such robust protection, might highlight changes that occur as motor neurones age that also makes them susceptible to degeneration in ALS. This could lead to insights into the key difference between neurones underlying their differential sensitivity in different neurodegenerative conditions.

3. Dendritic protection in *Wld^S* mice

3.1 Introduction

The theory of compartmental degeneration suggests that the morphologically and functionally distinct compartments of the neurone have specific molecular mechanisms responsible for their degeneration. A host of molecules have been shown to regulate apoptosis of the neuronal soma including key players such as Bcl-2 and the caspases. *Wld^S* has been shown to interfere with the molecular mechanism responsible for Wallerian degeneration of the axon. The axon terminal may have its own distinct pathway that regulates its destruction.

No evidence has been presented that indicates whether either of these destruction mechanisms (apoptosis or Wallerian degeneration) can occur within the dendrite, a fourth morphologically and functionally specialised neuronal compartment.

The present chapter aims to investigate any evidence for *Wld^S* protection in dendrite degeneration.

Dendrites and axons are both neuronal processes with a common developmental history. However they each perform different functions in the neurone and are morphologically specialized for these purposes, resulting in some key molecular differences. Some of the similarities and differences will be discussed below.

3.1.1 The development of axons and dendrites

Most evidence we have about the development of the axons and dendrites comes from the most commonly used system for examination of neuronal polarity; cultured hippocampal neurones. These develop one axon and several dendrites (as they would in situ) and maintain their characteristics at a structural and molecular level.

Dendrites and axons have a common beginning as neurites, growing from the newly differentiated neurone. After plating, hippocampal neurones develop protrusions

which grow to be short neurites all roughly equal in length. At this point it is not possible to identify special characteristics that could allow a prediction of which one will become the axon. There are no significant differences among the processes in light microscopic morphology or behaviour, in ultrastructural characteristics, in microtubule density or orientation, or in their content of markers that distinguish axons and dendrites in mature neurones (Arimura and Kaibuchi, 2005).

12- 24 hours after plating, one neurite will begin to grow rapidly (Esch et al, 1999) and will go on to become the axon. This growth immediately establishes the polarity of the neurone. A few days after the axon has begun its rapid growth, the remaining neurites elongate and acquire the characteristics of dendrites. About 7 days after plating, neurones form synaptic contacts and establish a neuronal network (Arimura and Kaibuchi, 2005).

3.1.2 Differences between mature axons and dendrites

Dendrites are often shorter than axons. They are thicker near their origin, at the cell body, and taper off, unlike the axon which has a similar cross section all along its length. When axons branch, this happens distally, near their target area, however, dendrites branch at semi-regular intervals.

Axons and dendrites differ further in the polarity of the microtubules they contain. In axons, the microtubules are uniformly oriented with the plus end pointed distally, whereas in dendrites, the orientation of microtubules is mixed (Craig and Banker 1994). This has important implications in the microtubule-based intracellular transport of molecules and organelles. Other molecular differences exist. Various cytoskeletal proteins, motor proteins, transmitter receptors, and ion channels are found exclusively or preferentially in axons or dendrites (Craig and Banker, 1994).

All of the organelles that are found in the soma, including ribosomes, endoplasmic reticulum (ER) and Golgi are also found in the dendrites. This allows protein synthesis to occur locally in dendrites. In contrast, ribosomes are scarce, if not

absent, in the axon and it is generally believed that there is no protein synthesis in the axon.

Both axons and dendrites have specialized endings for making synaptic contacts. The axon terminals have the machinery for transmitter release. The dendrites usually have dendritic spines, shaped like mushrooms, which contain the post-synaptic density, as well as the receptors and signaling complexes required to receive and transduce information.

3.1.3 Dendrite degeneration

There is evidence that there may be a degeneration mechanism specific to dendrites. Iyirhiaro et al, 2008 found that treatment with flavopiridol and minocycline provided long-term protection for cell bodies following global ischemia but dendrites were not spared (Iyirhiaro, 2008). Dendritic loss may be a survival strategy to contain viral infection in neurones. Expression of shorthairpin RNA (shRNA) against luciferase (which is not in the rat genome) in hippocampal pyramidal neurones in rat organotypic slice cultures triggers dramatic loss of dendritic spines and simplification of dendritic arbours (Alvarez et al, 2006). In both these cases dendrites appear to have degenerated independently of their neuronal cell bodies. Dendritic retraction also occurs in synapse remodelling, which occurs throughout the life of an organism (Alvarez and Sabatini, 2007). These suggest there may be a mechanism allowing destruction of dendrites without affecting the cell body.

Literature can be found supporting an apoptotic-like degeneration or a Wallerian-like degeneration of dendrites.

M. P. Mattson has produced a body of work demonstrating that apoptotic proteins are present in dendrites. Studies of cultured neurones, synaptosomes and postmortem brain tissue have shown that essentially all of the “cell death machinery” can be activated locally in synaptic terminals and dendrites. Such apoptotic cascades can cause several different changes in synaptic terminals. For example, Par-4 induction and caspase activation promote mitochondrial dysfunction, further caspase

activation, and release of factors into the cytosol that are capable of inducing DNA condensation and fragmentation (Mattson and Gleichmann, 2005; Gilman and Mattson, 2002). Studies of cultured hippocampal neurones and adult rats exposed to the seizure-inducing excitotoxin kainate, have shown that activation of synaptic glutamate receptors can induce apoptotic cascades that are initially localized to the postsynaptic dendritic compartments.

Mattson argues that these components of apoptotic pathways have physiological roles in synaptic plasticity in the dendrites and synapses of neuronal cells. But given that all the components are there, in pathological situations, they might be activated and cause degeneration [Figure 3.1 based on Mattson, 2004].

Alternatively taking the view that dendrite degeneration is not molecularly distinct from axonal degeneration, T. Koike et al (Koike et al, 2007; Ikegami and Koike, 2003; Yang et al, 2007; Touma et al, 2007) have produced a body of work which discusses axon and dendrite degeneration mechanisms in the same breath, referring to them collectively as neurites. Koike and colleagues work with cultured SCG neurones in which the axons and dendrites are not mature. They have described neurite death in response to disconnection from cell bodies, vinblastine treatment and cellular Zn^{+} chelation and demonstrated *Wld^S* protection against this neurite degeneration. This suggests that immature dendrites at least, may be susceptible to a form of degeneration that is not molecularly distinct from Wallerian degeneration in mature axons.

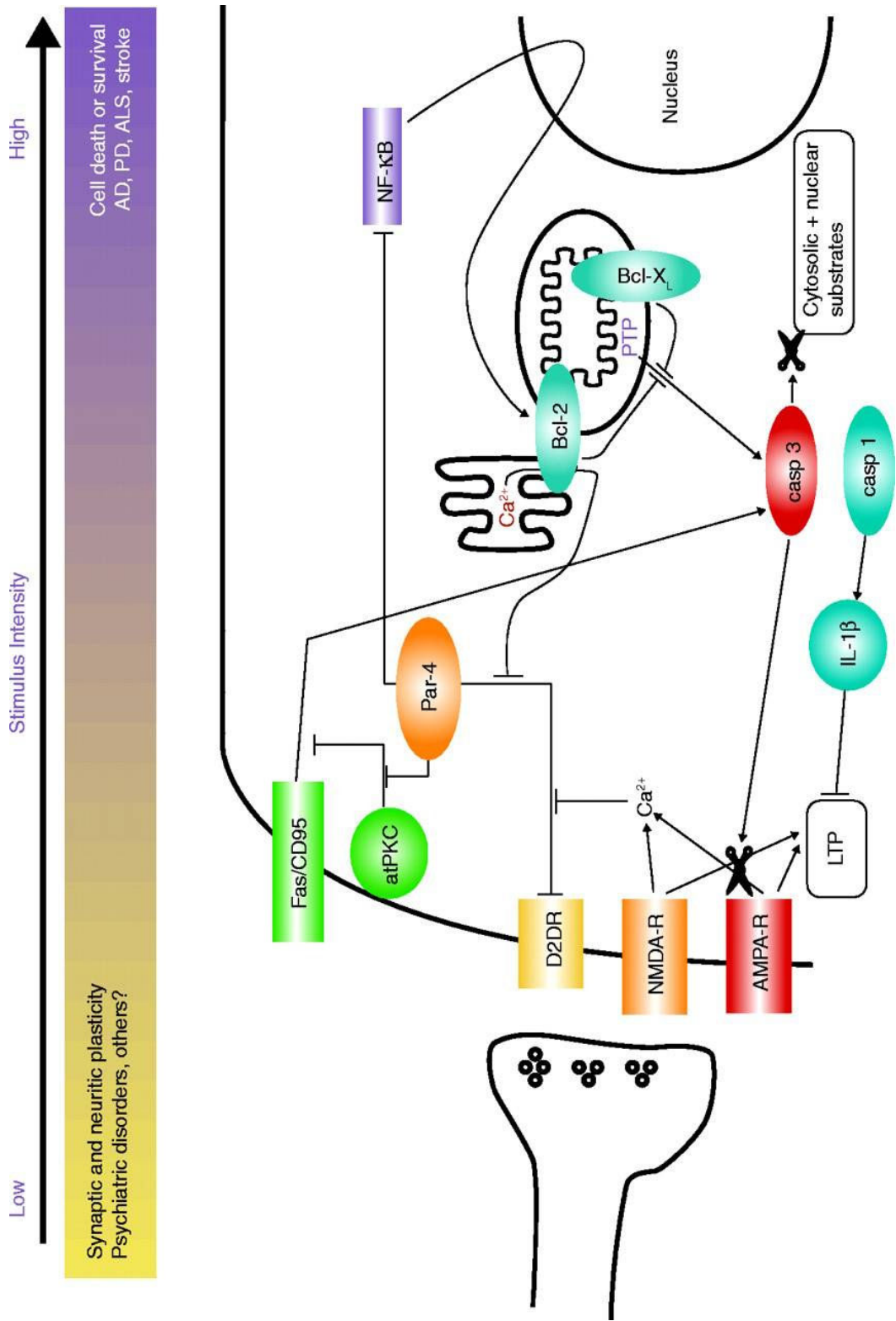


Figure 3.1 Interactions of apoptosis pathways with calcium signaling and synaptic transmission

This figure illustrates that molecules involved in apoptosis also have roles in normal synaptic function.

Examples include:

Par-4, which can transduce classical apoptosis pathways. However, Par-4 also interacts with the D2 dopamine receptor at synapses to inhibit downstream dopamine signaling. This latter interaction is antagonized by calcium-activated calmodulin.

Bcl-2, which inhibits apoptosis by regulating the permeability of the mitochondrial membrane. However it also enhances calcium leakage from the endoplasmic reticulum during synaptic transmission.

Activated caspase-3, an effector of apoptosis, can cleave GluR1, an amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor subunit, thereby modulating intracellular calcium levels.

[D2DR, dopamine 2 dopamine receptor; NMDA-R, NMDA receptor; AMPA-R, AMPA receptor, atPKC, atypical PKC (), LTP, long term potentiation; IL-1, interleukin-1 β ; casp 1, caspase 1; casp 3, caspase 3; PTP, mitochondrial permeability transition pore; NF- κ B, Nuclear factor-kappaB.]

3.1.4 Aim of chapter 3

My aim in this third chapter was to investigate whether mature dendrites were protected from degeneration by *Wld^S*. I first tried to do this using spinal cord slice cultures. This seemed to be the obvious choice as the motor neurone cell bodies are located in the spinal cord, and with characterisation of *Wld^S* protection of the motor neuronal soma (Bisby et al, 1995), axon (Lunn et al, 1989) and neuromuscular junction (Ribchester et al, 1995) it would complete the picture to look at motor neurone dendrites. However, although I was successful in culturing spinal cord for up to 14 days, the dendrites of the motor neurones were not clearly visible or amenable to injury in YFP-H mice. As an alternative I used retinal cultures where the dendritic trees of the few retinal ganglion cells (RGCs) that express YFP in the H-line present a spectacular and accessible appearance [Figure 3.3].

3.2 Spinal cord culture

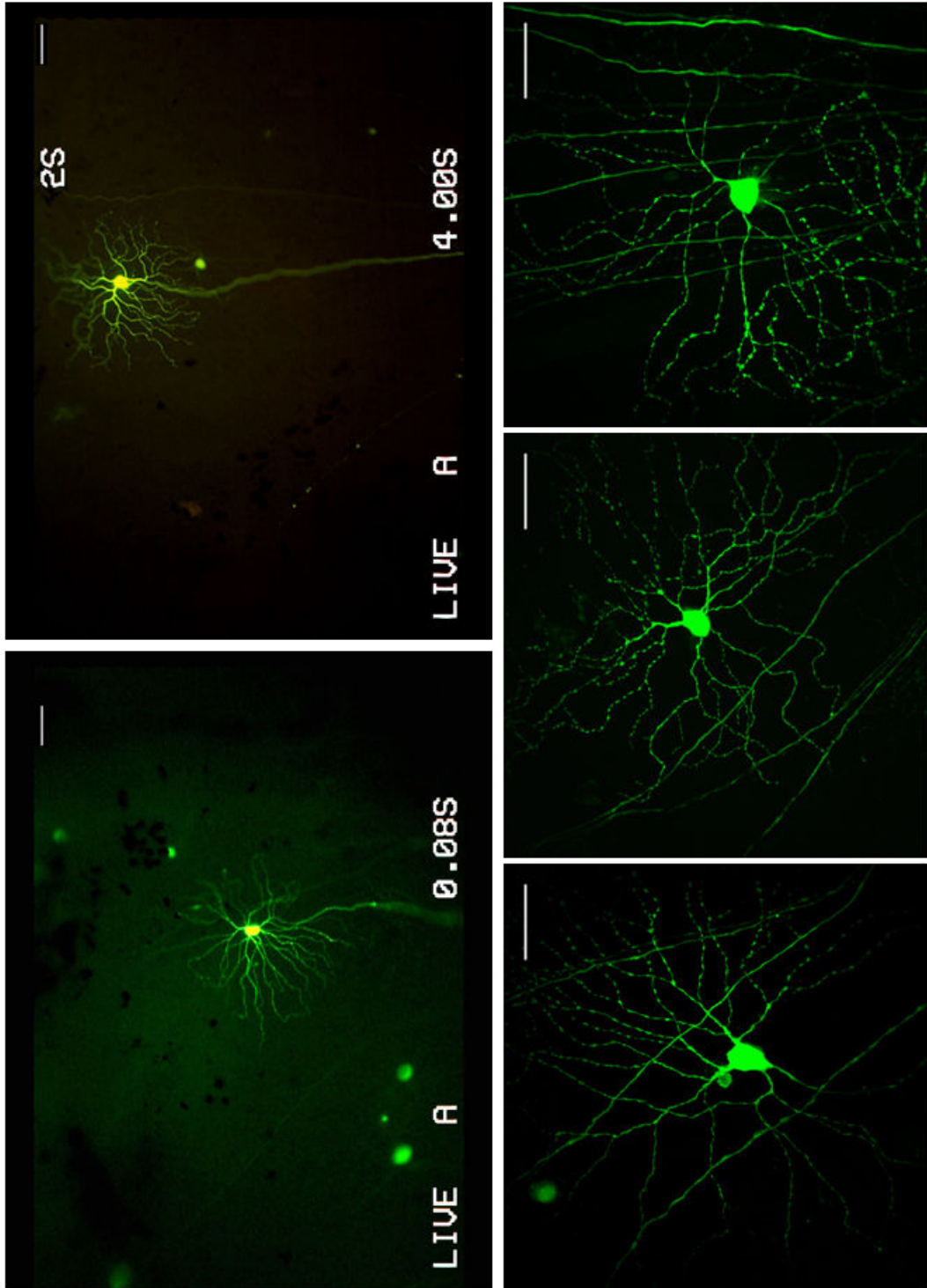
Spinal cord survived for up to 14 days in culture. However, motor neurones appeared in slices rarely and the dendrites were difficult to identify. With these issues, and the effect of length on *Wld^S* protection to contend with (Ribchester et al, 1995), I did not continue to try and investigate the protection of motor neurone dendrites.

3.3 Retinal culture

I originally attempted to culture the retina of *Wld^S* and wild type animals using the same culture media I had used to culture nerves. This resulted in the death and degeneration of the RGCs and probably the rest of the retina also. For what it might be worth, there were no discernable differences between the rates or pattern of degeneration of the soma or dendrites of RGCs in *Wld^S* retina in comparison to the degeneration of wild type retina [data not shown].

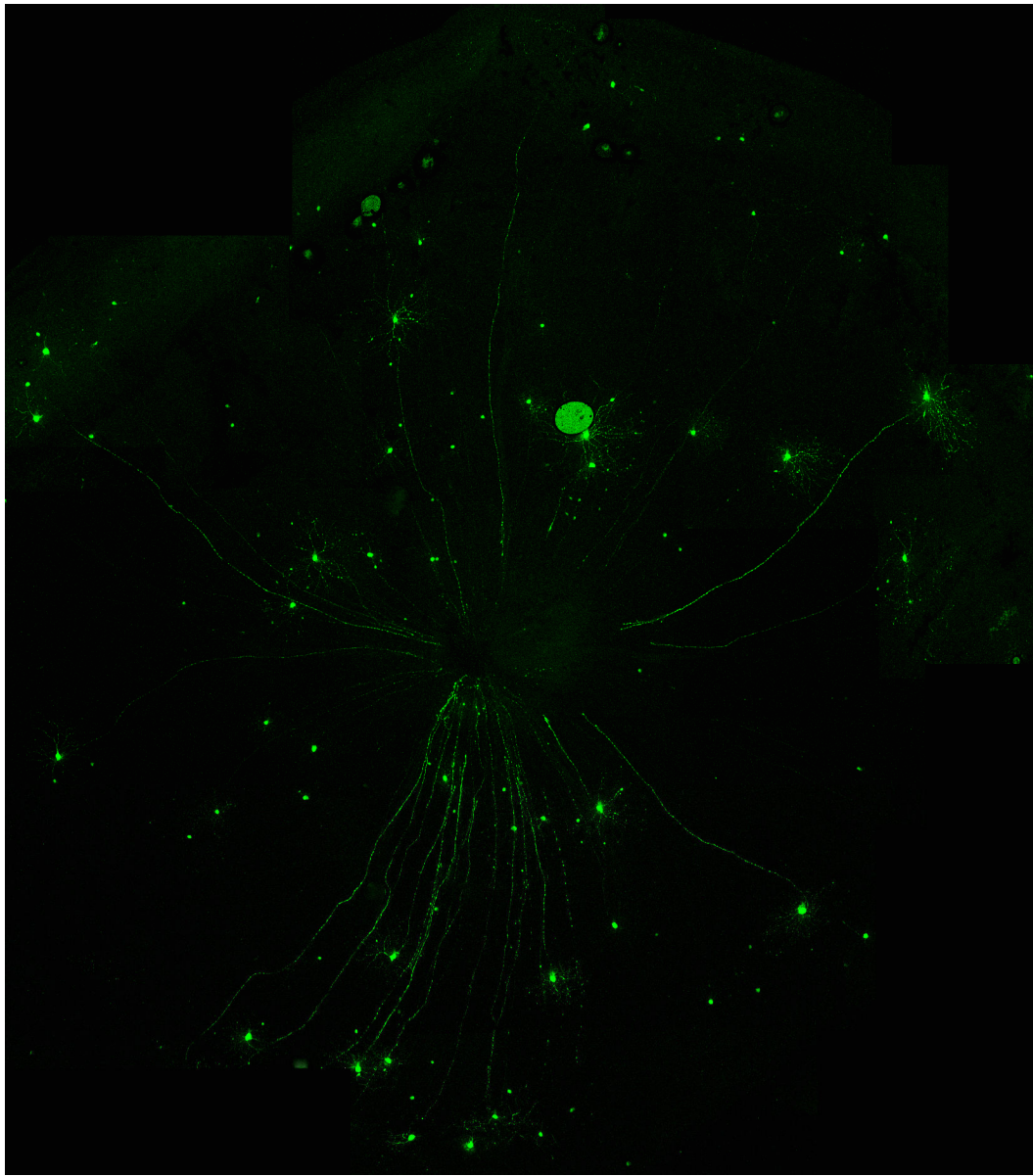
Once B-27 and glucose were added to the culture media, retinal explants appeared to remain healthy for up to 14 days in culture. Figure 3.2A shows RGCs retaining

distinctive morphology over time in culture and imaged with both the fluorescence and confocal microscope. Figure 3.2B shows a retina fixed after 2 days in culture still with healthy intact neurones and retaining the normal morphology. Retinal culture allowed for repeated visualisation of neurones as show in figure 3.3.



A

This is part of Figure 3.2. The figure legend follows.



B

Figure 3.2 Retinal morphology survives in culture

This figure extends over the 2 proceeding pages.

A) 5 images of RGCs in cultured retina. Two (top row) using conventional fluorescent microscope taken after 4 days in culture. Three images (bottom row) of fixed retina previously in culture for between 2 and 4 days, taken with the confocal microscope, scale bars= 50 μ m. B) Confocal microscope images reconstructed to show an entire retina, fixed after 2 days in culture.

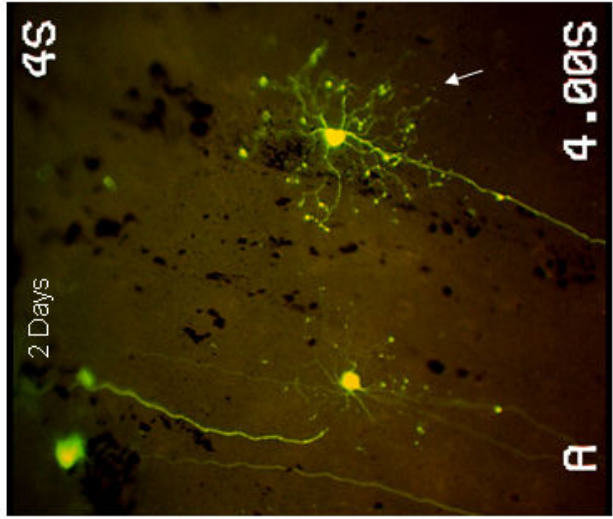
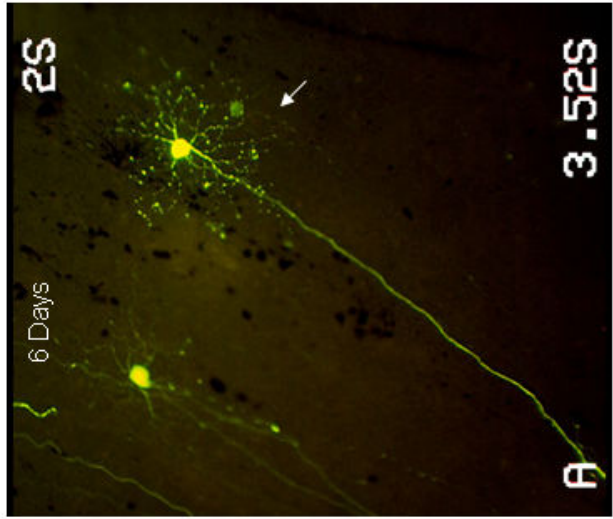
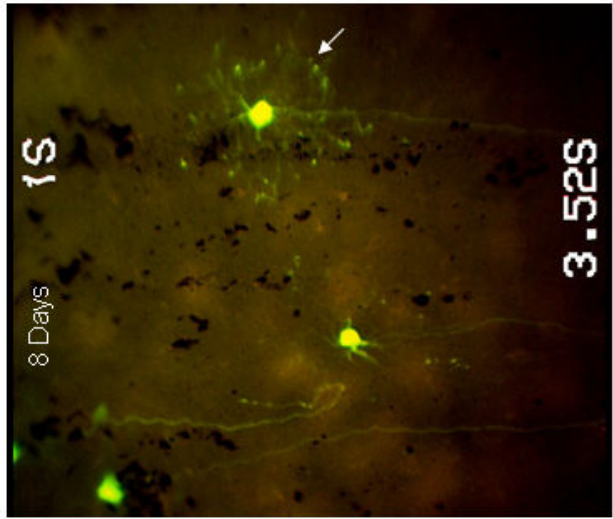


Figure 3.3 Survival of retinal explants in tissue culture

Three fluorescent microscope images showing two neurones surviving (from left to right) 2, 6 and 8 days in culture. The white arrow indicates the same neurone in each of the three pictures. Scale bars= 50µm

3.4 Dendrite degeneration after injury in *Wld^S* retina

I attempted to injure retinal ganglion neurones with needles or to separate the cell soma from the dendrites using razorblades. However, usually I found either no injury could be detected or the retina ceased to resemble retina. In two instances I was able to injure the neural cell body and follow up the neurone at later time points. In both cases on re-visualising the neurone I was unable to identify the cell body or dendrites, however the disconnected axon was visible. The more successful of these instances is shown in Figure 3.4. This suggests that dendrites degenerate much more rapidly than axon following a lesion to the RGC cell soma and therefore that *Wld^S* does not protect dendrites. However, this can not be confidently concluded from such limited and few examples.

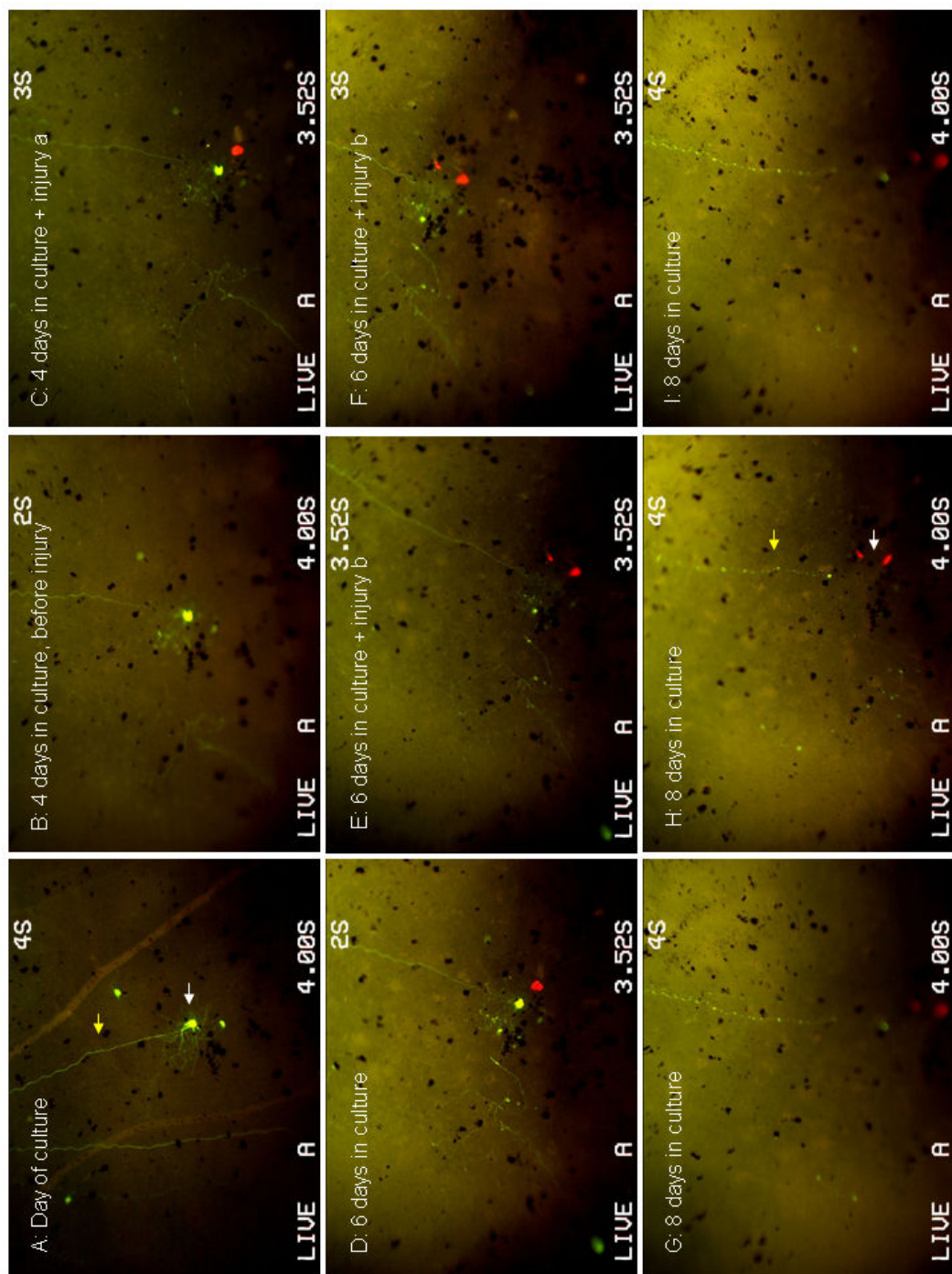


Figure 3.4 RGC dendritic protection after somal injury

A) taken on day of culture. B,C) taken after the retina was in culture for 4 days. B uninjured, C post injury. D,E,F) taken after 6 days in culture. No effect of injury was seen (D) so I attempted reinjury. This time damage to the cell body could be seen clearly (E,F). G, H) are taken after 8 days in culture. Here the neurone has vanished but the *Wld^S* protected axon remains. In G) the extent of the axonal protection can be seen. Scale bars on all picture equivalent to A, = 50µm.

The white arrow points at the cell body in A and at where the cell body would have been in H. The yellow arrow points at the axon in both A and H, where in H it is preserved despite loss of the cell body.

3.5 Discussion

My preliminary work has suggested that *Wld^S* does not protect the dendrites of the RGC. However, further work needs to be undertaken in order to confirm this result. I have found a suitable culture system which is amenable to repeated visualisation, in which dendrites are accessible for injury. However the tools needed to injure the retina need to be refined. Laser microablation, for instance using a multiphoton microscope, could be a fruitful approach. Limited time and resources precluded any attempt at this during the course of the present PhD project.

Identifying molecular players in dendrite degeneration is important clinically and scientifically. Dendritic membrane makes up a significant portion of any neurone. Different neurones vary significantly in their dendrite mass relative to axon mass (Craig and Banker, 1994) and for projection neurones such as motor neurones the axon mass may be several times larger than the dendritic mass. However, more than 80% of the cell membrane of a dentate granule cell (which has a short axon) is dendritic membrane (Craig and Banker, 1994).

Dendrites degenerate or retract during normal physiology for example during LTP and LTD and possible as a survival strategy to contain viral infection in neurones. There are also instances on the boundary of physiology and pathophysiology in which dendrites are lost. One example of this is dendritic retraction during stress. Apical dendrites of pyramidal neurones of the medial prefrontal cortex and hippocampus are susceptible to degeneration after sustained exposure to corticosterone, perhaps suggesting that dendrite degeneration occurs as a result of stress (Cerqueira et al, 2007).

Dendrites are lost in a range of diseases, both neurodevelopmental disorders, neurodegenerative and psychiatric diseases. Synaptic alterations in CA1 in mild Alzheimer's disease suggest loss of dendritic spines is one of the first stages in developing the disease (Scheff et al, 2007). Post-mortem studies have revealed

dendritic abnormalities of pyramidal neurones in the prefrontal cortex of subjects with schizophrenia. These abnormalities include decreased dendritic arbour size and complexity and reduced dendritic spine density on subpopulations on pyramidal neurones (Hill et al, 2006). Spilman et al, 2008 suggest after abnormal prion protein accumulation dendritic degeneration is the first thing that happens before neuronal cell death. Chronic ethanol exposure may cause degeneration and withdrawal of dendrites in the purkinje neurones of the cerebellum (Dlugos, 2008).

Understanding the mechanisms of dendrite degeneration and how to prevent it would enable therapeutic strategies to deal with dendrite loss in diseases where this is the correlate with clinical signs. In diseases where there is loss of entire neurones it may also be important to protect dendrites as protection of the cell body alone may not be enough to allow the neurone to retain its function.

D. Thesis Discussion

D.1 Summary

This thesis has extended the compartmental theory of neurodegeneration by using the *Wld^S* mouse to examine mechanisms of degeneration in axon terminals and in dendrites [Figure D.1].

Firstly, I have found that sensory nerve terminals are much better protected from Wallerian degeneration than motor nerve terminals in the muscles of *Wld^S* mice. I have also provided evidence that supports the idea of the axon terminal as a separate neurodegenerative compartment, through examination of the pattern of degeneration after axotomy. Secondly, I examined three possible explanations for the difference in levels of protection between motor and sensory axons and endings but neither protein expression level, activity nor branching appear to offer a satisfactory explanation. Thirdly, I carried out some preliminary experiments, examining dendrite protection in *Wld^S* retina. The data tentatively suggests that *Wld^S* may not protect retinal ganglion cell dendrites from degeneration, at least not as strongly as their axons. Implications of this work affect both clinical and scientific research.

Of interest to clinical research, it appears that sensory neuropathies may stand to benefit from therapy based on a *Wld^S* protective mechanism to a greater extent than motor neuropathies, particularly where age is an issue. The results also cast doubt on the assumption that axon terminals in the CNS would show decreased *Wld^S* protection in heterozygote and aged animals, as motor nerve terminals do. A demonstration that protection of nerve terminals in the brain was robust would make *Wld^S* research attractive to those working in CNS disorders. Also of interest to clinical researchers, it appears that the inhibition of Wallerian degeneration reveals a qualitatively different degeneration that may have more in common with the degeneration seen in some diseases. Finally, given the unexplained difference in *Wld^S*-mediated protection between the sensory and motor nerve terminals, this

increases evidence for a unique change in the constitution of motor nerve terminals as they age, and further examination of this might inform ALS research.

The findings of this thesis also suggest that scientific methods used to investigate the mechanism of *Wld^S* protection should take into account differences that may be due to the use of different classes of neurone in the assays. I.e: the neurites of the DRG may show more robust protection under a variety of conditions, whereas motor nerve terminals in vivo may be more sensitive to the specifics of *Wld^S* protection. The components of the mouse muscle spindle have been described, allowing researchers of the sensory ending to perhaps consider working with this genetically versatile creature. A primary tissue culture method for repeated visualisation of both peripheral nerve and explanted retina has also been demonstrated. The explanted retina preparation may be useful in understanding mechanisms of degeneration of mature dendrites.

Future directions suggested by this work include elucidating the difference between sensory and motor nerve terminal degeneration that is responsible for their differing levels of *Wld^S* protection after axotomy. That may also be key to understanding their different susceptibility to degeneration in a variety of disease and toxic insult scenarios. Examination of *Wld^S* protection of CNS synapses in old and heterozygote animals would be worthwhile. Further investigation into dendrite degeneration is necessary in order to confirm that *Wld^S* is not able to protect mature dendrites in the RGCs. Given the protection of *Wld^S* neurites in culture, it may be interesting to ask at what point the degenerative molecules become distinct and their relevance in development.

Having discussed the above in greater detail in the relevant chapters, I will use this discussion to review the compartmental theory of neurodegeneration, examining the likelihood of its evolution and looking critically at the assumptions and simplifications it is based in.

Axonal endings in the CNS

Synapses in the CNS have been demonstrated to be protected by *Wld^s* in young homozygote *Wld^s* mice. Whether this protection is robust in older and heterozygote mice is unknown. Results presented in this thesis suggest that not all axonal endings lose *Wld^s* protection in these circumstances.

Dendrites

Although neurites are protected by *Wld^s*, work presented in this thesis suggests that mature dendrites are not protected by *Wld^s*. However, more work is needed.

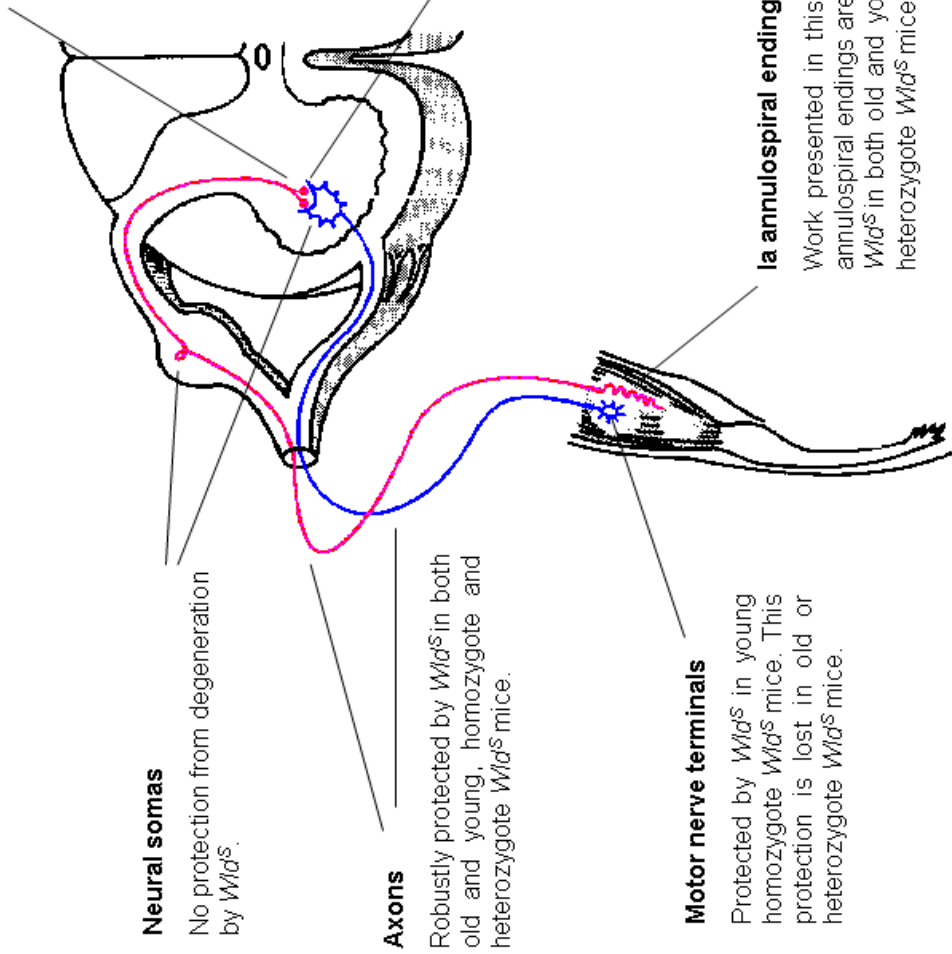


Figure D.1 Diagram of *Wld^S* protection of the different compartments of a neurone

The diagram shows the different neuronal compartments displayed in the reflex arc (based on Schade and Ford, 1965). The text describes what is known about the *Wld^S* protection of each compartment

D.2 The compartmental theory of neurodegeneration revisited

The compartmental theory of neurodegeneration, proposed by Gillingwater and Ribchester (2001), suggests that the morphologically and functionally distinct compartments of a neurone may also have distinct programmes of self destruction, governed by different molecular players. Dozens of proteins have been shown to control death of the cell body, examples include the Bcl-2 family and the caspases. Wallerian degeneration of the axon is regulated by *Wld^S* and as yet unconfirmed additional molecules.

The fact that motor nerve terminals are able to degenerate in the presence of both *Bcl-2* upregulation and *Wld^S* expression, as occurs in heterozygote and aged *Wld^S* mice, suggests a third putative neurodegenerative compartment; the axon terminal.

My research has added support for the axon terminal as a separate neuronal compartment. Although sensory nerve terminals investigated in this thesis were protected to an equivalent degree in old and heterozygote mice, the information from pattern of degeneration suggests they do degenerate by a separate mechanism to the axons in these animals. Further, I have proposed that dendrites also have a distinct mechanism for degeneration and the observations I have made hint that they are not protected by *Wld^S* despite their common neuritic origins.

D.3 Origins of compartmental degeneration of neurones

It would be evolutionarily beneficial for a neurone to have a molecularly distinct process by which to eliminate dendrites, axons or synapses only. There are many situations during the life time of an organism, in which it might be necessary to lose these neuronal compartments; however, it would be a disaster for the organism if the same mechanisms that lead to clearing away damaged axons could also lead to degeneration of the neuronal cell bodies. Maintenance of mature neurones is a priority as most neurones are postmitotic and, with the exception of olfactory

neurones and some hippocampal neurones (Taupin and Gage, 2002), neuronal cell bodies are generally not able to be replaced from any resident or dormant population ie: once lost, a neurone is lost forever.

D.3.1 As a mechanism for development

During development of the nervous system, there are many more neural connections created than exist in the mature animal. In order to establish this mature pattern of precise connectivity, the initial axonal connections must be pruned through retraction or degeneration.

For example, in an adult, one muscle fibre is innervated by an axon from a single motor neurone. However, during development multiple motor neurones make connections with single muscle fibres. The axon terminals compete at the neuromuscular junction on the muscle fibre and eventually all will retract except one. This process of synapse elimination is thought to occur throughout the nervous system.

During synapse elimination, axon terminals retract, however larger scale pruning of entire axons also occurs during brain development. In the CNS, growing axons typically extend well past their target, axon collaterals then form and some of these innervate the target region. Inappropriate collaterals and the length of axon which overshoot the target must subsequently be eliminated. The neural cell soma, however, remains intact (Luo and O' Leary, 2005).

D.3.2 Remodelling of connections throughout life

It is now well-established that post-synaptic structures fluctuate throughout life (Alvarez et al, 2007). Dendritic spines are constantly remodelled and although the dendritic arbour of most neurones is remarkably stable in the mature brain, there is a degree of remodelling which varies according to cell type (Alvarez et al, 2007). This has been suggested as a mechanism that might encode experience in the brain; by altering connectivity in neuronal circuits.

There is also increasing evidence that there is some structural rearrangement of pre-synaptic structures. De Paola et al report that subpopulations of presynaptic terminals appeared or disappeared over a few days in a study using long-term organotypic hippocampal slice cultures, and that the extent of this turnover is enhanced by synaptic activity (De Paola et al., 2003). More recent studies have found evidence for these presynaptic terminal rearrangements in vivo (De Paola et al., 2006; Stettler et al., 2006). The most recent of these studies is in functionally important presynaptic complexes formed by mossy fibre neurones in CA3 of the hippocampus. Galimberti et al, demonstrated that these pre-synaptic structures undergo rearrangement which occurs throughout the life of the animal and is influenced by experience (Galimberti et al, 2006). If it is necessary during adult life to structurally rearrange synapses, it is very important that the same molecules don't lead to a shutdown of the whole cell.

D.3.3 Response to stressors

In the PNS after injury it makes sense to remove any disconnected or partially disconnected and damaged axons, to provide uncluttered avenues for new axons to sprout and grow to reinnervate their targets and restore functionality.

As mentioned in chapter 3 it seems that dendritic retraction may occur in response to stressors as corticosteroids, toxic stress such as alcoholism and viral infection all have this effect. This dendritic retraction may occur in order to remove a neurone from a circuit when it is infected or incapable of normal function. It may also be to conserve resources, as the large membrane associated with a full arbour of dendrites requires a large amount of metabolic activity to maintain.

It seems possible that axon loss is also a protective response of a neurone that is somehow compromised. Maintenance of an axon may be beyond the capacity of a neurone in the presence of some toxic/ hormonal stressor but without the axon the cell might be able to survive. Once better conditions exist, at least in the peripheral nervous system, the neurone might then sprout a new axon.

D.4 Limitations of the compartmental theory of neurodegeneration

D.4.1 Polarity of the neurone

The idea of morphologically, and functionally distinct compartments is not universally applicable within the mammalian nervous system. The standard neurone with a dendritic arbour whose function is to receive and integrate information, a neuronal soma where the nucleus is located and protein manufactured, and one axon that carries information contained in action potentials to its target is certainly an oversimplification.

Functionally, it has been demonstrated that roles are not necessarily unique to one neuronal compartment. For example, there is now evidence for protein synthesis in all compartments of the neurone, although this is still contested in the case of the axon (Court and Alvarez, 2005). There are also examples of axo-axonic synapses which give axons the role of receiving input from other neurones. Also sensory axons receive signals which they then transmit towards the cell body.

Morphologically, there are some neurones that lack axons altogether such as the granule cells of the olfactory bulb. Some neurones may have more than one axon such as certain classes of amacrine cell, which have as many as six axons arising from different dendrites. Others, including certain classes of horizontal and amacrine cells, have shapes so unusual that it is uncertain if the terms axons and dendrites apply at all (Craig and Banker, 1994).

Even with classical polar compartmentalised neurones, there may be the potential within compartments to take on the morphology or function of another compartment. After injury some neurones can regrow their axon from a dendrite. Rose et al, 2001 have shown that unusual processes originating from distal dendrites of cat neck motor neurones after axotomy have ultrastructural and molecular features that are typically attributed to axons (Rose et al, 2001). The same group went on to demonstrate this in other classes of neurone (Fenrich et al, 2007).

Given the blurring between distinct functions and morphologies of separate compartments in some neural cell types, it is possible that the idea of compartmentalised degeneration does not apply universally in the nervous system.

D.4.2 Initiation may not occur in the same place as the destruction

The first sign of degeneration that is seen in various neurodegenerative diseases, or after injury, may not be a reliable indication of which part of the neurone is initiating the degeneration.

I showed in chapter one that axotomy in *Wld^S* mice caused degeneration of the sensory axon terminal, which occurred before the distal axon degenerated. This suggests that injury in the axon is able to cause degeneration in the terminal. Some molecular signalling must be involved in order for the spatially distinct nerve terminal to begin to undergo degeneration before the part of the axon directly connected to it.

There are further examples in the literature of the first sign of degeneration being spatially distinct from where it may have been initiated.

The induction of neuronal soma degeneration can be caused by excitotoxicity, ie: a signal received at the dendritic membrane. Similarly redundant neurones are lost during development seemingly by a “use it or lose it” mechanism. This suggests some signal generated at the post synaptic membrane must promote survival of the soma.

If it were true that dendritic retraction or axonal loss was a defensive response of a cell experiencing some kind of challenge, for example viral infection. It would seem likely that the cell soma was initiating the degeneration of its processes.

Perhaps, the signal might even be initiated in a different cell altogether. During synapse elimination it may be the post synaptic cell which signals to the innervating axon, either keeping it alive or bringing about its destruction.

So, just because the first sign of disease is degeneration of a particular compartment doesn't mean that there isn't something wrong elsewhere in the cell. If it were true that retraction or degeneration of processes was a protective mechanism for a neurone, then preventing the cell from shedding these processes might actually speed its demise.

D.4.3 Multiple forms of cell death

Up until now I have described Wallerian degeneration as an analogous form of self destruction for the axon as apoptosis is for the cell body. Though they appear to be molecularly distinct, there are similarities in principle. Firstly, each results in destruction of the neural compartment in response to a variety of insults, secondly each can be genetically manipulated.

However, the exclusive position of apoptosis as the genetically regulated form of cell death has been challenged. It appears there are several forms of genetically regulated programmed cell death (PCD), known as apoptotic-like PCD, necrotic-like PCD and autophagy, which differ to greater and lesser extents both morphologically and molecularly. Indeed even necrosis, which used to be thought of as the opposite of apoptosis- a disordered, unregulated 'accidental' destruction of a cell, which results in release of the contents of the cell into the cellular environment appears to sometimes occur after initiation of traditional apoptotic machinery and there are mechanisms which regulate this (Hetz et al, 2005). It seems likely that there are many ways for a cell to die, and much genetic machinery that could be involved. The precise form of cell death undertaken by a cell is likely to be related to the kind of event that has initiated the cell death, the type and maturity of the cell and the environment it is in.

Similarly, it seems likely that there is more than one form of axonal death and possibly nerve terminal degeneration and dendrite degeneration also. It could also be contemplated that some forms of axonal/dendritic or terminal degeneration are

specific to particular classes of neurone. This may explain selective degeneration in certain types of neurones in various neurodegenerative disease.

Evidence for more than one type of axonal death comes from Beirowski et al (2005). The evidence presented in this paper suggests that degeneration of the axon in *Wld^S* animals occurs by a qualitatively different pattern than Wallerian degeneration in wild type animals. Whether there are any situations in wild type mice when slow Wallerian degeneration occurs has not been shown.

However, another alternative form of axonal degeneration maybe responsible for axon elimination discussed in D.3. It seems unlikely that the branch elimination occurring either during development or throughout life, possibly to encode experience, is by a Wallerian degeneration mechanism or one might expect it to be abnormal in *Wld^S* mice and their behaviour to be affected, but they appear to be normal. However, the percentage of de novo branch formation and elimination in CA3 over 1 month of imaging was only 4% and elongations and retractions were balanced to result in a constant average branch length (Galimberti et al, 2006). This suggests that mice with a deficit in this area might appear normal without tests for subtle differences in behaviour. Also *Wld^S* mice are often killed young and live in un-enriched environments.

It seems likely that there are at least four, possibly related, mechanisms of axon loss; developmental axon loss, Wallerian degeneration after injury, Wallerian-like degeneration which occurs in disease and toxic challenge situations, and a slow axonal degeneration which occurs in the absence of Wallerian degeneration. These may or may not turn out to have molecular pathways in common.

Similarly, there is evidence for several mechanisms of axon terminal loss. The synchronous degeneration of terminals observed at wild-type denervated NMJs (Miledi and Slater, 1968) contrasts with the degeneration of *Wld^S* motor nerve terminals after axotomy. These withdraw in a piecemeal fashion ie: nerve terminals remove themselves from the endplate, bouton by bouton, until they form a bulbous

swelling at the distal end of the axon, detached from the endplate (Gillingwater and Ribchester, 2001; Ribchester et al, 1995). Similarly the degeneration of sensory terminals in *Wld^S* mice occurs progressively and retrogradely, with Ia annulospiral endings on muscle spindles degenerating before their parent axon. In contrast the degeneration of wild type endings is abrupt after a latent phase.

The ultrastructure of withdrawing neuromuscular terminals in axotomised *Wld^S* neurones is also distinctive (Ribchester et al, 1995; Gillingwater et al, 2000). However, the ultrastructure of degenerating nerve terminals in the CNS after cortical ablation appeared to be morphologically similar to that which occurs in wild type animals (Gillingwater et al, 2006b).

Nerve terminals that withdraw during synapse elimination look similar to the degenerating motor nerve terminals seen in *Wld^S* mice (Gillingwater and Ribchester, 2001). Developmental synapse elimination takes place at a normal rate in *Wld^S* mice, although axotomy in the neonate still delays degeneration (Parson et al, 1997).

The evidence comes together to suggest there are several mechanisms of axon terminal degeneration which may or may not be mechanistically related. One occurs in wild type animals in response to injury, another is unmasked in *Wld^S* animals and this may differ between CNS synapses and peripheral axon terminals. There is also a developmental mechanism which may share some features with the degeneration of nerve terminals that occurs in *Wld^S* mice after axotomy. Nerve terminal degeneration that occurs in dying back disorders may also share some features with the degeneration of nerve terminals that occurs in *Wld^S* mice after axotomy

Given the evidence for several distinct processes even within the compartmentalised model of neurodegeneration, it must be recognised that findings about degeneration of a particular compartment can't necessarily be generalised to other neuronal types or even the same neuronal type in different conditions.

D.5 Conclusion

There is increasing evidence supporting the compartmental theory of neurodegeneration. It is an evolutionarily viable theory with reasons for separate degenerative mechanisms for separate neuronal compartment that might explain their existence. It is important to understand the limits of this theory and that both simplifications and generalisations are examined.

This thesis has extended the information on this theory, using the *Wld^S* mouse as a tool and left open questions to be answered by future study.

Studies on PCD have demonstrated that it is difficult to obtain long lasting sustained neuroprotection in the face of chronic extrinsic stressor (Wang et al, 2002a). There is evidence that sustained inhibition of cell death in the continual presence of a chronic stressor can unmask alternative pathways of death (Iyirhiaro et al, 2008). Indeed, an alternative pathway of axonal and axon terminal degeneration is unmasked in *Wld^S* mice. This suggests that whatever compounds may be developed in the future to delay degeneration of different neuronal compartments, tackling whatever causes the harm to the neurone must be investigated.

However, delaying degeneration of the specific neuronal compartments involved in disease pathology has the potential to delay onset of disability and prolong lifespan. It may also give clinicians the time to locate the cause of neurodegeneration and treat this before the neural functions are lost.

It would be of great benefit in the study of neurodegenerative disease to make investigation into the degeneration of all compartments of the neurone central to the development of future therapies for these diseases.

A. Appendix

A.1 Introduction

Given the profound effect of the *Wld^S* mutation on the response of neurones to injury, it is remarkable that there is apparently very little else to say about the constitutive phenotype of the mutant mice. The mice have no overt behavioural abnormality which is why the axon-protection phenotype may have remained undiscovered until Brown and Perry's serendipitous discovery in 1989. However, two phenotypic characteristics reported to be specific to *Wld^S* mice, have been examined further in the appendix of this thesis. These are decreased pain sensitivity (Zhong et al, 1999) and increased hair loss, which has not been published, but can be seen regularly in laboratory mice.

A.2 Hair loss

Wld^S mice have bald patches especially around their eyes and nose but also behind their ears and on their backs. Having noticed what seemed to be decreased hair growth over the wound after by sciatic nerve transection in *Wld^S* mice compared to WT mice, I wondered if the loss of hair that has been noticed is due to a slower growth of hair in *Wld^S* mice.

A.2.1 Shaving

6 C57/B16 and 6 *Wld^S* mice were used. These were coded by ear punch and the genotype of the mice was kept secret until the experiment was over. Mice were anaesthetised by halothane inhalation and an area on the back of each of mouse was shaved with an approximate area of 2cm x 1.5 cm. Mice were photographed regularly up to 38 days after shaving. After 38 days when the majority of mice had a full covering of fur, the experiment was terminated and the identities of the mice revealed.

A.2.2 No discernable difference in the rate of hair growth between WT and *Wld^S* animals

From qualitative examination of the photos, there was no discernable difference in the amount of hair regrowth in WT and *Wld^S* mice at any day after shaving. Figure A.1 shows 2 mice from each group 26 days after shaving. This demonstrates that there is equivalent variation in both genotypes in hair regrowth.

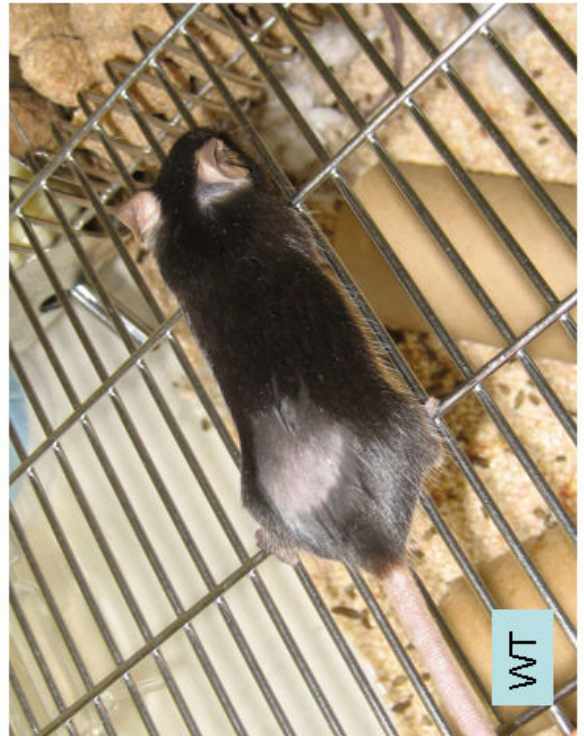


Figure A.1 No discernable difference in hair growth in *Wld^S* mice

Pictures to show 2 representative mice of each genotype 26 days after shaving. There was no discernable difference between the hair growth of *Wld^S* mice and WT mice

A.3 Pain sensitivity

Zhong et al used a temperature sensitivity assay to look at sensory impairments in IL-6 knock out mice before and after nerve crush. *Wld^S* mice were used as a comparison for IL-6 knock out mice in this experiment, as another mouse with reduced sensory regeneration after crush. Sensitivity to heat was tested by letting the paws of mice touch an aluminum hot plate set to 60°C for no more than 20 sec. Before the nerve was crushed, the mean paw withdrawal time of wild type mice was just over 4 seconds, whereas *Wld^S* mice took more than double that time to react to the same heat exposure with a mean paw withdrawal time of nearly 11 seconds (Zhong et al, 1999).

Nociceptors are bare nerve endings which are activated by stimuli which have the potential to cause tissue damage. These include strong mechanical stimulation, extremes of temperature and oxygen deprivation. In addition, chemicals released from damaged cells at the site of injury eg: ATP, proteases, and K⁺ ions can also be involved in pain. Elevation of extracellular K⁺ directly depolarises nociceptors, ATP causes depolarisation directly by binding to ATP-gated ion channels and proteases can break down an abundant extracellular peptide called kininogen into bradykinin which binds to receptor molecules and activates ionic conductance. Mast cells may also cause nociception, when activated by exposure to foreign substances eg: bee venom. They release histamine which binds to receptors on nociceptors to cause depolarisation (Bear et al, 2001).

It occurred to me that the chemical signals able to cause pain transduction may also be produced during Wallerian degeneration as this is a type of cellular injury. For this reason, it seems plausible that a mouse with inhibited Wallerian degeneration might have impaired pain transduction and therefore reduced sensitivity to pain. In addition, having demonstrated that the hair loss in *Wld^S* animals is not due to slower hair growth, another explanation could perhaps be overgrooming perhaps because the

recipient of the grooming failed to react to pain cues that might normally result in the termination of the activity before hair was lost.

A.3.1 P.A.M

I was unable to try the heat sensitivity assay, but was able to borrow equipment from another lab in the building who work on rheumatoid arthritis ie: joint pain, called the Pressure Application Measurement (P.A.M).

To use the P.A.M the mouse is scruffed and pressure is applied to the mouse knee joint with the experimenters finger and thumb through a transducer worn on the thumb. When the mouse is uncomfortable it withdraws its foot and the P.A.M gives a reading of the maximum pressure applied.

I did two experiments each with 12 mice, 6 *Wld^S* mice and 6 C57/Bl6 WT mice. These were coded by ear punch and their genotypes kept secret until the experiment was finished. In each trial I repeated the P.A.M three times. For each mouse I did 6 trials over the course of a few weeks.

A.3.2 Individual mice appear to have different sensitivities to pressure

In the first experiment each mouse the average standard deviation between trials in an individual mouse was much smaller than the standard deviation of all trials in all mice. This suggests that the P.A.M is able to detect individual differences in pressure sensitivity.

Experiment 1: Individual data

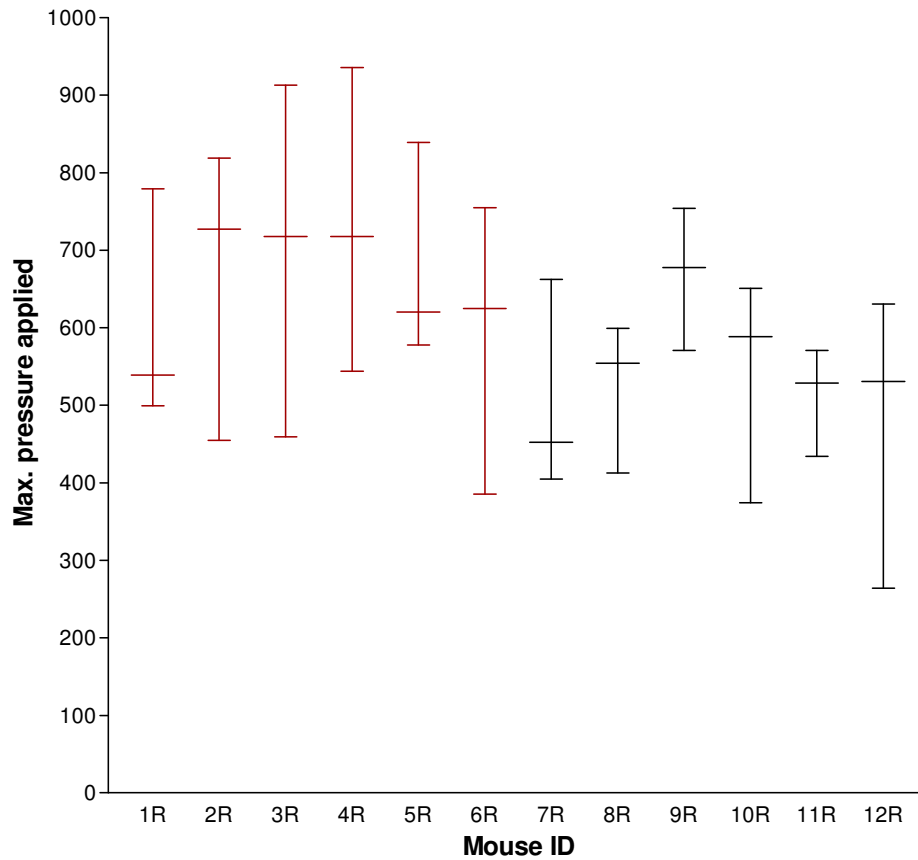


Figure A.2 Data from individual mice examined in experiment one

Data from the individual mice examined in experiment 1, shows that the P.A.M device can detect difference in individuals sensitivity to pressure. The range and standard deviations for each mouse over x trials is lower than the overall range and standard deviation of all trials.

A.3.3 No difference in pain sensitivities in *Wld^S* mice

The first twelve mice gave a highly significant difference between groups with $p=0.003$ and *Wld^S* mice appearing to have more sensitivity to applied pressure [Figure A.3]. This was surprising given that the Zhong paper reported a decrease in sensitivity to heat in *Wld^S* mice (Zhong et al, 1999). However, a repeat experiment gave no significant difference between groups and a non-significant difference in the opposite direction [Figure A.4].

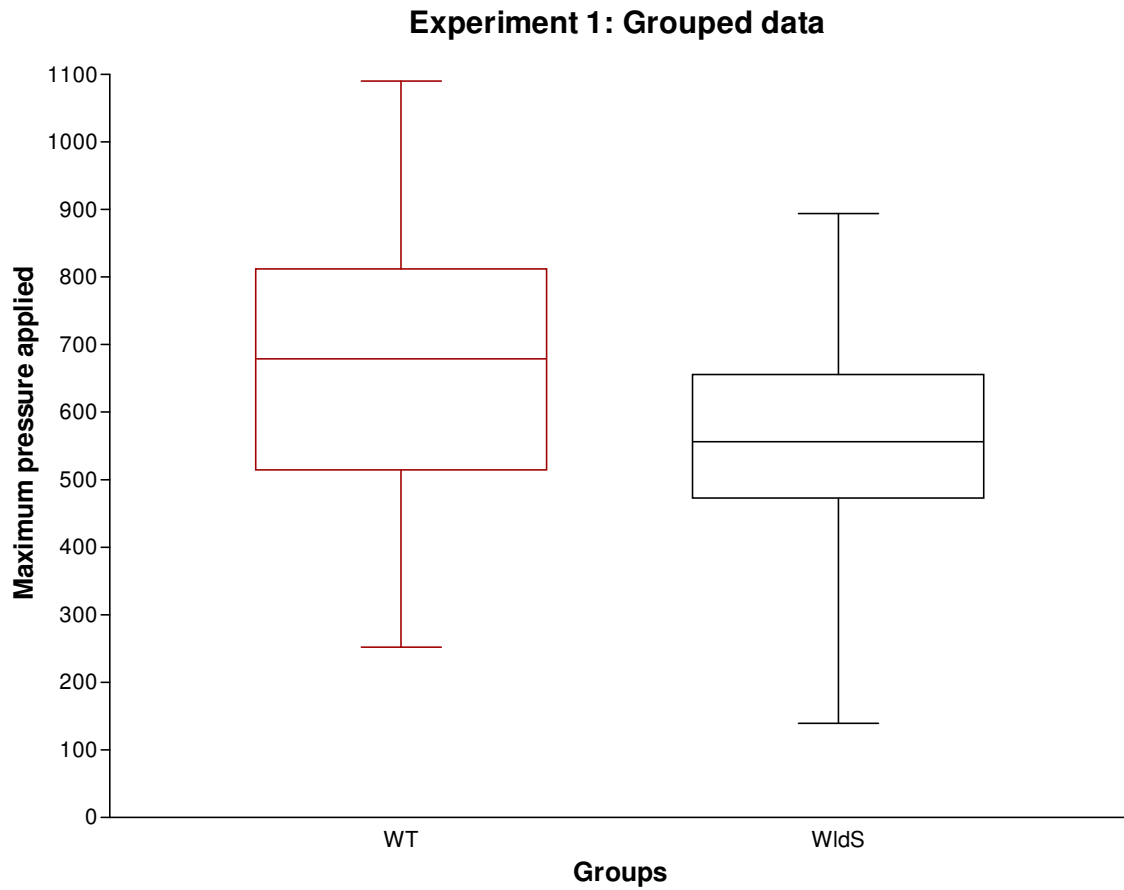


Figure A.3 Grouped data demonstrating a difference in pain reactions in *Wld^S* and WT mice

Grouped data from the twelve mice examined in experiment 1, shows a difference in the maximum pressure needed to cause a withdrawal of the foot in WT and *Wld^S* mice, $p=0.003$

Experiment 2: Group data

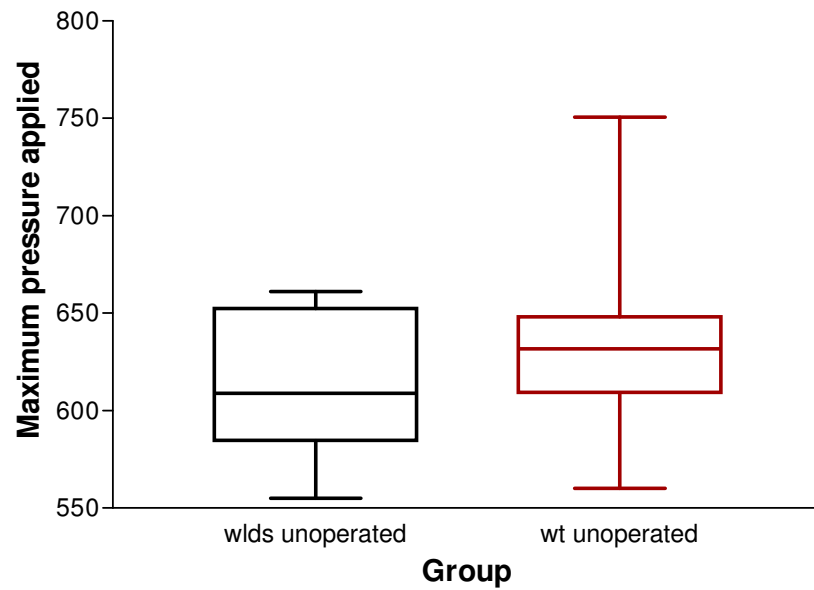


Figure A.4 Grouped data demonstrating no difference in pain reactions in *Wld^S* and WT mice

Grouped data from the twelve mice examined in experiment 2, shows no difference in the maximum pressure needed to cause a withdrawal of the foot in WT and *Wld^S* mice, $p=0.2925$. In addition there is a non-significant trend in the opposite direction from that in experiment 1.

A.4 Discussion

From the results presented about it appears that slow hair growth is not the reason for *Wld^S* alopecia. It also appears that there is no consistent difference between the sensitivity to pressure in *Wld^S* mice as compared to WT mice.

Further experiments that might be worth considering, include investigation of other possible reasons for the hair loss in older *Wld^S* animals. Perhaps this is a social problem due to over-grooming. It would also be interesting to repeat Zhong et al's (1999) heat sensitivity test, particularly as this was only done as a control in their experiment and was not examined.

The lack of obvious complications to living with the *Wld^S* phenotype further increases the interest in this gene as a potential molecular basis for therapeutic intervention in diseases in which axonal degeneration is important.

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