SYNAPSE WITHDRAWAL AT THE NEUROMUSCULAR JUNCTION IN A MOUSE WITH SLOW WALLERIAN DEGENERATION (WLD^S)

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DECLARATION

In accordance with postgraduate degree regulation 3.8.7 of the University of Edinburgh, I declare that the work described in this thesis is my own, except where otherwise indicated, and has not been submitted for any previous degree. I also acknowledge all assistance given to me during the course of these studies.

Richard John Mattison

ABSTRACT

Motor nerve injury results in a sequence of events known as Wallerian degeneration, which takes 1 - 2 days in rodents. Wallerian degeneration is significantly delayed in the naturally occurring C57BL/Wld^s mutant mouse. The ability to produce compound action potentials and the degradation of axonal material are delayed for up to 3 weeks following nerve injury.

This thesis aims to examine the processes that occur after nerve section at neuromuscular junctions of mutant Wld^s and normal mice. Functional morphological measurement of actively recycling synaptic vesicles at wild type nerve terminals using the vital dye FM1-43 indicated that the loss of the ability to recycle synaptic vesicles occurred within 24 hours, coinciding with disruption of neurofilament and SV2 proteins (stained using immunocytochemical methods). In contrast, the degeneration of nerve terminals in Wld^s mice following nerve injury was significantly delayed, and once initiated was further slowed, degeneration occurring between 3 and 10 days after axotomy. The morphological appearance of Wld^s nerve terminals after lesion differed greatly from that of degenerating wild type terminals: Wld^s terminals appeared to be retracting from the endplate region rather than simply degenerating. Electrophysiological recordings from degenerating junctions demonstrate that the delay of degeneration/withdrawal from nerve terminals of Wld^s mice is accompanied by a coincident loss of synaptic function. These data indicate that, as in normal mice, the first structural and functional changes to take place after nerve injury in mutant Wld^s mice is the disruption of nerve terminals at the NMJ. This thesis proposes that nerve terminal retraction in the mutant mouse is caused by a withdrawal mechanism similar to that observed during synapse elimination, a naturally occurring activity dependant developmental phenomenon, rather than by classical Wallerian degeneration as previously assumed. To test this hypothesis, botulinum toxin (which abolishes neurotransmitter release) was administered to the nerve terminal region at the same time as the nerves were lesioned. The results indicate that nerve terminal withdrawal still occurs, but that it is reduced and further delayed in muscles treated with botulinum toxin by 1.14 days, in comparison with denervated alone Wld^s muscles.

Additional studies assessed the role of Schwann cells in nerve terminal withdrawal. Terminal Schwann cells that overly the NMJ sprout in response to denervation, and have been shown to guide axonal processes back to endplates during reinnervation. Experiments were conducted to evaluate the Schwann cell reaction to the absence of degeneration after nerve section, as observed during the first few days after axotomy in the Wld^s mouse. Schwann cell sprouts were present at various endplates in the mutant mouse after nerve injury. However, when compared with denervated wild-type controls there were fewer Schwann cell sprouts in the Wld^s and sprouting in general appeared less extensive. Thus, the presence of an intact nerve terminal and spontaneous activity are sufficient to delay, but not abolish, Schwann cell sprouting in the Wld^s mouse after nerve lesion.

In sum, these studies provide an ideal opportunity for the further investigation of the molecular and cellular mechanisms of synapse withdrawal.

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ABBREVIATIONS AND NOTATIONS

BDNF	brain-derived neurotrophic factor
BoTX	botulinum toxin
CGRP	calcitonin gene-related peptide
CNTF	ciliary neurotrophic factor
EPP	endplate potential
FDB	flexor digitorum brevis muscle
FITC	fluorescein isothiocyanate
GAP-43	glial associated protein 43
GDC	granular disintegration of the cytoplasm
GFAP	glial fibrillary acidic protein
IGF	insulin-like growth factor
JE	monocyte chemoattractant protein-1
LIF	leukaemia inhibitory factor
MEPP	miniature endplate potential
mRNA	messenger ribonucleic acid
N-CAM	neural cell adhesion molecule
NF	neurofilament 2H3 protein
NGF	nerve growth factor
NMJ	neuromuscular junction
NT-4	neurotrophic factor 4
PBS	phosphate buffered saline
SNAP-25	synaptosome-associated protein of 25kDa
SV2	synaptic vesicle protein 2
TA	transversus abdominus muscle
TRITC	tetramethylrhodamine B isothiocyanate
TTX	tetrodotoxin

For my parents, Sid and Lesley Ann Mattison

CHAPTER 1

INTRODUCTION.

CHAPTER 1: INTRODUCTION.

The ancient Greek philosopher, Hippocrates, provided the first written description of the peripheral nervous system as early as the 4th Century B.C., and later Herophilus identified nerve tracts, tracing them to the spinal cord. In subsequent centuries, studies of the peripheral nervous system provided insights into the workings of nerves and nerve cells, but most scientists assumed that the nervous system, once developed, was 'hardwired' and unalterable and doubted that nerve healing was possible. Thus, without sufficient understanding of the anatomy, physiology and the regenerative capacity of the peripheral nervous system surgeons were unable to deal with nerve injuries and their subsequent repair. A plethora of studies in the past 150 years have considerably broadened our knowledge in this area leading to the development of various reconstructive strategies in treating nerve injury. One of the most fascinating elements of the both the peripheral and central nervous system remains the ability of axons and their endings, synapses, to respond to different stimuli with the remodelling of their anatomical and physiological characteristics. Adult peripheral axons are able to establish, withdraw, and re-establish synapses, grow and regenerate in response to damage, and otherwise continually transform their structure and function in response to different stimuli and to suit different environments. This thesis will attempt to address certain aspects of the remodelling of peripheral synapses with the use of a naturally occurring mutant, the C57BL/Wld^{s} mouse, in which the phenomenon of Wallerian degeneration is profoundly affected.

1.1 WALLERIAN DEGENERATION IN THE PERIPHERAL NERVOUS SYSTEM

Injury to axons results in an active process known as Wallerian degeneration. In the peripheral nervous system focal damage to a nerve trunk triggers Wallerian degeneration in the section of nerve distal to the site of injury. The nerve fibres proximal to the injured area, which remain in continuity with their cell bodies, not only survive but are also capable of regeneration. These principles were first documented 150 years ago by an English physician named Waller, and provided seminal insights into the organisation and function of nerves and nerve cells.

1.1.1 Historical perspective

It was once held that cells of the nervous system were intimately linked with one another, and it was not until the late 19th Century that the advent of appropriate staining techniques, pioneered by Camillo Golgi (1906) and advanced by Santiago Ramon y Cajal (1905), firmly established the concept of the cell as an individual unit. Thus, early neuroscientists considered the nervous system to be a syncytium of cells in which the cytoplasm of individual neurons are united in an intricate reticulum, and that separation of a section of axon from its cell body would not necessarily lead to degeneration of that axon. Indeed, the experiments of Burdach (1837) illustrate this point: he placed a ligature on the sciatic nerve of the frog and observed no alteration of nerve fibres either above or below the constriction. However, it is now widely accepted that severed distal axons of many types of neurons in mammals and some lower vertebrates completely degenerate within a few days of axotomy. In two classic reports Augustus Waller (1850, 1852) was the first to conclusively define the phenomenon of secondary axonal degeneration, now know as Wallerian degeneration. Waller demonstrated that a lesion to the frog hypoglossal nerve, which supplies the tongue, resulted in a loss of both muscular contraction and sensation by three days after nerve section. Histological examination of the nerve proximal to the lesion site, a technically challenging task at that time, revealed a coagulated medulla which eventually separated into pieces of various sizes which he termed *ovoid bodies*. The axons at this time appeared discontinuous and granulated. Waller ascribed these

observations to a degenerative phenomenon, hypothesising that the damaged fibres of the distal stump were of no use in the process of regeneration. He proposed that regeneration resulted from the production of new nerve fibres from the proximal, or central, stump which remained connected with their cell bodies in the spinal cord. It was their continuity and nutritive support that allowed the new fibres to rebuild the distal segment, and restore function. According to Waller, the death of the axons of the distal, or peripheral, stump was due to the separation of these axons from their cell bodies, or *trophic centres*.

Ranvier made the first detailed descriptions of Wallerian degeneration in 1871. He observed a contraction of myelin as early as two days after nerve section, which subsequently transformed into elongated drop shaped entities (ovoid bodies). The axons themselves became pale and granular, and fragmented into irregular spiral segments. He supposed that the destruction of this 'conducting filament' brought about both muscle paralysis and the transformation in the cells of Schwann: Schwann cell nuclei became enlarged and divided, surrounding themselves with a thick protoplasm. By 15 to 20 days all remnants of the axons had completely disappeared. At this time Ranvier noted the appearance of migratory leucocytes, whose function it was to collect the detritus of the old nerve. By 20 to 30 days Schwann cell nuclei had diminished in their number, abandoning the central axial zone for a more marginal position: thus forming a space to receive the newly generated axons. Ranvier called this series of events the 'functional degeneration of the peripheral stump'. Büngner (1891) carefully studied the mitotic phenomena of Schwann cells and observed that they joined together tip to tip to form cordons or longitudinal bands that fill in the old perimedullar sheath. He called these bands "Zellbander", and they have since become known as the *Bands of Büngner*. These conglomerations of Schwann cells are thought to provide an ideal growth substrate down which the regenerating axons can proceed.

The technical constraints of that era meant that it was often difficult to be sure of clearly staining degenerating axonal processes. Thus, the observations of Waller, Ranvier, and Büngner were by no means necessarily in full agreement with those of their colleagues or each other, and it was not until the elegant studies of Ramon y Cajal (1909, 1928) and his contemporaries that they were finally corroborated.

Ramon y Cajal, using a silver staining technique (reduced silver nitrate), confirmed many of the observations and theories of Waller and Ranvier, and conclusively demonstrated that nerve degeneration and regeneration are intimately linked. He also drew a distinction between the degeneration at the immediate site of the injury, and that which takes places further down the axon. Wallerian or *trophic degeneration*, he surmised, takes place in the peripheral stump when the stimulus from the nerve cell body or trophic centre is interrupted. *Traumatic degeneration* arises in both the central and distal stumps close to the vicinity of the nerve damage, and is a result of acute trauma and subsequent phlogosis. Therefore, Ramon y Cajal was of the opinion that in reality the distal segment presents two different types of degeneration: traumatic and Wallerian degeneration.

The work of this century has clarified and detailed the process of Wallerian degeneration, but the fundamental physiological mechanism remains to be validated. The initial studies of Wallerian degeneration at the start of this century were primarily morphological in nature and described the destruction of both axon and myelin sheath, division of Schwann cells, and infiltration of macrophages from the circulatory system. Subsequent investigations utilised electrophysiological techniques to demonstrate that one of the first changes is the failure of neuromuscular transmission and the loss of the ability to transmit a compound action potential along the distal stump, which occurs 3 days after denervation in mice (Lissak *et al.*, 1939). The advent of electron microscopy in the 1950s also yielded further insights, elucidating the disintegration of the axoplasm and its constituent components, and degradation and phagocytosis of myelin debris. Modern studies use molecular, immunocytochemical and a multitude of other techniques to examine molecular and cellular events that take place during Wallerian degeneration. These studies will be further addressed in context, in the subsequent sections.

1.1.2 Description of process

Wallerian degeneration is a term that describes a distinct series of biochemical and morphological events that occur following the separation of an axon from its cell body (see Figure 1.1). Physical damage to nerves produces a variety of degrees of injury. The effect of focal pressure on a single nerve fibre depends entirely on the severity of the pressure applied. The peripheral nerve is surrounded by longitudinally orientated collagen fibrils of substantial tensile strength, as well as basal lamina and the myelin sheath produced by Schwann cells. In this way, the delicate construction of a single axon is physically strengthened by its surroundings. Modest pressure applied briefly and focally to a nerve will result in the compression of cytoskeletal elements and a reduction in the calibre of the axon as a whole. This is a rapidly reversible process and does not result in degeneration of the distal portion of the axon. In some cases, focal pressure can distort the myelin overlying the nodes of Ranvier to produce a conduction block without causing the degeneration of the axon: this is known as *neurapraxia* (Seddon, 1943). Greater pressure applied to the nerve results in the displacement of both axonal constituents and myelin from the crush site, whilst the surrounding basal lamina and collagen remain in continuity. This type of injury causes the Wallerian degeneration of axons distal to the damaged region, and is often known as a crush injury. Regeneration after nerve damage of this type occurs more often because the extracellular matrix, comprising the basal lamina, remains intact and is an ideal substrate for axonal elongation - as such it provides a preferred route for regenerating axons. Transection of the entire nerve, including its connective tissue components and basal lamina as well as the axons themselves, invariably results in Wallerian degeneration. Nerve trauma of this kind often produces less favourable conditions for regeneration; peripheral nerves are under substantial tensile stress and after nerve section tend to separate a considerable distance. This gulf has to be crossed by the regenerating fibres of the proximal stump before reaching the facilitating growth substrate of the distal stump. Ramon y Cajal (1928) noted that, in comparison with crush injuries, axonal sprouts often stray over considerable distances and axon pathways are not as well defined after an injury of this type. Thus, regeneration and complete restoration of function occurs less

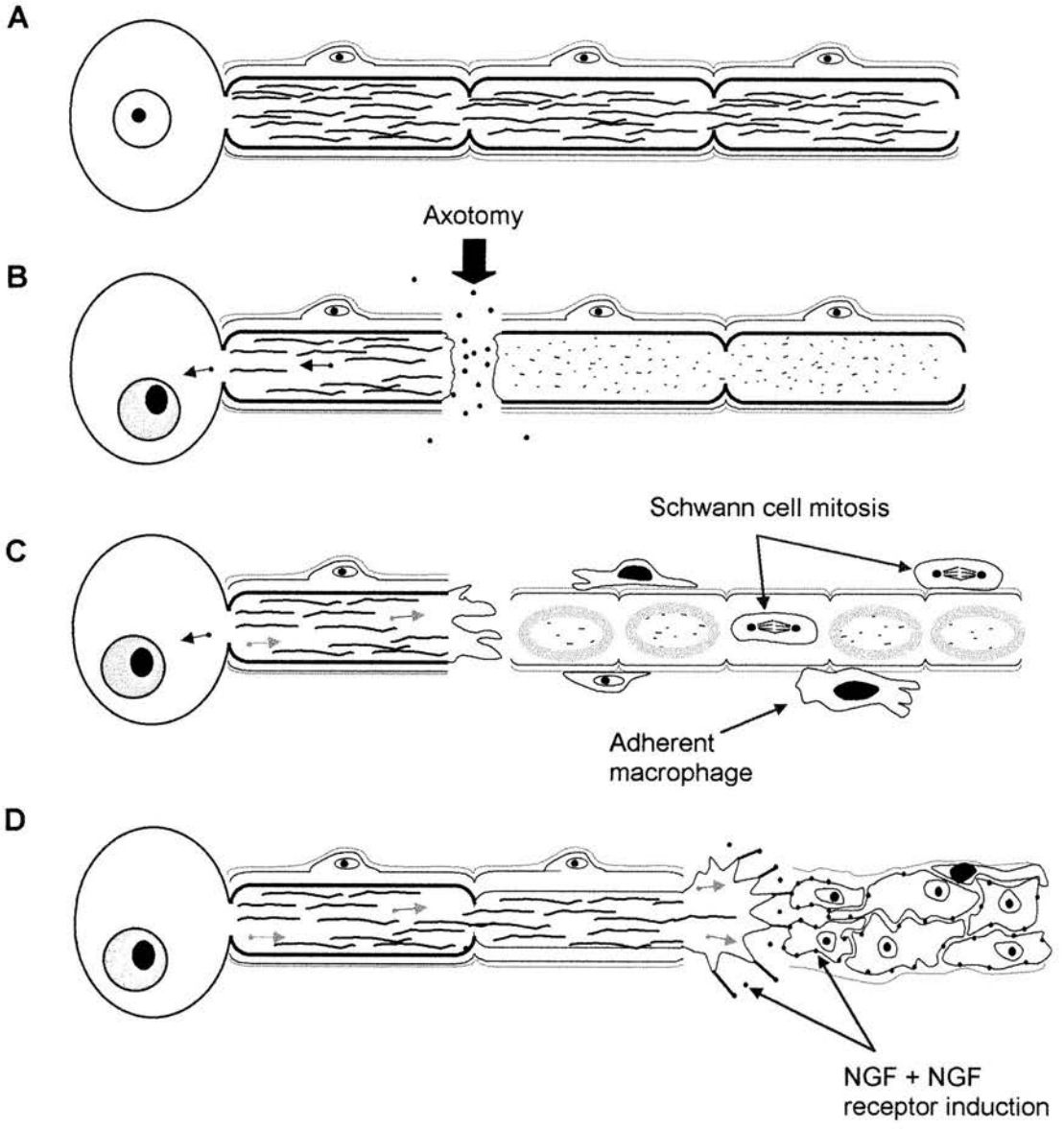
FIGURE 1: SCHEMATIC DIAGRAM OF THE CELLULAR AND MOLECULAR EVENTS OF WALLERIAN DEGENERATION.

A. Normal myelinated axon with intact axonal cytoskeleton. The axon is surrounded by myelin and associated with one Schwann cell per internode. The dotted line represents Schwann cell basal lamina. Note the dense distribution of neurofilament (NF), and the normal appearance of the cell body.

B. 24 hours after nerve injury. Nerve lesion creates a breach in axonal integrity, which results in the release of chemotactic factors at the lesion site. The ends of the distal and proximal axon rapidly reseal. The distal axon undergoes granular disintegration of the cytoplasm (GDC), whilst the proximal axon prepares for regeneration. Signals caused by the injury induce alterations in the cell body, that ultimately lead to changes in gene activation and protein expression; these prime the proximal axon for regeneration.

C. 48 hours after axotomy. The myelin sheath segments into structures known as 'early ovoids', which encapsulate the watery remnants of the axoplasm. Schwann cells undergo longitudinal division and begin to phagocytose myelin and axonal debris. Invading macrophages contribute to this process. The proximal axon sprouts and advances across the lesion site. The growth cone is supplied by newly synthesised proteins from the cell body.

D. From 7 to 14 days most myelin and axonal debris has been cleared and the division of Schwann cells form a longitudinal scaffold, called the 'bands of Büngner', which acts as a growth substrate to facilitate axonal elongation. Nerve growth factor (NGF) and the NGF receptor are upregulated and, upon contact with the growth cone, aid axon growth via receptor-mediated endocytosis.


A
Axotomy
B
Schwann cell mitosis
C
Adherent
macrophage
D
NGF + NGF
receptor induction

frequently after nerve trauma of this sort. This thesis will be confined to the discussion of Wallerian degeneration, without examining the phenomenon of neurapraxia.

After separation of the distal and proximal regions of the nerve, Wallerian degeneration takes place in the distal section whilst the proximal stump prepares for regeneration. Rupture of the axonal membrane results in the disruption of the ionic balance of the axonal cytoplasm (the axoplasm). At the site of injury, the axoplasm liquefies upon exposure to extracellular calcium (Hodgkin and Katz, 1949). This appears to be limited to the local region of damage - the axoplasm does not liquefy along the entire length of the axon, and is thus able to maintain structural integrity. A decreasing gradient of damage with increasing distance from the lesion site occurs, presumably influenced by cytosolic calcium buffering or sequestration/extrusion (Emery *et al.*, 1987; Gross and Higgins, 1987). Ionic gradients are re-established following the sealing of the axons, which occurs within 10-30 minutes. The re-sealing process has been shown to be dependent upon calcium ions activating phospholipase A2 (Schlaepfer and Bunge, 1973; Yawo and Kuno, 1983). Restoration of membrane integrity results in a return to an appropriate ionic equilibrium and the damaged axon is once more able to conduct compound action potentials by external stimulation (no intrinsic evoked activity occurs after the nerve has been severed or crushed).

Large swellings form on either side of the lesion site within 5 hours of nerve injury, formed by materials ferried along the axon by axonal transport and axoplasmic flow (Blumcke, Niedorf and Rode, 1966; Tomatsuri, Okajima and Ide, 1993). Mitochondria, vesicles and multivesicular bodies, and unidentified material accumulate adjacent to the site of injury in the sealed end of each axon segment. More pronounced swelling is apparent on the proximal side, where neurofilaments, microtubules and microfilaments recently synthesised by the cell body and destined for distribution along the length of the axon accumulate against the sealed membrane at the lesion site.

1.1.3 Spatio-temporal sequence of Wallerian degeneration

One of the first detectable events after nerve damage is the failure of neuromuscular transmission, in conjunction with the precocious degeneration of nerve terminals at the neuromuscular junction (Titeca, 1935; Lissak, Dempsey and Rosenbleuth, 1939; Birks, Katz, and Miledi, 1960; Salafsky and Jasinski, 1967; Miledi and Slater, 1970). Failure of neuromuscular transmission takes place after a period of quiescence, during which it is still possible to elicit a compound muscle action potential by stimulating the distal nerve. This is termed the *post-axotomal lag* or *latency period* and lasts 8 to 10 hours in the rat phrenic nerve (Miledi and Slater, 1970). During this time motor axons and their terminals appear functionally and morphologically normal. However, after this period nerve terminals begin to show signs of degeneration and neuromuscular transmission fails. This occurs from 8 to 10 hours onwards after nerve section in the rat phrenic nerve, until at 20 hours no endplates are able to transmit an action potential to the muscle (Miledi and Slater, 1970). Neuromuscular transmission fails abruptly at endplates, apparently without passing through an intermediate or weakened stage; stimulation of degenerating nerves produces an all on nothing response at the neuromuscular junction. The nerve action potential fails a day or two later, at which time it is possible to detect axonal disintegration in the nerve trunk (Weddel and Glees, 1941; Causey and Strattman, 1953; Luttges, Kelly and Gerren, 1976). The temporal distribution of Wallerian degeneration varies from species to species (see Table 1).

Table 1: TIME TO LOSS OF NERVE EXCITABILITY AFTER NERVE SECTION

Species	Nerve	Time to loss of nerve excitability (days)	Reference
Rabbit	Tibial	1½ to 2	Gutmann and Holubar, 1950
Rat	Sciatic	1 to 1½	Miledi and Slater, 1970
Mouse	Sciatic	1 to 2	Lunn *et al.*, 1989
Guinea Pig	Sciatic	1½ to 2	Kaesar and Lambert, 1962
Cat	Sciatic	3	Lissak *et al.*, 1939
Baboon	Lateral popliteal	4 to 6	Gilliat and Hjorth, 1972
Frog	Sciatic	6 to 7	Levenson and Rosenbluth, 1990
Human	Facial	4 to 7	Gilliat and Taylor, 1959

Adapted from Chaudhry, Glass and Griffin, 1992.

1.1.3.1 Stump length dependency

The length of the distal stump influences the duration of the interval before neuromuscular transmission fails (Eyzaguirre, Espildora and Luco, 1952; Luco and Eyzaguirre, 1955; Birks, Katz and Miledi, 1960; Miledi and Slater, 1970; Lubinska, 1982). Nerves with shorter distal stumps have a shorter post-axotomal lag before cessation of neuromuscular transmission. The converse applies to nerves with longer distal stumps. This length dependency phenomenon is not only determined by the length of the extramuscular nerve, but is also a result of the length of the intramuscular nerve branches: end plates further away from the intramuscular nerve trunk degenerate more slowly (Miledi and Slater, 1970). In the rat, the length of the latency period is increased by approximately 45 minutes for every additional centimetre of distal nerve stump. This value is about 10 times greater in the tenuissimus of the cat (Luco and Eyzaguirre, 1955).

The calibre and myelination of degenerating nerve fibres also influence the rate of degeneration. Axons which are unmyelinated degenerate first, followed by small myelinated fibres, with the largest myelinated axons degenerating later (Ranvier, 1878; Cajal, 1928; Weddel and Glees, 1941; Friede and Martinez, 1970; Lubinska, 1977). In comparison with large fibres, small fibres have a relatively larger surface

area to volume ratio and a high rate of metabolism with limited energy reserves (Gerard 1927; Brody, 1966), and therefore would be expected to degenerate first (LoPachin *et al.*, 1990).

1.1.3.2 Temperature dependency

The rapidity of Wallerian degeneration is also influenced by temperature. Nageotte (1932) demonstrated that fragmentation of injured axons *in vitro* develops only within certain temperature limits. Morphologic disruption of isolated fibres did not occur at lower temperatures, or after a nerve had been heated to 45 °C. Subsequent studies on the temperature dependency of Wallerian degeneration have provided additional support for this hypothesis (Joseph and Whitlock, 1972; Torrey, 1934). Wallerian degeneration is also altered in animals which are intrinsically cold-blooded: degeneration is slower in frogs than in mammals, reflecting the cooler nerve beds, and varies depending on the ambient temperature (Birks, Katz, and Miledi 1960). In some instances *in vivo* mammalian Wallerian degeneration can vary depending on body temperature. Long term survival of transected motor axons in the limbs of hibernating squirrels has been reported (Albuquerque, Deshpande and Guth, 1978), where the temperature is almost certainly low relative to 37 °C. The temperature dependence of Wallerian degeneration suggests that the process may be regulated by enzymatic degradation (see below, 1.1.4: Granular Disintegration of the Cytoplasm).

1.1.3.3 Age Dependency

Wallerian degeneration is affected by age. In the optic nerve of cats, the rate of degeneration is considerably faster in kittens than in adult cats (Cook *et al.*, 1974). Wallerian degeneration in mice also appears to be influenced in the same way: the sciatic nerves of mice show slightly decreased rates of degeneration and regeneration with advancing age (Tananka and Webster, 1991; Perry, Brown and Tsao, 1992; Tsao *et al.*, 1994).

1.1.3.4 Progression of nerve degeneration

The spatial sequence of degeneration within the nerve trunk of the distal stump has been difficult to define in mammals, largely due to the remarkable rapidity of the process, and remains a controversial issue. The debate arises as to whether axonal degeneration proceeds from proximal to distal (centrifugally), distally first with subsequent centrifugal spread, or simultaneously along the length of the axon. In the latter half of the 19th Century and the early part of this century several neuroanatomists sought to resolve this problem. Their studies merely had the effect of enhancing the controversy. Cajal (1909) and others (Schiff, 1856; Mitchell 1872) supported the view that Wallerian degeneration evolves simultaneously along the axon, and was contested most notably by the studies of Boeke (1916) who claimed that degeneration proceeded in a centrifugal manner. Latterly Parker (1933) and others (Causey and Strattman, 1953; Rosenbleuth and del Pozo, 1943), confirmed Boeke's observations by demonstrating that loss of conductive capacity occurred in a proximo-distal fashion. Yet more investigators provided evidence for simultaneous degeneration (Guttman and Holubar, 1950; Levenson and Rosenbleuth 1990; Miledi and Slater 1970; Salafsky and Jasinski, 1967). In a non-mammalian nerve, the olfactory nerve of the Garfish, this issue has been clearly resolved. Degeneration spreads in a proximo-distal fashion, with the axoplasm disappearing first near the site of lesion with a rate that corresponds to slow axonal transport (Cancalon, 1982). However, it appears that in this case the axon disappears as a result of continued emptying of the distal stump via axonal transport rather than by degeneration. In mammals, however, the origin of degeneration beyond the initial loss of nerve terminals has been more difficult to pinpoint. If indeed there is a spatio-temporal gradient in axonal breakdown it must have a very steep slope, and that the resolution of certain techniques will have to be enhanced to truly clarify the situation. Some confusion over the interpretation of results is to be expected however. Many studies which describe the progressive Wallerian degeneration of the nerve in a proximo-distal manner may in fact be describing the traumatic degeneration that inevitably takes place in the first 2-3 mm from the site of nerve injury. Donat and Wiśniewski

(1973) divide the degenerating nerve into 3 zones: the *traumatic zone* (the first 1-2 mm from the lesion site), the *peritraumatic zone* (the next 2-3 cm), and the rest of the nerve. In this study the traumatic and peritraumatic zones exhibit signs of organelle accumulations and dense pellets, but the rest of the nerve did not have any apparent gradient of degenerative changes before axonal degeneration, as would be expected if the centrifugal hypothesis were correct. It appears most likely that, in mammals at least, there is synchronous breakdown of the axoplasm along the length of the axon after the initial loss of nerve terminals at the neuromuscular junction. A recent electrophysiological analysis of injured human nerves supports this theory (Chaudhry and Cornblath, 1992).

In contrast, it is clear that the responses of Schwann cells to degeneration, such as the formation of ovoids and the clearance of myelin, proceed in a proximal to distal manner (Causey and Palmer, 1953; Lubinska, 1977).

1.1.4 Granular Disintegration of the Cytoplasm

One of the pivotal events in Wallerian degeneration is the reduction of the axoplasm, including cytoskeletal elements such as neurofilaments and microtubules, to granular and amorphous debris (Donat and Wiśniewski, 1973; Vial, 1958). This so-called granular disintegration of the cytoskeleton (GDC) underlies the failure of both neuromuscular transmission and nerve conduction, and the timing of GDC correlates well with the physiological data previously described. For example, GDC occurs abruptly along the length of the axon after a post-axotomal lag, in an identical timeframe to the failure of the nerve action potential (Guttman and Holubar, 1950; Levenson and Rosenbleuth 1990; Miledi and Slater, 1970; Salafsky and Jasinski, 1967). The latent period of GDC varies between species; axons of transected distal stumps in young rodents are reduced to granular and amorphous debris within 24 hours (Miledi and Slater, 1970), whilst in baboons and humans the same process takes several days (Gilliatt and Hjorth, 1972; Kaesar and Lambert, 1962). The axolemma degenerates at the same time, leaving a myelin sheath that contains only watery remnants of the axon. Microtubule dissolution precedes neurofilament degradation (Schlaepfer 1974). GDC is thought to be triggered by endogenous neutral proteases,

mediated by an injury induced rise in the free calcium concentration within the axoplasm. Such an increase in intracellular calcium could result from either the entry of extracellular calcium through calcium channels (George, Glass, and Griffin, 1995), by the reversal of the sodium exchangers (Lopachin and Lehning, 1997), by failure of calcium pumps, by breaches in the axolemma, or from the release of calcium that is sequestered within the axon. Schlaepfer (1971, 1974) has demonstrated that chelation of calcium (EGTA), or reduction of external calcium concentration prevented both primary axonal alterations and associated linear fragmentation of myelin sheaths of isolated nerves. Furthermore, degenerative changes were accelerated by the introduction of a calcium ionophore (A23187) and by raising extracellular calcium concentration (Schlaepfer 1971, 1977). Other investigators have identified a calcium concentration threshold for complete loss of neurofilament medium and heavy chain subunits as 0.20mM in organ cultures of mouse sciatic nerve (Glass, Schryer , and Griffin, 1994). This property of calcium is thought to be mediated by the activity of one or more calcium dependent proteases, known as calpains (Schlaepfer and Zimmerman, 1984; Kamakura *et al.*, 1983; Schlaepfer *et al.*, 1985; George, Glass, and Griffin, 1995).

1.1.5 Theory of Wallerian degeneration

The physiological mechanism by which Wallerian degeneration occurs remains subject to speculation. Scientists such as Waller (1850), Scott (1906) and Cajal (1929) assumed that the distal stump, upon separation from the trophic centre of the axon, the cell body, literally starved to death due to lack of nutritive support. According to this theory, motor nerve terminals degenerate after axotomy because their cell bodies can no longer supply them with the essential cellular components to ensure the maintenance of synaptic integrity and function. It has been well established that varying the length of the degenerating section of nerve alters the latency period and subsequently delays Wallerian degeneration. Miledi and Slater (1970) proposed that this effect was due to the migration of an as yet unidentified substance along the nerve which, upon arrival, influenced nerve terminal degeneration. It is probable that this putative factor arrives in the nerve terminal via an active transport mechanism. This

phenomenon can be explained in two ways. First, in support of the trophic theory, nerve terminals require an as yet unidentified factor (or factors), which is synthesised in the cell body and transported via fast axoplasmic transport down the axon. Use or loss of this substance at the neuromuscular junction would have to be balanced by its supply from the cell body. Interruption of the nerve would therefore isolate the nerve terminal from the source of this factor and when the concentration falls below a critical level, nerve terminal degeneration would ensue. A larger nerve stump would contain more of the factor being transported to the nerve terminal and as such prolong its survival. Miledi and Slater also outlined a different interpretation of this result, namely that a deleterious process or signal engendered at the site of injury is transported to the nerve terminal, and once there effects Wallerian degeneration. Presumably this 'wound substance' would initiate an active disassembly and degeneration of the nerve terminal, rather than death by starvation. Apoptosis, a naturally occurring form of programmed cell death, is an active process that removes inappropriate, senescent, damaged or abnormal cells that could interfere with organ function or develop into tumours (Kerr, Wyllie and Currie, 1972). Raff and colleagues (Jaconson, Burne and Raff, 1994) demonstrated that apoptosis can take place in the absence of a nucleus, via a mechanism that probably involves the activation of cellular proteases such as calpains (Squier *et al.*, 1994). This has led some investigators to hypothesise that Wallerian degeneration takes place by mechanism akin to cytoplasmic apoptosis, (Lapper *et al.*, 1994; Ribchester *et al.*, 1995). In support of this theory, Wallerian degeneration and apoptosis bear many similarities. First, apoptosis is genetically regulated, and is thought to arise from an imbalance between positive and negative regulators (such as *Bcl-2* and *Bax*) which in turn leads to downstream signalling and the activation of cellular proteases and endonucleases. Wallerian degeneration may also be genetically regulated, as degeneration is delayed in a naturally occurring mutant, the C57BL/Wld^{s} mouse. Second, Wallerian degeneration appears to be a calcium dependent process involving calpains (Schlaepfer and Zimmerman, 1984), and some forms of apoptosis are also dependent on calcium and calcium-activated proteases (Squier *et al.*, 1994). Third, the ovoid bodies that develop in the distal nerve as a result of Wallerian degeneration are reminiscent of 'apoptotic

bodies', which form during apoptosis as a result of the packaging of degenerating cellular constituents into membrane bound entities that are destined for phagocytosis by neighbouring cells or macrophages. Finally, the time course of Wallerian degeneration is similar to that of apoptosis; anucleate cells die within approximately 48 hours after the initiation of apoptosis (Jacobson, Burne, and Raff, 1994).

1.1.6 Cellular responses of Schwann cells and macrophages to Wallerian degeneration

Schwann cells undergo prompt changes in the synthesis of myelin components following axotomy. There is a dramatic reduction in the synthesis of myelin lipids within the first 12 hours in the rat (White *et al.*, 1989). After two days there is a marked fall in mRNA content for many of the major myelin proteins, such as P_o and myelin basic protein (Trapp, Hauer and Lemke, 1988; Mitchell *et al.*, 1990), reaching basal levels by 5 days after denervation. These changes demonstrate that the presence of an intact axon is required for signalling myelination and myelin maintenance by the Schwann cell; in nerves that fail to degenerate after injury (see below, 1.3: The C57BL/Wld^s mutant mouse), Schwann cells maintain their myelin and synthesise new myelin (Thomson *et al.*, 1991). In response to denervation, reactive Schwann cell changes result in the withdrawal of Schwann cell cytoplasm and the collapse of the myelin sheath. One of the earliest changes is the retraction of the myelin sheath from the nodes of Ranvier (Causey and Palmer, 1953; Lubinska, 1977). The myelin sheath separates into several short segments, which then collapse and fold around the cavity originally occupied by the axon. These entities are called ovoid bodies. Each section of myelin that forms an internode, belonging to one Schwann cell, collapses into six to 10 ovoids. After several days the ovoids condense and become separated by intervening Schwann cell cytoplasm. This process takes place in the proximal region of the distal stump first, before spreading distally (Causey and Palmer, 1953; Lubinska, 1977). However, although the general advance of degeneration is proximo-distal, there is no directional trend within individual internodes: ovoid bodies form in the middle near the Schwann cell nucleus, and only later does the entire internode fragment.

Most of the degenerating elements of the distal nerve are cleared by phagocytosis three weeks after nerve injury in the rodent. However, there remains some controversy as to the origin of the phagocytic cells which mediate this process. Previously it was assumed that Schwann cells digested the remnants of the axon and myelin sheath (Büngner, 1891; Satinsky *et al.*, 1964; see review: Sunderland, 1978). In recent years it has generally been accepted that both macrophages and Schwann cells may play a part in removing the end products of Wallerian degeneration. The extent to which these cell types were responsible for myelin clearance was a difficult issue to examine: investigators were unable to reliably distinguish between these two cell types using morphological criteria alone (Abercrombie and Johnson, 1946; Gibson, 1979). This led to speculation that Schwann cells and macrophages were merely different morphological forms of the same cell (Weiss and Wang, 1945). However, in the 1980s it became possible to selectively label macrophages with antibodies such as F4/80 (Austyn and Gordon, 1981). With the use of this antibody, Perry *et al.* (1987) demonstrated that there is a small population of irregularly scattered resident macrophages in peripheral nerves, and that these were supplemented by the infiltration of more macrophages from the circulatory system. The recruitment of macrophages is thought to be initiated by the release of the immediate early gene product monocyte chemoattractant protein-1 (JE) which is synthesised by an as yet unidentified cellular subpopulation within the zone of trauma, in response to nerve injury (Carroll, and Frohnert, 1998). JE is first detected at 1.5 hours after lesion, reaching maximal levels by 24 hours and persisting to 18 days. Other studies demonstrate that in the rat sciatic nerve Schwann cell cytoplasm contains small amounts of myelin debris at a time before macrophage recruitment (Stoll *et al.* 1989), indicating that Schwann cells digest at least some myelin before the arrival of macrophages, and in co-cultures with dorsal root ganglion neurons Schwann cells are able to degrade myelin in the absence of macrophages (Fernandez-Valle, Bunge, and Bartlett Bunge, 1995). In opposition to this, the experiments of Beuche and Friede (1984) demonstrated that when degenerating nerve segments were incubated within millipore chambers whose pore sizes were too small to admit macrophages (0.22 μm) myelin debris remained for up to eight weeks. In contrast,

nerves held within chambers that had pore sizes large enough to permit macrophage entry (5.0 μm) had normal myelin clearance. These investigators therefore claimed that Schwann cells do not remove any myelin debris. Although this issue has not been clearly resolved, a plausible rationalisation for these conflicting results would involve the clearance of myelin debris in a biphasic manner, with Schwann cells digesting the myelin sheath in the early stages of degeneration and the later stages of phagocytosis and myelin lipid processing being performed by recruited macrophages (Ballin and Thomas, 1969; Bigbee *et al.*, 1987; Stoll *et al.*, 1989; Goodrum *et al.*, 1994; Perry and Brown, 1992). The recognition of degenerating myelin by phagocytic cells appears to be extremely specific - in a partially damaged nerve myelin that belongs to undamaged axons is not phagocytosed, nor are the membranes of resident Schwann cells, of which myelin is composed (Perry *et al.*, 1987).

The importance of myelin clearance is twofold. First, 'pathway clearance' mediated by Schwann cells and macrophages is an important step in allowing axonal regrowth. A substance presented on the surface of the myelin sheath, called myelin-associated glycoprotein (MAG), has been shown to be inhibitory for neurons in the central nervous system (McKerracher *et al.*, 1994) and degeneration of myelin, containing MAG, is an essential prerequisite to nerve regeneration in the peripheral nervous system – MAG inhibits nerve regeneration and antibodies to MAG reverse this inhibitory effect (Schafer *et al.*, 1996; Torigoe and Lundborg, 1998). Second, phagocytosis of myelin debris recycles the lipids present in myelin for the purpose of remyelination, after regeneration has occurred. Both Schwann cells and macrophages phagocytose myelin, but most of the hydrolysis of myelin lipids takes place within macrophages, and yields cholesterol and phospholipids (Rawlins *et al.*, 1970, 1972; Goodrum *et al.*, 1995). These are complexed to a lipid carrier molecule called apolipoprotein E, and transferred to Schwann cells for reutilization in the synthesis of new myelin (Ignatius *et al.*, 1987; Boyles *et al.*, 1989; Goodrum *et al.*, 1995).

1.2 PERIPHERAL NERVE REGENERATION

1.2.1 Historical perspective

Although Waller's contemporaries did not doubt his observations of degeneration after nerve trauma, many questioned his supposition that function was restored by the regeneration of nervous fibres arising from the central stump. Indeed, Waller's hypotheses initiated a debate that lasted nearly 60 years. The dispute centred around two polarised doctrines, which were as follows: the theory of continuity or *monogenist* hypothesis and the theory of discontinuity or *polygenist* hypothesis. The adherents to the monogenist doctrine up held Waller's principles, believing that the regenerated fibres of the periphery were formed by the elongation of the intact fibres of the central stump. Polygenists maintained that regenerated fibres were formed by the differentiation and transformation of the cells of the distal stump. This theory states that these cells produce segments of axons which constitute a chain, and that during the process of regeneration these links ultimately unite to form continuous conductors. Phylippeaux and Vulpian (1859) were the first polygenists to contest Waller's theories. They asserted that in young mammals and birds the distal stump regenerated after nerve lesion, but was at all times anatomically separated from the proximal stump. Phylippeaux and Vulpian concluded that the distal stump was therefore able to regenerate independently. They named this phenomenon *autogenous regeneration*. Bethe (1899), agreed with these findings and contrived further experiments by which he claimed proved this theory beyond reasonable doubt. After sectioning the nerve he arranged the severed ends so that there could be no physical contact and therefore no possibility of physiological continuity. Once regeneration had taken place he examined the region between the proximal and distal stumps and observed no new nerve fibres bridging the gap. Stimulation of the distal stump produced contractions in the muscle, whilst stimulation of the central stump in the same preparation produced no response: Thus, the distal portion of the severed nerve had regenerated independently of the proximal stump. Many investigators had observed the division of Schwann cells within the distal stump after nerve injury, and

Bethe claimed that the newly formed nerve fibres arose from the differentiation of these cells. The debate was only settled with the advent of the reduced silver nitrate staining technique, which was the first reliable axonal stain. The polygenist doctrine was firmly refuted by the experiments of Ramon y Cajal (1909), who clearly observed axon sprouts emerging from the central stump and growing into the distal stump, and Harrison (1910), who was the first to demonstrate the new growth of axons in tissue culture.

1.2.2 Regeneration of the peripheral nervous system

Wallerian degeneration leads to the removal and recycling of axonal and myelin derived material to create the appropriate local environment for the regeneration of axons from the proximal stump. As previously mentioned, before nerve regeneration can proceed the remaining debris, collapsed myelin and the remnants of the axon, must be cleared away. This is a pivotal step in the regeneration of peripheral nerves. Subsequent changes in Schwann cells and macrophages lead to the presentation of a favourable environment for the regrowth of axons from the proximal segment. Alterations in the molecular machinery of the cell body downregulate proteins normally associated with cellular stability and upregulate proteins necessary for growth, readying the proximal axons for elongation.

1.2.2.1 Schwann cell division

Following degradation and extrusion of their myelin sheaths Schwann cells de-differentiate and divide (Cajal 1928; Abercrombie and Johnson, 1946). Cell division occurs in two waves. The first wave peaks at around four days in cats and rodents (Pellegrino *et al.*, 1986; Clemence *et al.*, 1989). The second wave of Schwann cell mitosis becomes evident 14 days after the time of injury (Pellegrino and Spencer, 1985). The total number of divisions depends upon the original distance between Schwann cell nuclei; Schwann cells that were originally in close proximity divide only a few times whilst those that were further apart divide many more times (Thomas, 1948). Therefore, Schwann cells of myelinated fibres with long internodes produce more daughters cells. These observations suggest that mitosis continues until a relatively short distance between Schwann cells is achieved, and their cytoplasmic

processes become interlaced (Spencer *et al.*, 1979). The first Schwann cell division takes place in the central area of the internode close to the Schwann cell perikaryon, and divides the old internode into two cells. Subsequent divisions take place until a continuous tract is formed in line with the original basal lamina, consisting of inter-linking Schwann cells processes. This can be observed as a longitudinal band (the Bands of Büngner) along which regenerating axons grow (Thomas, 1964), and is thought to provide a favourable substrate for axonal elongation (see below).

The precise signal that stimulates Schwann cell mitosis is unknown, although it is thought that both nerve degeneration products and macrophage infiltration stimulate the first mitotic peak. In cultures, axonal membrane and myelin debris can promote Schwann cell mitosis (Salzer *et al.*, 1980; Yoshino *et al.*, 1984; Meador-Woodruff *et al.*, 1985) Macrophages that have digested myelin membrane release a soluble Schwann cells mitogen (Baichwal *et al.*, 1988), and TGF-β, which is a macrophage product, stimulates the Schwann cells division in vitro (Ridley *et al.*, 1989). The second wave of Schwann cell mitosis probably occurs in response to nerve regeneration and stimulated by axonal contact (Salzer and Bunge, 1980; Pellegrino and Spencer, 1985).

1.2.2.2 Cell body responses

At the same time as these changes are taking place in the distal stump, the neuronal cell bodies activate and prepare for regeneration. Within the first few hours the early response genes *c-fos* and *c-jun* and the low affinity nerve growth factor receptor are expressed (Jenkins and Hunt, 1991; Wu, 1996). The main early morphological events are *chromatolysis*, which is the dispersal of the Nissl substance due to the disintegration of large granular condensations of rough endoplasmic reticulum, and the migration of the nucleus to an acentric position (Barr and Hamilton, 1948; Watson, 1968). These observations are indicators of increased mRNA and protein synthesis and signify that the neuron is entering a growing state. Following nerve injury cellular concentrations of tubulin and actin, structural proteins involved in axonal growth, and growth associated proteins such as GAP-43 increase (Skene *et al.*, 1986; Tezlaff *et al.*, 1988). The pattern of synthesis of cytoskeletal components recapitulates that observed during development. It is probable that these changes are

initiated when a putative trophic factor is no longer conveyed via fast retrograde transport to the neuronal cell body (reviewed in Cragg, 1970; Richardson and Verge, 1986), or from the arrival of an injury-induced factor (Ambron and Walters, 1996). These changes have the effect of preparing the cell body and proximal axon for regeneration, and if a subsequent lesion is made at this time the growth response is faster than if only a single lesion had been created originally (McQuarrie, 1973, 1979; Lankford, Waxman, and Kocsis, 1998). In this paradigm the second lesion is known as a *conditioned lesion*.

1.2.2.3 Morphological response of the axon

Axons begin to send out processes from the terminus of the proximal stump as early as 5 hours after axotomy (Tomatsuri, Okajima and Ide, 1993). The first sprouts usually originate from the terminal nodes of Ranvier, through the gap left by the partial retraction of Schwann cells (Friede and Bischausen, 1980). Within a few days regenerating axons grow down the endoneurial sheath at a rate of approximately 4mm/day in the rat sciatic nerve (Forman and Berenberg, 1978). This is equivalent to the rate of slow axoplasmic transport, and has led some investigators to propose that axonal growth is limited by the supply of actin reaching the growth cone (Wujek and Lasek, 1983).

Neurites are known to be attracted by degenerated peripheral nervous system tissue (Kuffler, 1986) and the distal stump may provide trophic support during axonal re-growth (Politis, Ederle, and Spencer, 1982; Politis, 1985). Schwann cells are also thought to play a major role in the guidance and support of regenerating axons. In some cases, Schwann cells form a cellular bridge spanning the gap between the ends of the severed nerves (Abercrombie and Johnson, 1942). *In vitro* experiments have demonstrated that several components of the Schwann cell basal lamina sheath, such as laminin, fibronectin and the heparin sulfate proteoglycan complex, provide growth substrates for regenerating axons (Rogers *et al.*, 1983; Jessel, 1988). Schwann cells produce neurotrophic factors which promote the survival and extension of axons re-growing into the distal stump. Schwann cells synthesise NGF and NGF receptors, peaking at 24 hrs and persisting for at least 2 weeks after nerve injury (Heumann *et al.*, 1987). This synthesis is stimulated by the presence of the cytokine interleukin-1,

produced by infiltrating macrophages (Lindholm *et al.*, 1987). NGF is a trophic factor that supports axonal regeneration in specific susceptible neurons, and it is thought that NGF receptors immobilise NGF protein on the Schwann cell plasmalemma to facilitate axonal elongation (Johnson, Taniuchi, and Di Stefano, 1988; Taniuchi *et al.*, 1988). Schwann cells also express brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) which have been shown to promote axon growth and may act as lesion factors after nerve injury (Acheson *et al.*, 1991; Stockli *et al.*, 1989; Sendtner *et al.*, 1992, 1994). BDNF increases to several times the normal level by 4 days after axotomy, reaching maximal levels 4 weeks later (Meyer *et al.*, 1992). CNTF, however, is present at a high level in Schwann cells of intact nerves, and is downregulated after axotomy (Rende *et al.*, 1992; Sendtner *et al.*, 1992). CNTF disappears from Schwann cells 30-60 days after denervation and re-appears with re-innervation (Smith *et al.*, 1993). This suggests that CNTF is only synthesised when axonal contact is re-established. The administration of both these factors, by injection or osmotic minipump, improves the functional regeneration of the rat sciatic nerve (Lewin *et al.*, 1997; Cheng *et al.*, 1998). In a recapitulation of the developmental environment, Schwann cells re-express certain cell adhesion molecules such as L1 and N-CAM, which have been shown in cell cultures to improve the rate of axonal growth (Martini and Schachner, 1988). Tenascin-C, an extracellular matrix glycoprotein, is strongly expressed by Schwann cells after nerve section and appears to be essential for the growth of axons in the distal stump (Fruttiger, Schachner, and Martini, 1995), and a lipid carrier molecule, apolipoprotein E, can modulate neurite outgrowth (Handelmann *et al.*, 1992). Therefore, it is apparent that Schwann cells are a fundamental component in the support and growth of axons along the distal stump during peripheral nerve regeneration.

1.3 THE C57BL\Wld^s MUTANT MOUSE

Wallerian degeneration is a rapid and concise process that occurs after nerve damage. The physiological mechanism by which axons degenerate after nerve lesion remains subject to speculation. The two main theories favour either passive degradation due to loss of some trophic factor, or a more active mechanism in which the axon initiates its own demise following nerve injury. One aspect of degeneration has recently come to light - Wallerian degeneration is under genetic control. Lunn *et al.* (1989), during their investigation into the role of macrophages in Wallerian degeneration, discovered by chance that the strain of mouse they were using, C57BL/Ola, had remarkably retarded rates of Wallerian degeneration. This naturally occurring mutant mouse strain, now called C57BL/Wld^s, carries a mutation which considerably slows the degenerative phenomena that occur after nerve section. The normal allele has been named 'Wld' for Wallerian degeneration and the mutant is called 'Wld^s', where *s* denotes 'slow Wallerian degeneration'.

1.3.1 Retarded rates of degeneration

Distal Wld^s axons appear structurally intact for up to one month (Glass and Griffin, 1991; Glass *et al.*, 1993) and can conduct compound action potentials for up to 3 weeks after nerve lesion (Lunn *et al.*, 1989; Perry *et al.*, 1990b; Tsao *et al.*, 1994). The mutation occurred spontaneously at Harlan-Olac Laboratories (Bicester, U.K.) and does not appear to exert any harmful effects on the mice. In fact, the C57BL/Wld^s mouse is indistinguishable from the C57BL strain from which the mutation arose - both strains have identical appearance, behaviour, histocompatibility and genotype of more than 50 microsatellites and restriction fragment length polymorphisms (Lunn *et al.*, 1989; Coleman *et al.*, 1998). The developmental phenomenon of synapse elimination, the removal of excess synapses from neuromuscular junctions, also appears to be normal (Parson, MacKintosh and Ribchester, 1997). In sum, Wld^s mice have considerably retarded rates of Wallerian degeneration, but the studies to date indicate that apart from this aberration the C57BL/Wld^s mouse is unaffected by the

mutation in any other way.

Initial experiments suggested that the delay in Wallerian degeneration in Wld^s mice was caused by a defect in the recruitment of macrophages from circulatory system (Lunn *et al.*, 1989). In wild type mice denervation results in an influx of myelomonocytic cells, which contribute to the phagocytosis of the degenerating debris of Wallerian degeneration (Perry, Brown and Gordon, 1989). In Wld^s mice, transection of peripheral nerves results in extremely retarded rates of macrophage recruitment. To investigate the possibility that the lack of macrophage recruitment resulted in slow Wallerian degeneration, Lunn *et al.* (1989) prevented recruitment of myelomonocytic cells by administration of a selective monoclonal antibody against the complement type 3 receptor in normal mice. The authors observed that this had the effect of slowing axonal degeneration, thus validating the theory that monocyte recruitment causes slow Wallerian degeneration. A subsequent report suggested that certain environmental factors influence the rate of degeneration in Wld^s mice, probably via an inflammatory mechanism, further implicating monocyte recruitment in Wallerian degeneration (Perry *et al.*, 1990b). However, a series of studies have demonstrated that the mutation affects an intrinsic property of nerve axons. Perry and colleagues (1990a) replaced bone marrow in Wld^s mice with that from histocompatible C57BL/6J mice in order to determine whether the slow recruitment of monocytes was due to the failure of the nerve to produce a chemotactic signal, or the failure of monocytes to respond. If the lack of recruitment was due to a defect in Wld^s monocytes then a bone marrow transplantation would correct for this, and normal recruitment would ensue after nerve injury. However, the authors observed that Wld^s mice with C57BL/6J bone marrow still had retarded rates of monocyte recruitment. When Wld^s bone marrow was transplanted to 6J mice, and the nerve lesioned, normal rates of recruitment were observed during degeneration. The response of Wld^s myelomonocytic cells to a range of inflammatory agents and chronic infection was tested and found to be similar to that of 6J mice. Furthermore, C57BL/Wld^s mice were used for a study of delayed hypersensitivity, and once more were found to respond as normal (Rosen, Milon and Gordon, 1989). Demyelination of axons by an intraneural injection of lysophatidyl choline results in normal rates of

macrophage recruitment in Wld^s mice (Hall, 1993). To further preclude the involvement of macrophage recruitment in the phenotype of Wld^s mice isolated segments of nerve were placed in culture medium for several days, whereby recruitment of monocytic cells was not possible (Perry *et al.*, 1990a). Wld^s nerves still degenerated much more slowly than wild type nerves. In subsequent study, pre-degenerated sections of Wld^s nerve sheath were transplanted into 6J mice, and the axons allowed to regenerate before being sectioned once more (Glass *et al.*, 1993). Lesioned axons from 6J mice degenerated at a normal rate. The converse situation was also applied – 6J nerve sheaths were transplanted into Wld^s mice, the regenerated nerve sectioned, and axonal degeneration was retarded. These observations suggest that the intrinsic cells of Wld^s nerve sheaths are not responsible for the slow rates of degeneration. Finally, *in vitro* experiments using primary cultures of dorsal root ganglion and superior cervical ganglion showed that transected Wld^s axons were able to survive for up to seven days (Glass *et al.*, 1993; Buckmaster *et al.*, 1994; Deckwerth and Johnson, 1994). In sum, slow Wallerian degeneration in C57BL/Wld^s mice is an intrinsic property of the axon.

1.3.2 Molecular nature of the mutation

Investigations into the molecular nature of the mutation have yielded further insights as to the mechanism of slow Wallerian degeneration in the Wld^s mouse. The Wld^s mutation is thought to be due to the naturally occurring spontaneous alteration of an as yet unidentified autosomal dominant gene (Perry *et al.*, 1990b). Further studies mapped the genetic locus, using conventional methods and genetic markers, to the distal end of mouse chromosome 4, near the locus of proantriodilatin (Lyon *et al.*, 1993). The most recent investigation suggests that the mutation is due to an 85-kb tandem triplication that arose from illegitimate or nonhomologous recombination between short homologous sequences (Coleman *et al.*, 1998). Interestingly, a form of Charcot-Marie-Tooth disease (CMT2A), which is characterised by axonal loss, has been mapped to a homologous locus in humans, 1p36 (Ben-Othmane *et al.*, 1993). Coleman *et al.* (1998) envisaged three ways in which the Wld gene could be affected by a tandem triplication. First, if the entire Wld gene and all its regulatory components

was located within the triplicated region, then it is conceivable that the gene product would be increased threefold resulting in an increase in the steady state level of the protein. Second, if the Wld gene was split by the triplication then this might result in a truncated protein product, or the formation of a hybrid protein with a novel function. Third, the gene could remain intact but come under the control of a different regulatory element or transcription factor. This could in turn affect the transcription of the gene; the rate of transcription could increase even more than threefold, and could affect genes adjacent to the triplicated region. A further complication arises from the possibility that the 85-kb region may encode for more than one gene, on the basis that there is an average spacing of one gene per 30-60 kb in the mouse genome. The authors note that further studies are required to provide formal proof that the triplication is indeed the cause of the Wld^s mutation. To truly characterise the molecular nature of the Wld^s phenotype it is necessary to construct a detailed map of the Wld locus, and to use a functional model to transfer the candidate Wld^s gene to wild type neurons.

It is generally accepted that the Wld^s mutation declines in its effectiveness with advancing age. Various studies have demonstrated that degeneration in older Wld^s mice takes place more rapidly, but nevertheless still slower than wild type degeneration (Perry Brown and Tsao, 1992; Tsao *et al.*, 1994; Ribchester *et al.*, 1995; *personal observation*). This phenomenon is interesting because in wild type mice the converse applies - older nerves degenerate more slowly (Tanaka and Webster, 1991; Perry Brown and Tsao, 1992; Tsao *et al.*, 1994). However, a conflicting study by Crawford and colleagues (1995) reported that the rates of degeneration in young and old Wld^s nerves remains constant. A possible explanation for the discrepancy may arise from the use of the British and American stocks respectively in the studies of C57BL/Wld^s mice, which may differ slightly in their phenotypic characteristics, although this is unlikely (Crawford *et al.*, 1995). This issue remains to be definitively resolved.

1.3.3 Biochemical and cellular events during delayed axonal degeneration

The first study of the C57BL/Wld^s mouse revealed that axonal degeneration after peripheral nerve section is considerably delayed in comparison with normal, rapid Wallerian degeneration (Lunn *et al.*, 1989). Wallerian degeneration is also delayed in the central nervous system (Perry, Brown and Lunn,, 1991; Ludwin and Bisby, 1992). The distal stump of lesioned peripheral Wld^s nerves can transmit a compound action potential for up to 3 weeks (Lunn *et al.*, 1989; Perry *et al.*, 1990b; Tsao *et al.*, 1994) and retain structural integrity for as long as one month (Glass and Griffin, 1991; Glass *et al.*, 1993). Neuromuscular transmission fails earlier, from 3 days to two weeks, and this coincides with the morphological degeneration of nerve terminals (Ribchester *et al.*, 1995). Furthermore, the overall rate of degeneration in Wld^s nerves is dependent upon the length of the distal stump, similar to wild type mice, although the rates are still of a slower order in wild type mice. Neuromuscular transmission of Wld^s nerves fails one to two days later per additional centimetre of distal nerve, as compared with 45 minutes per centimetre in normal mice (Ribchester *et al.*, 1995; Miledi and Slater, 1970). Axonal disintegration in the C57BL/Wld^s mouse is dependent upon calcium as in normal mice, and several studies have identified that calpains are present - proteases which can degrade the axonal skeleton in the presence of calcium (Glass, Schryer and Griffin, 1994; Tsao *et al.*, 1994). However, it appears that more calcium is needed to effect axonal disintegration in Wld^s axons (Glass, Schryer and Griffin, 1994): the threshold calcium concentration for the complete disintegration of the cytoskeleton is 0.2mM in wild type mice, whilst some Wld^s axons are still preserved at 0.25mM as assessed by neurofilament immunoreactivity.

The remarkable preservation of Wld^s axons after nerve injury led some investigators to speculate that axonal transport may be slowed or stopped. However, fast axonal transport of organelles in isolated nerve segments proceeds at normal anterograde and retrograde rates for up to eight days post lesion (Smith and Bisby, 1993). Slow axonal transport of neurofilaments, tubulin and actin continues for up to 14 days post axotomy (Glass and Griffin, 1991, 1994; Watson Glass and Griffin 1993).

In addition to slowed Wallerian degeneration, C57BL/Wld^s mice also exhibit a slowing of the rate of retrograde degeneration of retinal ganglion cells after optic

nerve section (Perry, Brown and Lunn, 1991). This was an intriguing result as it had previously been assumed that cell body degeneration occurred via apoptosis, a quite distinct phenomenon from Wallerian degeneration. Axotomy of a neonatal peripheral nerve results not only in the Wallerian degeneration of the distal axons, but also retrograde cell death of motor neuron cell bodies via apoptosis. In the Wld^s mouse, apoptotic neuronal cell death after neonatal nerve injury was delayed by several days, but not prevented (Lapper, Brown and Perry, 1994). This result led to the speculation that Wallerian degeneration and apoptosis are related phenomenon (Lapper, Brown and Perry, 1994; Ribchester *et al.*, 1995). However, Deckwerth and Johnson (1994) demonstrated that, *in vitro* at least, cell body death appears to take place independently of axonal degeneration. In this study, trophic support was withdrawn from sympathetic neurons of Wld^s explants. In normal mice this would result in death of cell body and neurite simultaneously but in Wld^s explants, whilst the soma died by apoptosis, the neurites survived and remained metabolically viable for up to seven days after axotomy. Based on these results the authors proposed that neuritic degeneration occurs independently and autonomously of cell body death, and thus via separate mechanisms.

The fact that granular disintegration of the cytoplasm and myelin breakdown in Wld^s mice does not occur until after a long lag period has compounding effects on the other changes that are subsequent to Wallerian degeneration. It is known that the delay in Wallerian degeneration in C57BL/Wld^s mice is an intrinsic property of the axon. The original report of slow Wallerian degeneration by Lunn *et al.* in 1989 demonstrated that recruitment of macrophages after nerve injury was retarded in Wld^s mice. Therefore, it is sensible to assume that the delay in the recruitment of myelomonocytic cells must be attributable to the prolonged integrity of the axonal cytoskeleton. A recent study demonstrated that Monocyte Chemoattractant Protein-1 (JE) may be implicated in the recruitment of macrophages (Carroll and Frohnert, 1998). JE is a potent chemoattractant for macrophages (Schall and Bacon, 1994) and JE mRNA is initially strongly expressed in an endoneurial cell population at the site of nerve injury (Carroll and Frohnert, 1998). The expression pattern of JE mRNA changes, becoming widespread along the distal segment by 16 days post axotomy, coincident with the

distribution of infiltrating macrophages. However, JE is not expressed after nerve trauma in the C57BL/Wld^s mouse, in which macrophage recruitment is almost non-existent. These data suggest that JE is released initially at the site of injury to attract circulatory myelomonocytic cells, and then subsequently released along the length of the distal stump once macrophage invasion has taken place. The absence of JE expression in the axotomized Wld^s nerve suggests that Wallerian degeneration is required to initiate the production of JE from endoneurial cells. Schwann cell division and myelin degradation are also delayed in the Wld^s mouse (Lunn *et al.*, 1989; Brown *et al.*, 1991b), and down regulation of the myelin protein gene for P_0 does not occur until after nerve degeneration takes place (Thomson *et al.*, 1991). Furthermore, the muscle wastage that takes place as a result of denervation in wild type animals does not occur in Wld^s muscle (Brown *et al.*, 1991a).

1.3.4 Nerve regeneration in the Wld^s mutant mouse

Surprisingly, regeneration of peripheral nervous system motor axons to the soleus muscle takes place as rapidly and comprehensively as in other mice (Lunn *et al.*, 1989; Perry *et al.*, 1992b). In the sciatic nerve, which supplies the soleus muscle, regenerating motor axons are thought to associate with Schwann cells that normally surround unmyelinated axons (Brown, Lunn and Perry, 1992). In contrast, sensory axons do not regenerate as quickly as wild type axons after nerve injury (Bisby and Chen, 1990; Brown, Lunn and Perry, 1991,1992). However, sensory axons are able to elongate as normal in culture (Buckmaster *et al.*, 1994), and the regenerative capacity of both motor and sensory Wld^s neurons *in vivo* remains intact: Brown *et al.* (1994) observed a copious sprouting reaction at the site of injury in both motor (phrenic) and sensory (saphenous) nerves. Furthermore, the initial increase in c-jun and GAP-43, and the decrease in medium weight neurofilament mRNA expression, were similar in facial motor neurons and dorsal root ganglion neurons of Wld^s and 6J mice (Gold, Storm-Dickerson and Austin, 1993; Brown *et al.*, 1994; Bisby, Tetzlaff and Brown, 1995). Interestingly, the subsequent recovery of c-jun and GAP-43 mRNA to normal levels, which occurs after target innervation in normal mice, is delayed in Wld^s neurons (Bisby, Tetzlaff and Brown, 1995). These findings suggest

that the initial cell body response to axotomy in Wld^s mice is similar to that of normal mice, and that Wld^s neurons do not have a reduced capability to regenerate. Therefore, it is possible that Wld^s nerve sprouts are prevented from regenerating down the distal stump by the lack of a permissive environment, caused by the delay of Wallerian degeneration. Several factors may contribute to this.

Apolipoproteins are upregulated after nerve injury in normal mice, and are thought to facilitate myelin breakdown and remyelination (Ignatius *et al.*, 1986; Boyles *et al.*, 1989). In the Wld^s mouse, apolipoprotein E production is confined to regions where myelin destruction and phagocytosis occur (Saada *et al.*, 1995). Furthermore, regeneration of Wld^s axons only occurs where myelin associated glycoprotein, a factor thought to be inhibitory to axonal growth, has been cleared or disrupted (Schafer *et al.*, 1996). Likewise, tenascin-C, which supports neurite outgrowth, is only expressed after axonal degeneration in Wld^s mice, and regrowing axons were invariably associated with tenascin-C expression (Fruttiger, Schachner and Martini, 1995).

Therefore, the success of regeneration in peripheral nerves of the C57BL/Wld^s mouse appears to be partly dependent on the degeneration of axotomized distal axons. Without this event it is probable that sprouts face a hostile and non-permissive environment in which it is difficult to proceed. The success of motor axon regrowth to the soleus muscle can be explained by the fact that the unmyelinating Schwann cells of the sciatic nerve provide an alternative environment for growth of motor axons (Brown, Lunn and Perry, 1992): motor axons do not regenerate in the phrenic nerve, where there are no unmyelinated axons and as such no associated unmyelinating Schwann cells (Brown *et al.*, 1994).

1.4 AIMS

This thesis explores the phenomenon of slowed Wallerian degeneration in C57BL/Wld^s mice, and in particular the effect of peripheral nerve lesion on motor nerve terminal structure and function. The majority of the studies of the C57BL/Wld^s mutant mouse to date have specifically examined Wallerian degeneration in the peripheral nerve trunk. The conclusion of these reports is that Wallerian degeneration of distal axons is suspended for a limited period of time following nerve injury. This remarkable finding suggests that Wallerian degeneration may be under genetic control. How does the mutation that results in slowed Wallerian degeneration of peripheral nerves affect neuromuscular synapses? Preliminary investigations of Wld^s neuromuscular junctions indicate that Wallerian degeneration is delayed for up to 2 weeks (Ribchester *et al.*, 1995). However, many other possibilities remain to be explored. For instance, are axotomized neuromuscular synapses maintained as normal during the delay period, or are there subtle changes in the morphological and physiological characteristics of motor nerve terminals preceding the initiation of degeneration? Are persistent nerve terminals functionally efficacious? Does Wallerian degeneration proceed as normal in motor nerve terminals once initiated, or does some other process take place? What is the role of spontaneous activity in persistent axotomized Wld^s neuromuscular synapses? How are terminal Schwann cells, which normally respond rapidly to Wallerian degeneration, affected by the persistence of intact neuromuscular junctions following nerve damage? This thesis attempts to address these questions, and others, with the use of several techniques. The first series of experiments, in Chapter 2, further characterises the effect of peripheral nerve damage on Wld^s motor nerve terminals at the neuromuscular junction. These studies resulted in unexpected findings, which formed the basis of subsequent experiments (Chapters 3 and 4). Possible reasons for these surprising findings, discussed in Chapter 2, resulted in the formulation of hypotheses which were tested in Chapter 3.

Chapter 2 comprises a detailed study of the changes in nerve terminal morphology and function following axotomy. The structural changes that occur following nerve axotomy at the neuromuscular junction are examined using two techniques for

assessing morphology: vital staining, utilising dyes that label living preparations, and immunocytochemical techniques to visualise fixed preparations. Both of these techniques employ fluorescent imaging. Functional changes at C57BL/Wld^s neuromuscular junctions were measured using three electrophysiological recording techniques.

Chapter 3 is a study of the effect of activity on the changes that take place at axotomized Wld^s neuromuscular junctions. Botulinum toxin was used to block neurotransmitter release from Wld^s nerve terminals following axotomy. Morphological changes in motor nerve terminals were quantitatively examined using immunocytochemical labelling of nerve terminals, preterminal axons, and postsynaptic acetylcholine receptors.

Chapter 4 entails an investigation into the effect of persistent motor nerve terminal structure, which occurs following lesion of Wld^s nerves, on the phenomenon of Wallerian degeneration-induced reactive Schwann cell sprouting. The effect of the intrinsically slow axonal degradation on associated cell types has been studied to some extent in the peripheral nerve trunk. However, there have been no studies that address the consequences of intact peripheral nerves and nerve endings on associated cell types at the neuromuscular junction, which have distinct and characteristic reactions to normal Wallerian degeneration.

The discussion in Chapter 5 focuses on the implications of the findings as a whole, and suggests directions for future research.

CHAPTER 2

NERVE TERMINAL RESPONSES TO AXONAL LESION IN WLD^S AND WILD TYPE MICE.

CHAPTER 2: MOTOR NERVE TERMINAL RESPONSES TO AXONAL LESION IN WLD^S AND WILD TYPE MICE.

2.1 INTRODUCTION

The vertebrate neuromuscular junction is arguably the most comprehensively studied and accessible synapse, and as such is one of the leading preparations for the analysis of synaptic plasticity. The mammalian neuromuscular junction, which will be the focus of this thesis, can be simplified to three different cell types: motor neuron, muscle fibre, and Schwann cell. All three cell types are highly specialised, and at the synapse there are high concentrations of organelles and molecules that are found in low concentrations outwith the region of the neuromuscular junction.

The motor nerve terminal lies in a shallow gutter on the muscle fibre, and capped by terminal Schwann cells. The major function of the motor nerve terminal is the release of neurotransmitter to elicit muscle action potentials and thereby induce muscle fibre contraction. The mammalian motor nerve terminal is polarised into two halves, into which specialised organelles are organised. The presynaptic region of the nerve terminal, which is directly adjacent to the synaptic cleft, is dominated by a high density of 50-nm-diameter synaptic vesicles, that are packaged with the neurotransmitter, acetylcholine. Synaptic vesicles are clustered around dense areas of the presynaptic membrane (active zones) where they fuse with the membrane to release their contents. Active zones are associated with voltage-dependent potassium and calcium channels, which regulate calcium-dependent secretion of synaptic vesicles (Robitaille *et al.*, 1993; Sugiura *et al.*, 1995; Day *et al.*, 1997). The mechanism of synaptic vesicle fusion has yet to be fully elucidated, but involves the interaction of several vesicle-specific proteins such as SV2, synaptophysin, syntaxin and SNAP-25 (reviewed in Sudhof, 1995; Calakos and Scheller, 1996). Active zones lie directly adjacent to postsynaptic specialisations called junctional folds, which are capped by acetylcholine receptors. Vesicle fusion results in the release of acetylcholine, which diffuses across the synaptic cleft and acts on postsynaptic receptors to induce muscle contraction. Thus, synaptic 'strength' is primarily a function of the number of quanta released on presynaptic excitation (the *quantal*

content) (Katz and Thesleff, 1957). The more proximal region of the nerve terminal, which is adjacent to the overlying Schwann cell, is occupied by largely occupied by mitochondria, which are necessary for the synthesis and release of transmitter, with some microtubules and neurofilaments. The preterminal axon collateral consists mainly of microtubules and neurofilaments and contains very few vesicles and mitochondria, and no active zone (Yee *et al*, 1988).

The postsynaptic endplate on the muscle fibre is geared to respond rapidly and reliably to acetylcholine released from the nerve terminal. Junctional folds, which are highly invaginated, are juxtapositioned to active zones in the nerve terminal. At the top of the junctional folds there is an extremely high density of acetylcholine receptors (>10,000/μm^2), which are associated with a number of signalling molecules, and which maintains the high postsynaptic density of acetylcholine receptors (Moscoso *et al.*, 1995; Valenzuela *et al.*, 1995; Martin *et al.*, 1996). The density of acetylcholine receptors decreases in extrasynaptic areas of the muscle fibre membrane to > 10/μm^2 (Salpeter *et al.*, 1988). Sodium channels and N-CAM are concentrated in the depths of the junctional folds (Covault and Sanes, 1986; Flucher and Daniels, 1989). An elaborate network of cytoskeletal proteins support the junctional folds, with rapsyn, utrophin and α-dystrobrevin-1 at the top and ankyrin, α-dystrobrevin-2, and dystrophin at the bottom, and are thought to maintain the different domains within them (Sealock *et al.*, 1984; Covault and Sanes, 1986; Flucher and Daniels, 1989; Wood and Slater, 1998; Peters *et al.*, 1998). The organisation of the postsynaptic junction, with acetylcholine receptors at the crest of the junctional folds, and sodium channels clustered in the depths of the folds, is thought to enhance the efficacy of synaptic transmission (Wood and Slater, 1997).

One or more terminal Schwann cells caps the motor nerve terminal, surrounding the entire nerve terminal with finger-like cytoplasmic projections. Terminal Schwann cells are thought to provide the nerve terminal with trophic sustenance (Bunge, 1993), and also play a major role in innervation and reinnervation of muscle fibres by growing axons, and during other forms of synaptic remodelling. These will be reviewed in depth in Chapter 3.

Damage to the nerve results in disruption to these structures, which will be further explored below.

2.1.1 Nerve terminal degeneration in wild type mice

One of the earliest events that occurs after a peripheral nerve lesion is the degeneration of motor nerve terminals at the neuromuscular junction. This degeneration manifests itself in two ways: loss of neuromuscular transmission coincident with morphological disruption of nerve terminal structure.

2.1.1.1 Morphological degeneration

The first degenerative changes in the ultrastructure of nerve terminals take place as early as three hours after nerve lesion, and are characterised by disruption of mitochondria, a reduction in the number of synaptic vesicles, an increase in neurofilamentous material, and the presence of membrane 'whorl' structures surrounding vesicular aggregations. Immediately after nerve injury there is a 'lag period', between 3 and 8 hours in rodents, during which no obvious structural changes take place, and all nerve terminals appear normal (Miledi and Slater, 1970; Manolov, 1974; Winlow and Usherwood, 1975).

After nerve section, mitochondria become circular in shape, increase in diameter to ~0.4-0.5μm, and the cristae become disorganised and fragmented (Birks, Katz, and Miledi, 1960; Miledi and Slater, 1970). Disrupted mitochondria can be detected in some terminals as early as 3 hours post axotomy (Manolov, 1974). By 6 hours after nerve injury, many nerve terminals have swollen mitochondria. By 12-18 hours all mitochondria appear abnormal, and most have degenerated into dense bodies. By 24 hours post lesion no mitochondria can be detected (Winlow and Usherwood, 1975).

In normal preparations, synaptic vesicles are densely packed in nerve terminals and the overall population density varies between 56 and 209 μm^{-2} (Winlow and Usherwood, 1975). Synaptic vesicles are generally more concentrated near the presynaptic membrane than in the rest of the nerve terminal (Hubbard and Kwanbunbumpen, 1968; Winlow and Usherwood, 1975). The total number of synaptic vesicles declines from 6 hours post axotomy onwards, but the overall distribution does not appear to change significantly (Winlow and Usherwood, 1975).

During this time, synaptic vesicles sometimes cluster together to form multivesicular bodies; the tendency for vesicles to aggregate is more apparent in terminals with low vesicle populations. By 12 hours post lesion very few synaptic vesicles remain, and the nerve terminal is dominated by multivesicular bodies, membranous whorls, dense granules, degenerating mitochondria and an accumulation of neurofilamentous material (Manolov, 1974; Winlow and Usherwood, 1975).

At this time, the nerve terminal membrane becomes fragmented and several small axon profiles appear in the synaptic groove. Terminal Schwann cells, which overlie the neuromuscular junction, engulf and digest nerve terminals during Wallerian degeneration. Glial processes begin to penetrate nerve terminals by six hours after nerve section (Manolov, 1974). By 24 hours after axotomy terminal Schwann cells have completely replaced the nerve terminal in the synaptic gutter, and lie adjacent to the muscle membrane. It appears that Schwann cells phagocytose nerve terminals in varying stages of degeneration. Miledi and Slater (1970) described the engulfment of an almost intact nerve terminal, whilst in other preparations nerve terminals which were clearly in the final stages of degeneration remain distinct from terminal Schwann cells. After 48 hours post axotomy no distinguishable remains of the nerve terminal can be recognised, and Wallerian degeneration at the neuromuscular junction is complete.

It is important to note that during the course of these studies the investigators observed many nerve terminals in varying stages of degeneration, even at the same timepoint. This variability seems to indicate that nerve terminal degeneration occurs asynchronously. It is interesting to note that Miledi and Slater (1970) reported a lag period of 8-10 hours, during which nerve terminals remained morphologically normal, whilst Manolov (1974) described mitochondrial disruption as early as 3 hours post axotomy. Two factors could account for this apparent discrepancy between the studies. Manolov (1974) took the presence of swollen and abnormal mitochondria to indicate the first signs of degeneration after nerve lesion. However, Winlow and Usherwood (1975) demonstrated that even normal nerve terminals contain mitochondria that appear to be swollen and disorganised (up to 19%). Therefore, some care must be taken when interpreting the presence of disrupted mitochondria in motor nerve terminals. Miledi and Slater (1970) demonstrated that

the duration of nerve terminal degeneration is dependent upon the length of the distal nerve stump. This may account for the differences between various studies: different experimental procedures may leave shorter lengths of distal nerve, which would account for the observation of more rapid alterations in nerve terminal morphology.
In sum, Wallerian degeneration of nerve terminals takes place from approximately 3 to 24 hours post axotomy and involves gross organelle disruption, neurofilament accumulation, reduction in the number of synaptic vesicles, and nerve terminal membrane fragmentation followed or preceded by Schwann cell phagocytosis.

2.1.1.2 Functional degeneration

Degeneration-induced disruption of the ultrastructural morphology of the neuromuscular junction closely parallels the changes that occur in the functional state of nerve terminals. Concurrent with the structural disintegration of the nerve terminal, there is a decline in the ability to release transmitter.

In the rat diaphragm, all nerve terminals retain the ability to transmit impulses for 8-10 hours after nerve section (Miledi and Slater, 1970). This lag period, in which nerve terminals remain functionally normal, is similar to the lag period that occurs during morphological degeneration of nerve terminals. After this time some end-plates lose the ability to produce endplate potentials (EPPs) after stimulation of the distal stump. Failure of EPPs occurs abruptly, without proceeding through a transitory weakened state. Failure of spontaneous MEPPs occurs at the same time as EPP failure. However, Miledi and Slater (1970) presented evidence to indicate that before MEPP failure occurs the overall frequency of MEPPs seems to decline. Nerve terminals functionally degenerate asynchronously: often nerve terminals transmitting as normal neighbour endplates which are functionally silent (Miledi and Slater, 1970). This reflects the morphological data, in that degeneration does not occur simultaneously at all neuromuscular junctions. From 8 hours post axotomy onwards, the number of endplates failing to respond to nerve stimulation progressively increases, until at 20 hours no endplates can transmit (Miledi and Slater, 1970). Various studies have demonstrated that this is dependent on the length of the distal stump (Eyzaguirre, Espildora and Luco, 1952; Luco and Eyzaguirre, 1955; Birks, Katz and Miledi, 1960; Miledi and Slater, 1970; Lubinska, 1982).

2.1.2 Nerve terminal degeneration in mutant C57BL/Wld^s mice

Wallerian degeneration in the C57BL/Wld^s mouse is considerably delayed (Lunn *et al.*, 1989). Severed distal nerves are able to conduct compound action potential for up to 3 weeks, and retain structural integrity for up to one month (Lunn *et al.*, 1989; Tsao *et al.*, 1994; Glass and Griffin, 1991; Glass *et al.*, 1993). In normal mice the structural and functional integrity of nerve terminals declines at an earlier stage than in the main nerve trunk. Is this relationship represented similarly in the Wld^s nerve? Preliminary reports suggested that normal neuromuscular junction and normal terminal morphology, visualised with silver stain, were present up to five days after nerve section in the Wld^s mouse (Crawford *et al.*, 1995). In the preceding report to this thesis, Ribchester and colleagues (1995) demonstrated that neuromuscular transmission, as evaluated from tension measurements and intracellular recordings, was comparable with control levels 3 days after nerve section. The number of innervated fibres declined from this point until, after two weeks, no endplates responded to stimulation. Spontaneous activity persisted even after the loss of EPPs, a phenomenon distinct from that observed in wild type mice (cf. Miledi and Slater, 1970). The physiological evidence for the maintenance of functionally normal nerve terminals was corroborated by FM1-43 imaging. FM1-43 is a styryl dye that labels actively recycling synaptic vesicles. Motor nerve terminals up to 7 days denervated retained the ability to stain and destain with FM1-43, indicating that synaptic vesicles were able to recycle for this period (Ribchester *et al.*, 1995). Furthermore, nerve terminals labelled with zinc iodide osmium (ZIO) to visualise nerve terminal structure appeared normal for up to 7 days after axotomy. After 8 days, however, nerve terminals appeared fragmented and abnormal. Electron micrographs of neuromuscular junctions 5 days after nerve section indicated that mitochondria were morphologically unaltered and that synaptic vesicles appeared normal. Interestingly, one micrograph showed clustering of vesicles close to the plasma membrane. Some terminals also displayed accumulation of neurofilamentous material that was not present in control images. As in wild type mice, the rate of degeneration was dependent on nerve stump length. However, the nerve stump length dependent delay in Wld^s mice was 1-2 days per centimetre of nerve as opposed to ~45 minutes per

additional centimetre of nerve in wild type mice.

To conclude, nerve terminal degeneration in C57BL/Wld^s mice is considerably delayed. Nerve damage results in alterations in motor nerve terminal structure and function after 3 days, progressing until approximately 2 weeks post axotomy when few terminals persist. In the main nerve trunk at this time most axon profiles remain, and Wld^s nerves are still able to conduct a compound action potential after 2 weeks. Therefore, degeneration of nerve terminals in Wld^s mice is considerably delayed, but appears to take place faster than axonal disintegration in the main nerve trunk. However, the precise characterisation of events that occur after nerve section at the neuromuscular junction remains to be elucidated.

The experiments detailed in this chapter address aspects of degeneration in both wild type and Wld^s mice. First, a serial study was undertaken to assess in detail the ability of Wld^s motor nerve terminals to recycle synaptic vesicles for prolonged periods after nerve section. A parallel study investigated the same phenomenon in wild type terminals for comparison. Further experiments analysed Wld^s and wild type nerve terminal morphology after axotomy, using immunocytochemical techniques. The purpose of this study was to elaborate in detail on the similarities and differences between wild type and Wld^s motor nerve terminals after axotomy. A 24 hour time-period study investigated the rate of degeneration in Wld^s nerve terminals *ex vivo*. Morphological data was corroborated with information about the decline of transmitter release in Wld^s nerve terminals post axotomy. Standard electrophysiological techniques were used to investigate quantal content in Wld^s terminals after nerve section, using two independent techniques. This analysis was combined with a study of MEPP frequency post axotomy. To consolidate the morphological and electrophysiological findings a study of both structure and function at the same neuromuscular junction was undertaken.

2.2 MATERIALS AND METHODS

2.2.1 Animal Care

C57BL/Wld^s mice bred in the Department of Medical Microbiology in Edinburgh. This colony (formally designated C57BL/6/EUMM) shares the property of slow Wallerian degeneration with the colony of C57BL/Wld^s mice maintained in Oxford, on which the initial experiments were performed (Lunn *et al.*, 1989). Both of these stocks were originally derived from Harlan-Olac, and appear to be identical (Ribchester *et al.*, 1995, Parson *et al.*, 1997). Animals were ordered direct from Harlan-Olac for the immunocytochemistry studies. Experiments were performed on mice aged 3 weeks to 2 months; previous studies indicated that the property of slow Wallerian degeneration in Wld^s peripheral nerves gradually lessens after 3 months of age, so that that rate of degeneration approaches that of wild-type mice (Perry, Brown and Tsao, 1992; Tsao *et al.*, 1994; Ribchester *et al.*, 1995).

The 'wild type' strains used in these experiments were CBA or C57BL/6J. Experiments were performed on mice age-matched with Wld^s animals, in order to minimise variation.

The structure and function of neuromuscular junctions was examined in *transversus abdominus* (TA) and *flexor digitorum brevis* (FDB) after nerve injury to the intercostal nerves or the tibial (lateral plantar) nerve respectively. The TA muscle is only 2-3 fibres thick in some regions, which has the effect of minimising background fluorescence, and therefore was selected for imaging purposes (vital staining and immunocytochemistry). The TA muscle is innervated by 6 intercostal nerves, distributed over a relatively large area, and as such yields many endplates suitable for detailed morphological examination. The FDB muscle has relatively short muscle fibres (~500μm), which have been shown to be electrically isopotential along their length (Bekoff and Betz, 1977; Ribchester, 1989). This facilitates intracellular recording and subsequent analysis of transmitter release as a correlate to the quantal content of nerve terminals.

2.2.2 Surgery

Mice were anaesthetised by inhalation of halothane (2-5% in N_2O/O_2; 1:1), until heavy sedation and the loss of reflex responses had occurred. The animals were placed on a heat blanket to minimise heat loss during the surgical procedure. The relevant area was shaved and sterilised to reduce risk of infection. All surgical procedures were performed in accordance with UK Home Office Regulations. The animals had free access to water and feed at all other times.

2.2.2.1 Transversus abdominus

Unilateral sections of the left second to eighth intercostal nerves were carried out with small spring scissors. A section of nerve (3-5mm) was removed to ensure complete axotomy. The nerves were severed close to the vertebral column (~0.5cm), in an attempt to equalise the length of the distal portion, and the wound closed with 6/0 Mersilk suture. Occasionally, the intercostal muscle was accidentally breached, causing a pneumothorax to develop. These mice were stabilised and usually recovered immediately after the anaesthetic had worn off. In general, the mice recovered rapidly and did not appear to suffer any breathing difficulties or restriction in movement. The animals were sacrificed 6 hours to 10 days after axotomy, by stunning and dislocation of the cervical vertebrae. The experimental and contralateral muscle and nerve preparations were dissected out, typically leaving the ribcage attached to the muscle, and bathed in normal physiological saline (aerated with 95% O_2 and 5% CO_2) (see Appendix A). These preparations were attached with steel pins to the base of a 3 cm Sylgard-lined chamber and the nerves were checked for signs of axotomy.

2.2.2.2 Flexor digitorum brevis

Mice were prepared as above, and unilateral sections to the tibial nerve of the right hind limb were carried out. A segment of tibial nerve was resected to ensure complete axotomy. Mice were allowed to recover, and sacrificed as above 3 to 13 days after axotomy. Dissected isolated nerve-muscle preparations were bathed in normal physiological saline, aerated with 95% O_2 and 5% CO_2, and pinned in a Sylgard-lined dish by the tendons.

2.2.3 FM1-43 Imaging

FM1-43 is a vital aminostyryl dye that selectively labels synaptic vesicles all species examined to date (for review, see Cochilla, Angleson and Betz, 1999). FM1-43 inserts into the outer leaflet of the vesicular membrane during the endocytic phase of synaptic vesicle recycling, in response to a depolarising stimulus (Figure 2.1). Once loaded, preparations remain visible for several hours. The loading process is reversible, and synaptic vesicles labelled with FM1-43 can be destained by promoting transmitter release over a period of several minutes at high frequencies. Background fluorescence in stained preparations is usually very minimal because FM1-43 is virtually non-fluorescent in aqueous medium, and is considerably more fluorescent when bound to vesicular membranes. This dye is particularly useful because it enables visualisation of living tissue, and facilitates the assessment of synaptic vesicle recycling in motor nerve terminals.

Isolated TA nerve-muscle preparations were first incubated with 5μg/ml TRITC conjugated α-bungarotoxin in normal physiological saline for 20 minutes. Bungarotoxin stains muscle endplates by irreversibly binding to acetylcholine receptors. This facilitates the localisation of neuromuscular junctions, whose nerve terminals may be in a state of degeneration. The α-bungarotoxin solution was then rinsed off and the muscle washed for a further 15 to 20 minutes. The preparation was then bathed in a 2μM solution of FM1-43, in physiological saline containing elevated levels of potassium (50mM; see Appendix A); to compensate, the sodium concentration was reduced by 45mM. Elevating the levels of potassium in the bathing solution has the effect of depolarising the nerve terminal membrane. Depolarisation causes neurotransmitter release, maximally stimulating exocytosis and endocytosis, thus enabling optimal loading of the terminal with FM1-43. Also, vesicles stain even though nerve conduction may be blocked proximal to the neuromuscular junction. In effect, all endplates that are able to recycle synaptic vesicles are loaded with FM1-43, regardless of any other loss of functionality. The preparations were incubated for 4 minutes, then washed with normal saline for a further 20 minutes before imaging.

FIGURE 2.1: STRUCTURE AND FUNCTION OF FM1-43

STRUCTURE OF FM1-43

N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl)pyridinium dibromide

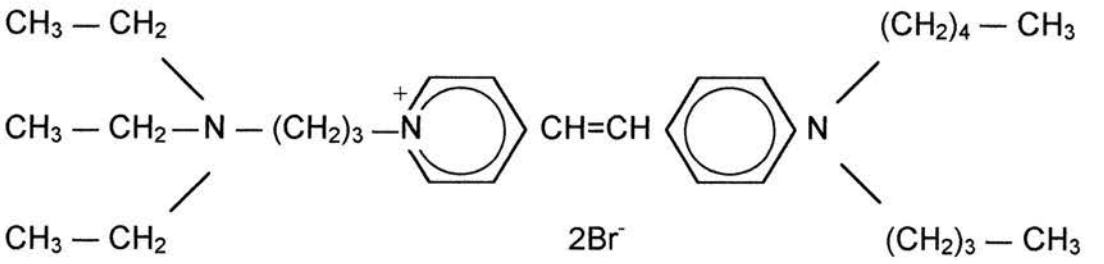
MECHANISM OF ACTION

A. Schematic representation of a mammalian neuromuscular junction. (NT denotes nerve terminal.)

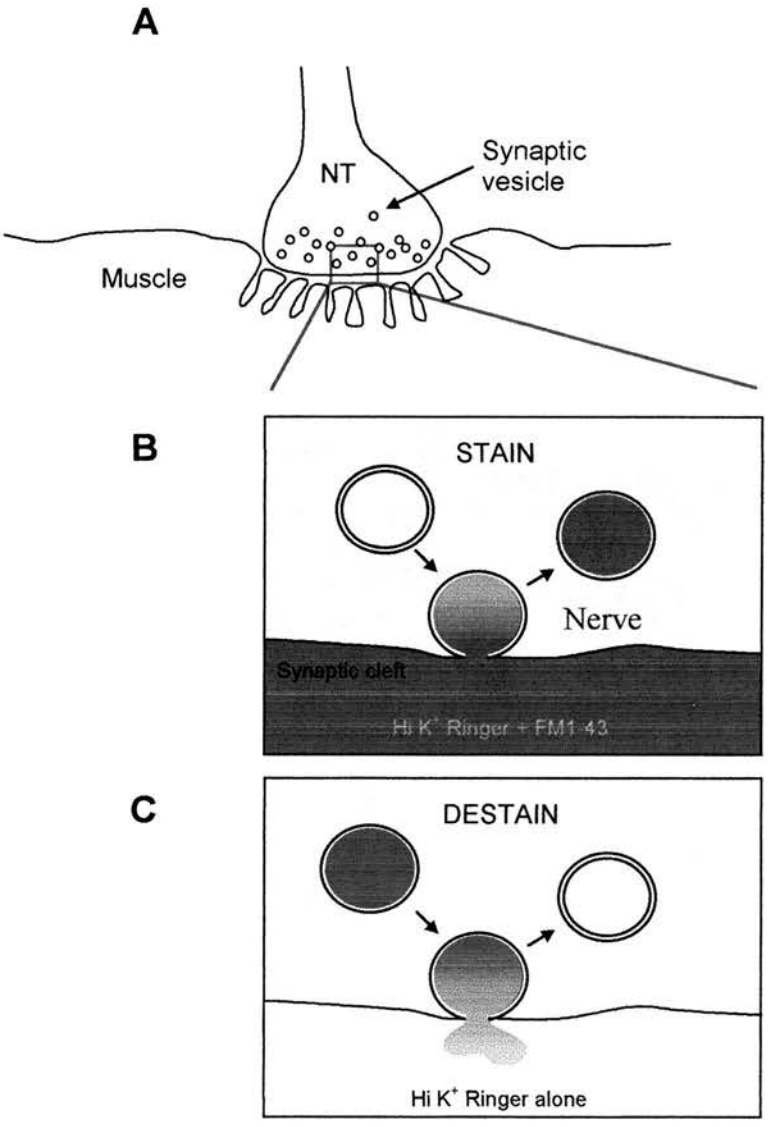
B. FM1-43 staining protocol. Inset depicts synaptic vesicles loading with dye. Isolated nerve muscle preparations were bathed in FM1-43, in physiological saline containing elevated levels of potassium to promote synaptic vesicle exocytosis and endocytosis. The dye is taken up during endocytosis and preferentially partitions into the vesicular membrane.

C. FM1-43 destain. Inset shows dye leaching out of vesicles. Preparations are washed in physiological saline containing elevated levels of potassium, to induce vesicle exocytosis. This allows FM1-43 to diffuse out into the synaptic cleft, down a concentration gradient. Synaptic vesicles destain.

STRUCTURE OF FM1-43



MECHANISM OF ACTION



The preparations were then viewed with a fluorescence microscope using a 100W mercury lamp, and the appropriate filter block. FM1-43 was visualised using a Nikon filter block with a 435 nm excitation filter, 455 nm dichroic mirror and a 515 nm interference barrier filter. TRITC conjugated α-Bungarotoxin was imaged using a Nikon filter block fitted with a 540 nm excitation filter, 580 nm dichroic mirror and a 590 nm interference barrier filter. Images were captured through either a Falcon LTC1160 SIT camera (yielding black and white images) or a Hamamatsu 3 CCD camera (colour images). The video gain and offset were set optimally for the range of fluorescence intensity at each endplate, in order to maximise the visualisation of each neuromuscular junction studied. These images were processed on a Powermac 8500/150 using Openlab, Photoshop or NIH Image (public domain software) software packages. Occupancy, size and area analyses were carried out using Openlab. This program enables the viewer to digitally surround the boundary of the object, which yields a range of precise information about the object such as area, perimeter, shape, pixel intensity, and a number of other parameters. This tool was used in the offline analysis of endplates. Photographic quality images were prepared using Photoshop and printed using a Kodak ColorEase printer.

2.2.4 Immunocytochemistry

Nerve-muscle wholemount preparations were dissected, and treated as follows:

1. Fix for 10 minutes in 4% paraformaldehyde in 1x phosphate buffered saline (PBS) solution.
2. Wash for 1 hour in PBS.
3. Incubate with TRITC conjugated α-Bungarotoxin (5μg/ml) for 20 minutes, followed by 20 minute PBS wash.
4. Place preparation in 100% methanol at –20 °C.
5. Wash 3x10 minutes PBS.
6. Preblock (0.3% Triton-X, 0.2% Bovine Serum Albumin, 0.1% Sodium Azide in PBS) for 30 minutes.
7. Incubate overnight with primary antibodies (goat anti-rabbit NF 1:500, SV2 1:250 in preblock solution) at 4 °C.
8. Wash 3x10 minutes with preblock solution.

9. Incubate >1 hour with secondary antibody (rabbit anti-mouse FITC 1:250 in preblock solution). Cover preparation with tinfoil from this point onwards.
10. Wash 3x10 minutes PBS.
11. Mount and coverslip preparation with Vectashield. Store at 4 °C.

Endplates were imaged and analysed as described in 2.2.3.

2.2.5 Repeated visualization

Nerve-muscle preparations were dissected and stained with FM1-43 and α-Bungarotoxin 3 days after axotomy, as described above. A superficial scan of the preparation yielded the appropriate area for visualisation, and an *en face* endplate was chosen for closer inspection. Neutral density filters of 0.3 and 1.0, which reduce transmitted light by 50% and 90% respectively, were employed to minimise the risk of photobleaching and phototoxicity that may occur as a result of prolonged exposure to fluorescent light. The preparation was superperfused with physiological saline at all times, and both the Sylgard dish and the saline were heated to 37 °C to mimic *in vivo* conditions as closely as possible. The physiological saline was changed every 3 to 4 hours. Endplates were visualised every 2 hours up to 10 hours post dissection, and thereafter at 24 hours. It was sometimes necessary to restain the nerve terminal with FM1-43 after prolonged periods. However, this had the effect of raising the background fluorescence considerably.

2.2.6 Intracellular recording

Intracellular recordings were performed on isolated FDB nerve-muscle preparations 3 to 13 days post axotomy. The preparations were initially bathed in normal physiological saline aerated with 95% O_2 and 5% CO_2, and attached to the base of a Sylgard-lined recording chamber using fine steel pins.

Focal recordings of endplate potentials (EPPs), action potentials and spontaneous miniature endplate potentials (MEPPs) were obtained using standard intracellular recording techniques. Muscle fibres were impaled with glass microelectrodes of approximately 40 MΩ resistance, filled with 3 M potassium chloride. The frequency of MEPP occurrence was monitored for each fibre in one or more 10 second sweeps

on a slow oscilloscope time base. The muscle nerve was electrically stimulated using a suction electrode with pulses of 0.2 ms duration and up to 100 V in amplitude. Action potentials and EPPs evoked by nerve stimulation were recorded. For each muscle, recordings from at least 20 unambiguous muscle fibres were obtained. Muscle fibres apparently unresponsive to nerve stimulation were included in subsequent analyses. Intracellular recordings were digitised using a modified SONY PCM 701ES digital pulse code modulator and recorded on VHS videotape. Measurements and analyses were carried out either online or offline, using a CED1401+ interface and a personal computer, using the WCP software package. All intracellular recordings were performed at room temperature (17-22 °C).

2.2.6.1 Quantal analysis using high magnesium saline

Intracellular recordings were obtained as above. Normal physiological saline was substituted for saline containing elevated levels of magnesium and lowered levels of calcium (high magnesium saline: see Appendix A). An estimate of quantal content can be made by analysing the postsynaptic EPP (see below). Neuromuscular transmission requires entry of calcium ions into the nerve terminal, and therefore is weakened by suppressing transmitter release with solutions containing reduced calcium ions. Furthermore, magnesium ions antagonize the effect of calcium by blocking calcium channels. Thus, muscles bathed in high magnesium/low calcium solution have suppressed neurotransmitter release, which prevents the generation of action potentials leaving EPPs. The quantal content can subsequently be measured from EPPs in the muscle fibre. This method has limitations: the use of high magnesium suppresses the overall transmitter release from the nerve, and therefore measurement of quantal content may result in a reduced estimate. A more accurate measure of quantal release can be made by using μ-conotoxin, which suppresses the muscle action potential without affecting the EPP.

Only muscle fibres that responded to nerve stimulation were further investigated. The response of axotomized and contralateral muscles following repetitive stimulation of the nerve (100 times; at 2Hz) was recorded as above. These responses were subsequently processed offline, and an assessment of transmitter release was made using the 'failures method' of quantal content analysis. This method uses the

following equation: M = ln (no. trials/no. failures), where M denotes the quantal content. This formula estimates the probability of transmitter release based on the number of failures to respond to repetitive nerve stimulation. Another method of analysing the quantal content uses the 'variance' method, described as follows: $M = 1/(cv)^2$, where cv = standard deviation (σ)/ mean amplitude. This method takes into account the variability of EPP amplitude during repeated stimulation. A comparison of the two methods demonstrated that in most cases these analyses yielded similar results, and the failures method was chosen for the analysis of quantal release at axotomized Wld^s neuromuscular junctions.

2.2.6.2 Endplate potential analysis using μ-conotoxin

Intracellular recordings were obtained as detailed above. μ-conotoxin (2μM) was added to normal physiological saline at a concentration sufficient to abolish the muscle action potential. The preparation was bathed in this solution for 15 minutes or until all muscle action potentials ceased before intracellular recording was performed. Only muscle fibres that responded to nerve stimulation were further investigated. The response of axotomized and contralateral muscle to repetitive nerve stimulation (100 times) was recorded, and assessed offline for the number of failures.

2.2.6.3 Intracellular recording from identified junctions

TA nerve terminals 4 days post axotomy were stained with 2μM FM1-43 as detailed above (see 2.2.3). Labelled nerve terminals were briefly visualised, and a suitable endplate chosen for intracellular recording. Recording microelectrodes were filled with potassium chloride solution. The microelectrode was carefully guided towards the selected muscle fibre under the microscope. TA nerve muscle preparations were bathed in physiological saline containing μ-conotoxin (see above, 2.6.1.2) in order to abolish muscle action potentials. Intracellular recordings were performed as detailed above (see 2.6). Recordings were only made from neuromuscular junctions that were occupied by a visibly stained nerve terminal. Several muscles fibres were recorded from in this manner, and then the preparation was stained with TRITC conjugated α-Bungarotoxin (see 2.2.3) in order to label acetylcholine receptors. An analysis of endplate occupancy was performed in order to determine direct correlations between

nerve terminal structure and function. Electrophysiological recordings in part of this study were performed by Ellen Costanzo.

2.3 RESULTS

2.3.1 Functional and morphological assessment of C57BL/Wld^s nerve terminals after nerve section

The morphology of motor nerve terminals in C57BL/Wld^s mice after nerve injury was examined in three ways. First, FM1-43 loading combined with TRITC conjugated α-Bungarotoxin allowed the visualisation of recycling synaptic vesicles and acetylcholine receptors respectively. This technique permitted the investgation of living neuromuscular junctions. Second, nerve terminals in fixed preparations were examined using immunocytochemical techniques to stain NF and SV2 proteins. Third, nerve terminals were visualised over a 24 hour period to investigate the rate of morphological changes after nerve section.

2.3.1.1 FM1-43 imaging

Motor nerve terminals of Wld^s mice were stained with the vital dye FM1-43 and TRITC α-Bungarotoxin to assess the ability of synaptic vesicles to recycle over a period of 3 to 10 days after nerve lesion.

Unoperated Wld^s nerve terminals loaded with FM1-43 were identical to unoperated wild type mouse motor nerve terminals, indicating that there were no obvious morphological abnormalities of adult motor nerve terminals due to the Wld mutation. Low power observations of nerve muscle preparations revealed that motor nerve terminals were characteristically distributed in a wide band across the middle third of the muscle. Within this central band, neuromuscular junctions were segregated into a series of endplate regions, concordant with the segmental innervation of the TA muscle by the intercostal nerves 2 to 6. In most muscles, approximately a third of all endplates were clearly visible and *en face*, such that the majority of nerve terminal and acetylcholine receptors staining were visible. These junctions were selected for further investigation. The staining pattern of FM1-43 almost exactly matched the distribution of acetylcholine receptors. The complete overlap of staining pattern

between the dyes was therefore designated '100% occupancy', to represent complete occupancy of the synaptic gutter by the nerve terminal (Figure 2.2).

FM1-43 staining confirmed that synaptic vesicles in axotomized Wld^s nerve terminals remain able to recycle for a relatively long period of time (cf. Ribchester *et al.*, 1995). At 3 days nearly all terminals examined displayed 100% occupancy, that is the FM1-43 staining pattern completely corresponded to the receptor distribution. From this time onwards the number of nerve terminals stained with FM1-43 declined until, at 10 days post axotomy, no terminals remained stained with the dye (Figure 2.3). The loss of FM1-43 terminal staining occurred asynchronously throughout the nerve-muscle preparation – some endplates presented complete terminal stain whilst neighbouring junctions had very little or no FM1-43 stain (Figure 2.3). Furthermore, the loss of FM1-43 stain did not occur abruptly over the entire junction but rather appeared to decline in a fragmentary or piecemeal manner (Figure 2.4). Thus, within a nerve terminal some regions appeared to lose recycling synaptic vesicles earlier than other regions. The loss of FM1-43 stain did not occur randomly, but appeared to localise to specific regions or boutons whose synaptic vesicles were simultaneously affected. The incidence of fragmented nerve terminals (placed into bins as follows: 0%, 1-24%, 25-75%, 76-99% and 100% occupancy) transiently increased from 3 to 5 days post axotomy, thereafter declining (Table 2.1 and Figure 2.5).

The number of endplates that did not exhibit any FM1-43 staining, denoted as '0% occupancy', increased in a linear fashion up to 10 days, at which time no labelled terminals remained. The linearity of the 0% occupied series suggests that the rate of complete loss of terminal staining is relatively constant between 3 and 10 days post axotomy. Assuming this, approximately 12.5% of endplates degenerate per day over this time period (Figure 2.6). Furthermore, 26% of all endplates are 100% unoccupied by 4 days after axotomy, whereas at 3 days nearly all endplates had no morphological alterations. This observation, coupled with the estimation of the overall rate of FM1-43 loss, implies that after a variable lag period loss of recycling synaptic vesicles in motor nerve terminals of C57BL/Wld^s mice takes less than 24 hours once initiated.

Many nerve terminals developed a more punctate staining pattern from 4 to 5 days

post axotomy in comparison with wild type terminals. This consisted of highly stained 'bright spots', usually localised to small regions of the nerve terminal (Figure 2.7). This phenomenon was particularly noticeable in nerve terminals that were in advanced stages of loss of vesicular staining.

Endplate size and shape was correlated with the extent of withdrawal that had occurred. Size analysis was conducted to establish whether larger endplates withdrew faster. This theory was proposed in light of the fact that in older Wld^s mice axotomy-induced changes take place at a faster rate (Perry, Brown and Tsao, 1992; Tsao *et al.*, 1994; Ribchester *et al.*, 1995). Older mice in general have larger endplates (see Sanes and Lichtman, 1999). Therefore, it is possible that larger endplates withdraw faster than smaller ones. Morphometric analysis of endplates between 3 and 10 days post axotomy indicated that fragmentation and loss of FM1-43 staining was independent of either size or shape. Both these parameters were measured in the Openlab software package by drawing a digital lasso around the endplate region. Overall, neither large nor small endplates were especially predisposed to loss of vesicle recycling (Figure 2.8, 2.9). Using the Spearman Rank Correlation test, at 7 and 9 days post axotomy smaller nerve terminals were significantly more persistent than larger terminals, although the correlation coefficient of these regressions were relatively low (-0.243 and -0.3524 respectively, 1 designates perfect correlation for persistent large endplates, -1 vice versa). In addition, the shape of a particular endplate had little influence on the loss of FM1-43 staining (Figure 2.8, 2.10). The shape coefficient was calculated from the formula 4π Area/Perimeter^2, which produces a value of 1.0 for a perfect circle. A value farther from 1.0 would describe a more irregularly shaped endplate. According to the Spearman Rank Correlation, at 6 days post axotomy irregularly shaped endplates were significantly persistent over more regular shaped ones (-0.251, 1 designates perfect correlation for persistent regularly-shaped endplates, -1 vice versa). At 8 and 9 days post lesion the converse was true – regular shaped nerve terminals were significantly more persistent (0.345 and 0.4788). At all other days examined, neither round nor irregularly shaped endplates were significantly predisposed to loss of vesicle recycling.

Although the nerve terminal staining pattern clearly changed, the distribution and fluorescent intensity of α-Bungarotoxin stained acetylcholine receptors did not

appear to change over the period examined. Morphometric quantification indicated that the fluorescent intensity of acetylcholine receptor staining did not significantly alter during the period of loss of synaptic vesicle recycling in Wld^s nerve terminals (Figure 2.11). Staining of endplates 3 to 10 days post axotomy was assessed by measuring the pixel intensity of acetylcholine receptors labelled with TRITC α-Bungarotoxin. The pixel intensities of receptors in occupied and unoccupied regions at the same neuromuscular junction were compared. Note that the receptor measurements were gathered from preparations that had been stained only with TRITC conjugated α-Bungarotoxin, then latterly loaded with FM1-43 and the same endplates relocated to assess the extent of loss of FM1-43 stain. This method removed the possibility of 'bleed through' of FM1-43 dye into the α-Bungarotoxin emission spectrum, which would affect quantitative measurement of pixel intensity. The results indicate that the pixel intensity of receptors underlying regions of actively recycling vesicles did not differ significantly from the pixel intensity of receptors below unoccupied regions (paired t-test: $p = 0.434$; see Figure 2.11).

To summarise, Wld^s motor nerve terminals undergo a piecemeal reduction in actively recycling synaptic vesicles from 3 days post axotomy onwards, resulting in the complete loss of all staining by 10 days. Decline in actively recycling vesicles is randomly initiated after a variable time delay, and once begun appears to proceed in a linear fashion until complete. Acetylcholine receptor distribution does not change significantly during this period.

TABLE 2: FRACTIONAL OCCUPANCY OF AXOTOMIZED ENDPLATES

Days post axotomy	Occupancy (as % of total no. endplates)					Total no. endplates/ animals
	0%	1-24%	25-75%	76-99%	100%	
3	0	0	0	9.3	90.7	129/4
4	26.2	2.4	19.0	19.0	33.3	112/4
5	28.2	16.7	12.8	16.7	25.6	118/6
6	58	15	8	9	10	157/6
7	54.5	13.0	11.7	15.6	5.2	87/5
8	56.1	26.8	7.3	4.9	4.9	101/4
9	80	14.3	5.7	0	0	98/3
10	100	0	0	0	0	146/4

FIGURE 2.2: FM1-43 STAINING AT WLD^s NEUROMUSCULAR JUNCTIONS.

FM1-43 staining at unoperated control TA Wld^s neuromuscular junctions.

Images **A** and **C** show acetylcholine receptors labelled with TRITC-conjugated α-Bungarotoxin.

Images **B** and **D** show recycling synaptic vesicles loaded with the vital dye FM1-43. Faint preterminal staining can be detected in **B**.

Note the precise overlap between the distribution of FM1-43 stained synaptic vesicles and postsynaptic acetylcholine receptors. This is denoted as '100% occupancy'.

Scale bar = 20μm

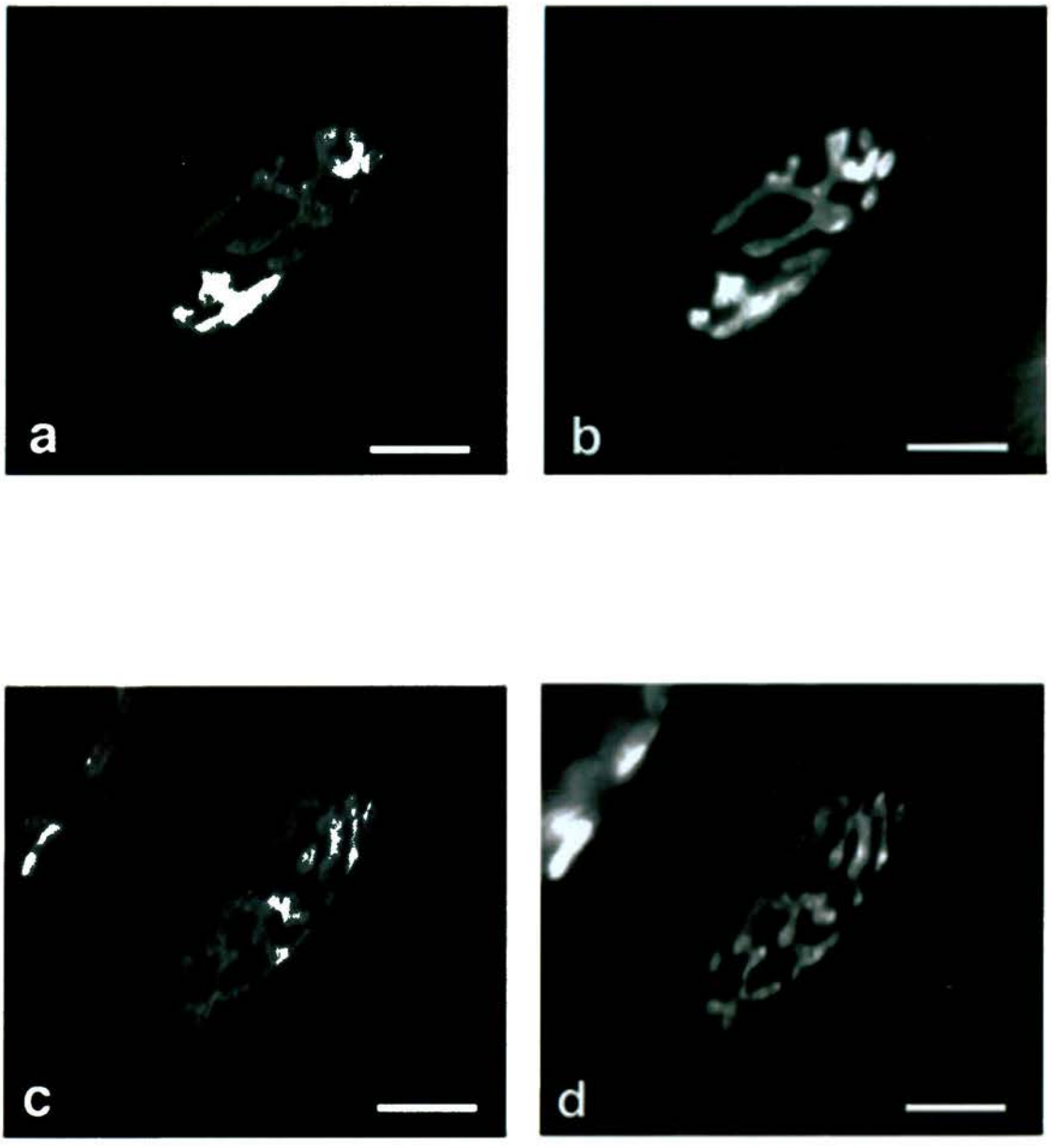

a
b
c
d

FIGURE 2.3: OCCUPIED AND UNOCCUPIED ENDPLATES AT AXOTOMIZED WLD^S NEUROMUSCULAR JUNCTIONS.

Axotomized Wld^S TA neuromuscular junctions, showing complete absence of FM1-43 staining (0% occupancy), and adjacent occupied endplates.

A. and **B**. Acetylcholine receptors labelled with TRITC-conjugated α-Bungarotoxin (**A**), and FM1-43 staining (**B**) from the same neuromuscular junction 8 days after axotomy. There was no FM1-43 staining detected at this junction, suggesting that all synaptic vesicles had stopped recycling. This endplate was denoted as 0% occupied.

C. and **D**. Acetylcholine receptors labelled with TRITC-conjugated α-Bungarotoxin (**C**), and FM1-43 staining (**D**) at two neuromuscular junctions 7 days after nerve lesion. At one junction (located in the middle of the image), no vesicular staining was detected and this endplate was classed as 0% occupied. An adjacent endplate displayed robust FM1-43 staining.

Scale bar = 20μm

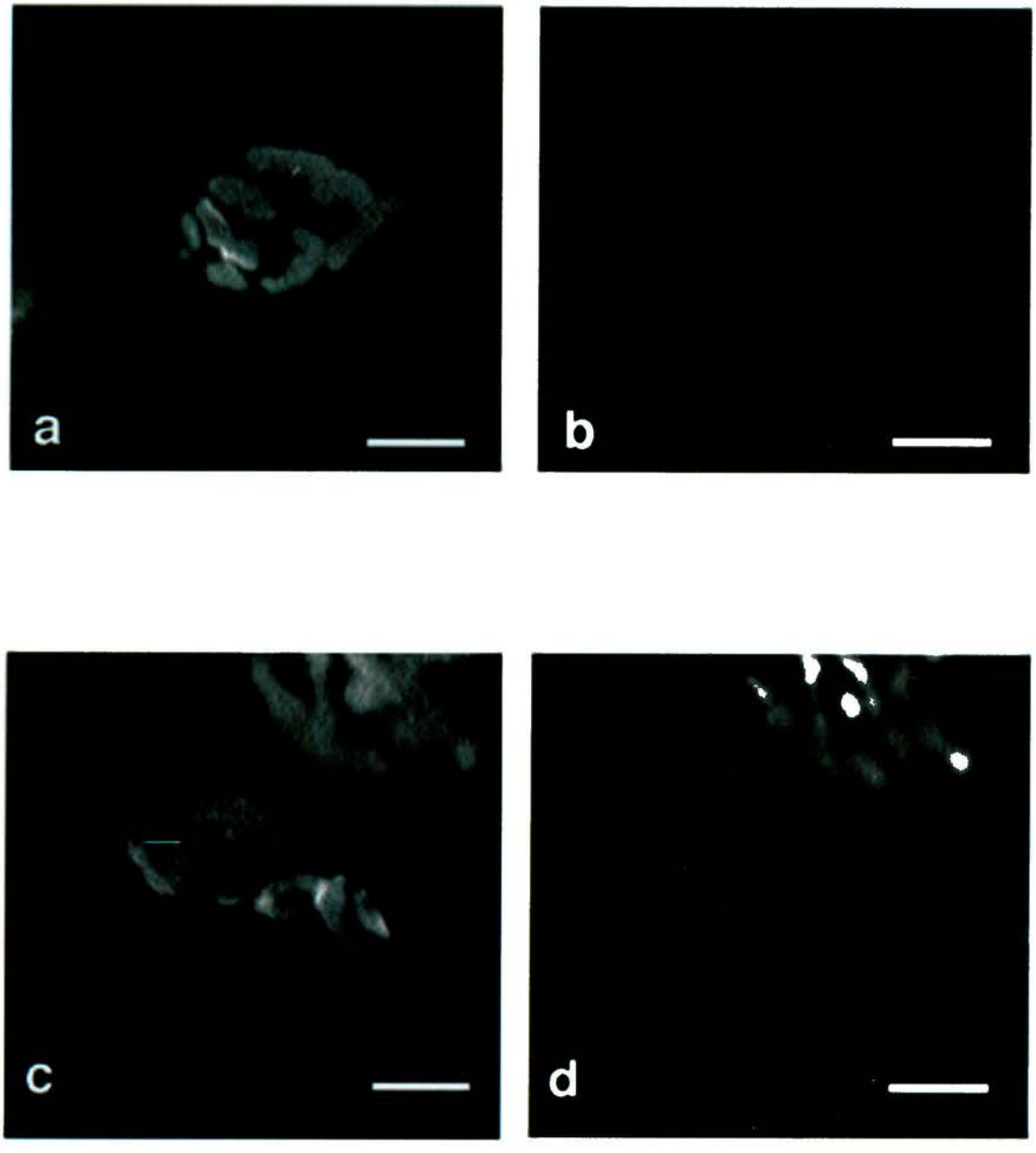

a
b
c
d

FIGURE 2.4: PARTIAL REDUCTION IN NERVE TERMINAL STAINING AT AXOTOMIZED WLD^S NEUROMUSCULAR JUNCTIONS.

Axotomized Wld^S TA neuromuscular junctions, showing piecemeal reduction in endplate occupancy by nerve terminal processes stained with FM1-43.

A and **B**; **C** and **D**; **E** and **F**: Acetylcholine receptors labelled with TRITC-conjugated α-Bungarotoxin (**A**, **C** and **E**), and FM1-43 staining (**B**, **D** and **F**) from the same neuromuscular junction.

A and **B**. Reduction of FM1-43 nerve terminal staining at one bouton 7 days after axotomy (see **B**, red arrow), leaving the underlying acetylcholine receptors unoccupied (see **A**). The preterminal axon is clearly stained.

C and **D**. Wld^S neuromuscular junction 9 days after axotomy. Morphometric measurements indicate that this endplate has 76% occupancy.

E and **F**. Wld^S neuromuscular junction 8 days after axotomy. Large regions of the nerve terminal are not stained with FM1-43 (see **F**). This endplate is 54% occupied.

Scale bar = 20μm

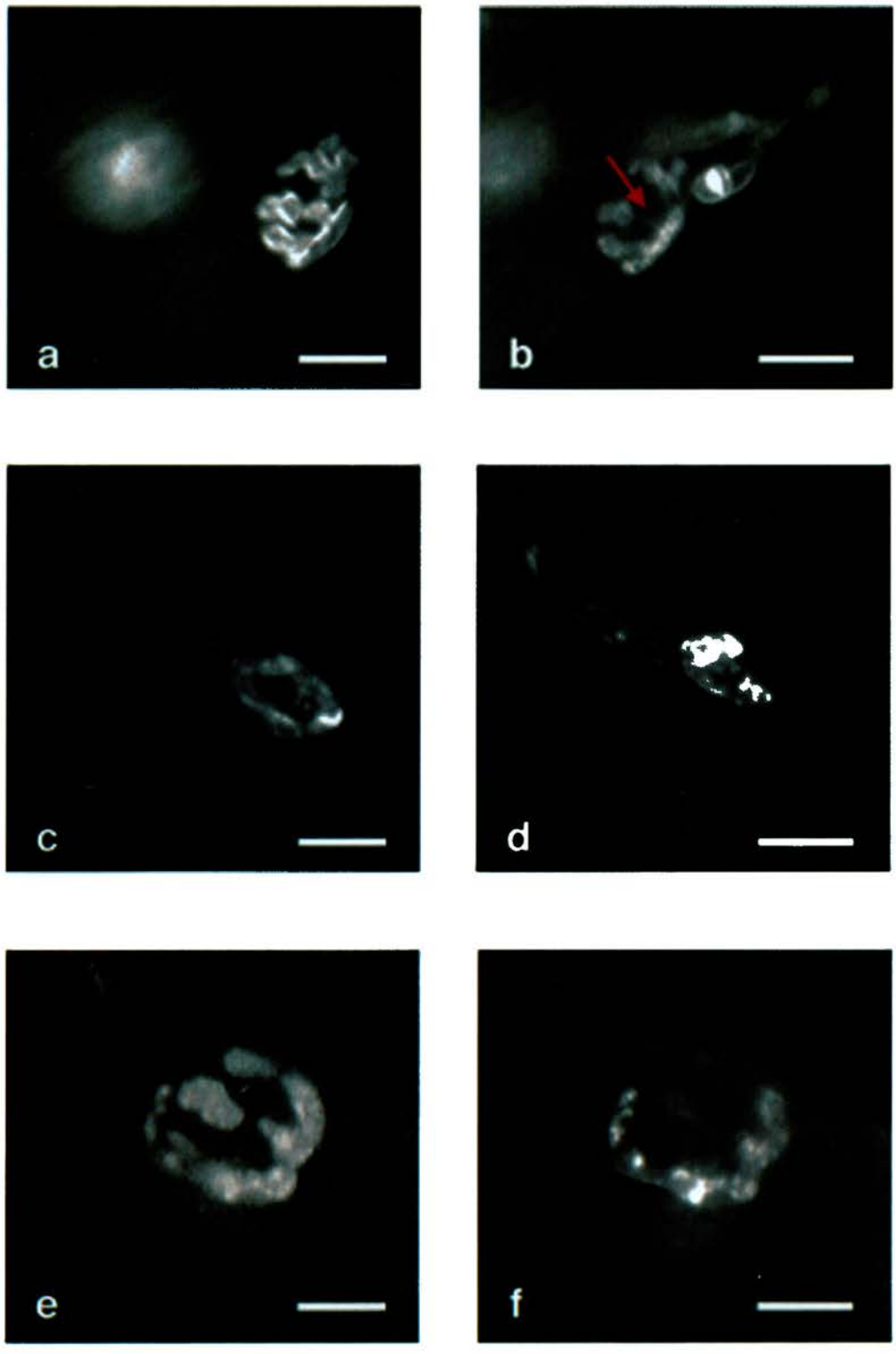

a
b
c
d
e
f

FIGURE 2.5: FRACTIONAL OCCUPANCY OF AXOTOMIZED WLD^S ENDPLATES AS ASSESSED BY FM1-43 STAINING.

Loss of actively recycling synaptic vesicles at axotomized TA neuromuscular junctions. 948 endplates and 36 animals were morphometrically assessed for occupancy criteria. The average occupancy of an endplate is correlated with days following axotomy.

At 3 days post axotomy the average endplate is 99% occupied, and largely remains morphologically unaltered.

From 4 days post axotomy onwards the average occupancy of endplates declines, until at 10 days an average of only 1% of the muscle endplate was covered by actively recycling vesicles, as assessed by FM1-43.

Error bars represent the standard error of the mean.

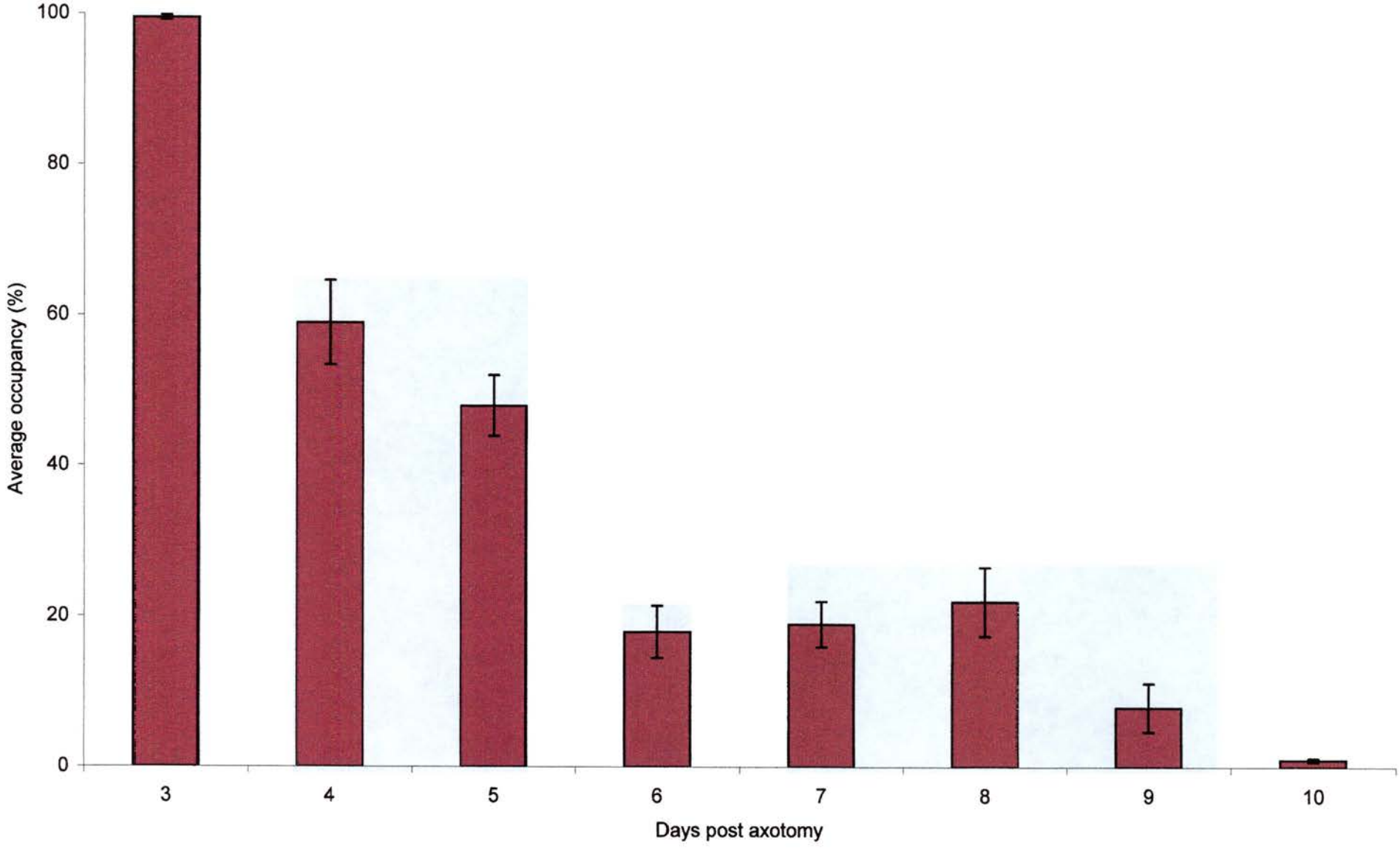

100
80
60
40
20
0
Average occupancy (%)
3
4
5
6
7
8
9
10
Days post axotomy

FIGURE 2.6: RATE OF LOSS OF FM1-43 STAINING AT AXOTOMIZED WLD^S NEUROMUSCULAR JUNCTIONS.

An estimation of the rate of loss of FM1-43 staining at Wld^S TA neuromuscular junctions. Rate of endplates entering 0% occupied state (total loss of FM1-43 stain). The number of endplates that are 0% occupied, taken as a percentage of the total number of endplates examined, is plotted against the number of days post axotomy. The data represent pooled results from 948 endplates and 36 animals. The linearity of the data was assessed using the non-parametric Spearman Rank Correlation. The correlation coefficient, r, was calculated as 0.9286 ($p=0.0022$), which indicates that the number of endplates which become completely unoccupied is directly proportional to the number of days post axotomy. This predicts that these data are nearly perfectly linear. Therefore, the rate of increase in 0% occupied endplates can be calculated using the slope of the linear regression. From this, it is estimated that the number of endplates that become fully unoccupied increases by 12.49% per day from 3 days post axotomy to 10 days post axotomy. Conversely, the number of endplates that remain either fully occupied or partially occupied declines by 12.49% every day. Assuming this rate, 99.92% of nerve terminals entirely lose actively recycling vesicles between 3 and 10 days post axotomy.

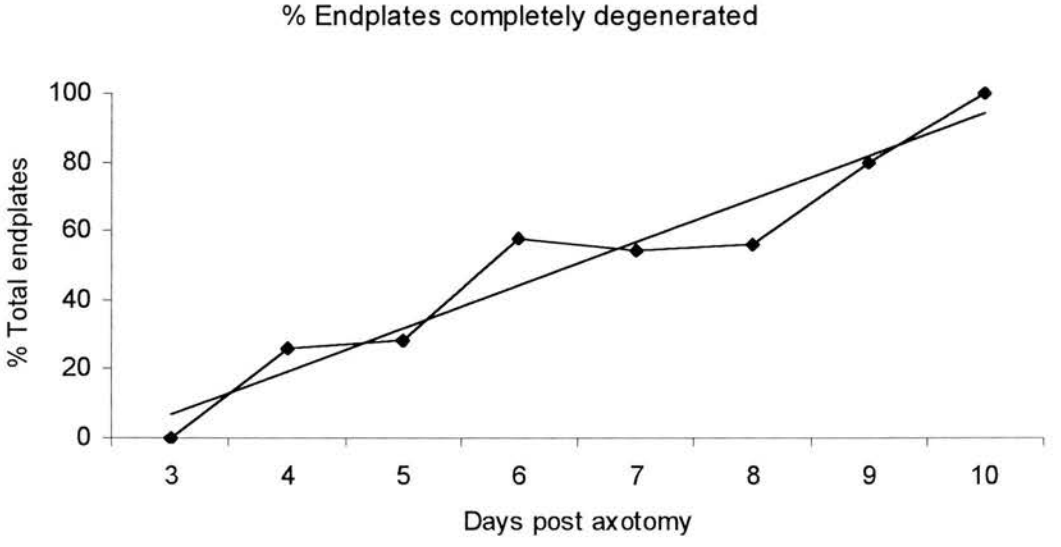


FIGURE 2.7: PUNCTATE NERVE TERMINAL STAINING AT AXOTOMIZED WLD^s NEUROMUSCULAR JUNCTIONS.

Axotomized Wld^s TA neuromuscular junctions in advanced stages of loss of actively recycling vesicles often developed a pattern of highly localised FM1-43 staining.

Acetylcholine receptors labelled with TRITC-conjugated α-Bungarotoxin (**A** and **C**), and FM1-43 staining (**B** and **D**) from the same neuromuscular junction.

A and **B**. Wld^s neuromuscular junction 7 days after axotomy. This nerve terminal is in the advanced stages of loss of FM1-43 staining. Intensely stained regions of synaptic vesicles (see **B**) are represented as bright spots in this image.

C and **D**. Wld^s neuromuscular junction 6 days after axotomy. One bouton appears saturated with FM1-43 dye, perhaps indicating a clustering of recycling synaptic vesicles.

Scale bar = 20μm

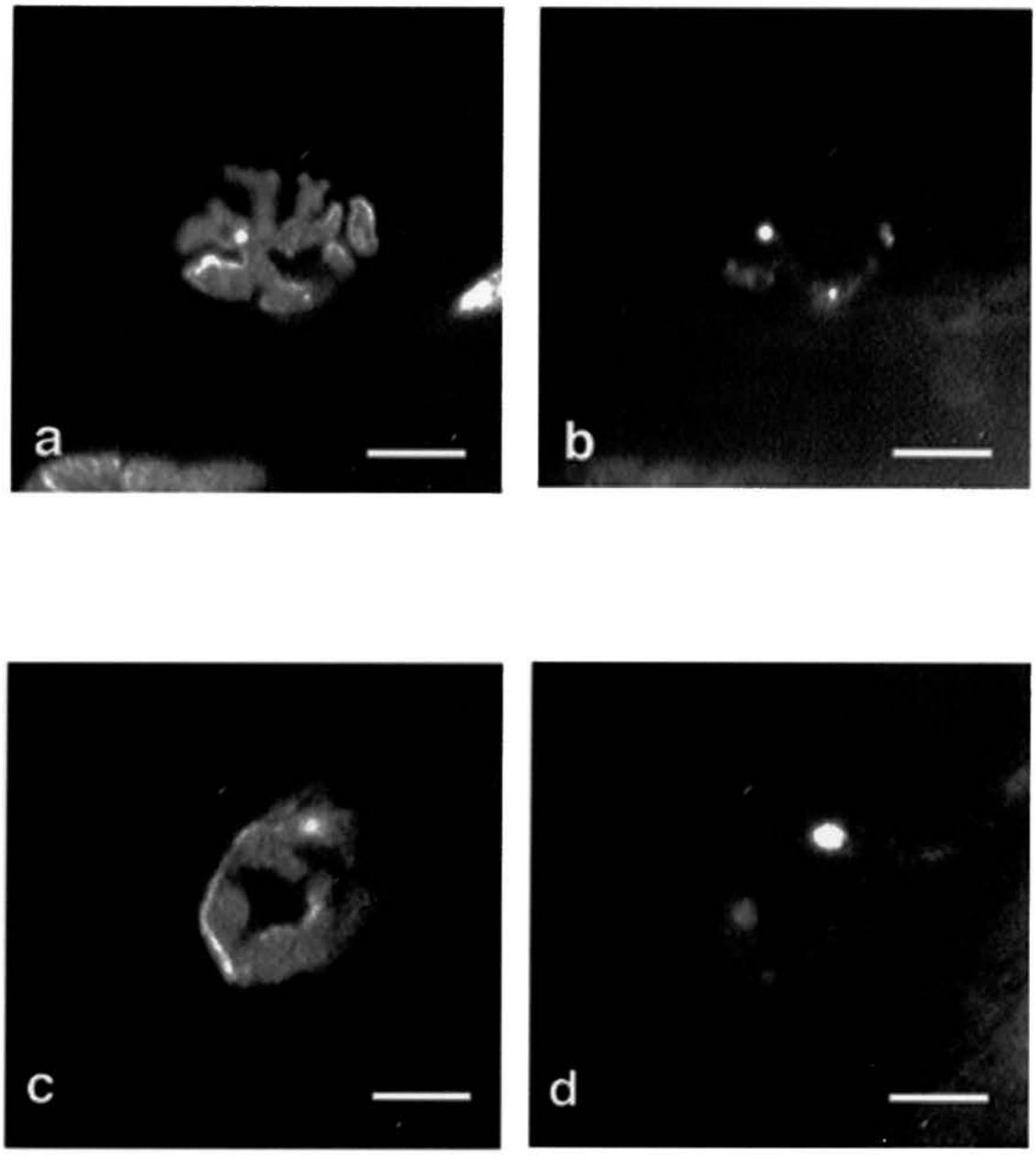

a
b
c
d

FIGURE 2.8: VARIATION IN SHAPE AND SIZE OF NEUROMUSCULAR JUNCTIONS.

In the TA muscle, neuromuscular junctions were of extremely variable shape and size. Distinctly different junctions are represented below.

Acetylcholine receptors labelled with TRITC-conjugated α-Bungarotoxin (**A** and **C**), and FM1-43 staining (**B** and **D**) from the same neuromuscular junction. Both junctions are from control, unoperated Wld^S TA muscles.

A and **B**. Small, circular-shaped neuromuscular junction. Note the compact distribution of nerve terminal boutons. This endplate has a shape coefficient of 0.98.

C and **D**. Large, irregular-shaped neuromuscular junction. This junction is distributed over a large area, with a dispersed patterning of nerve terminal processes and underlying acetylcholine receptors. The shape coefficient for this endplate is 0.20.

Scale bar = 20μm

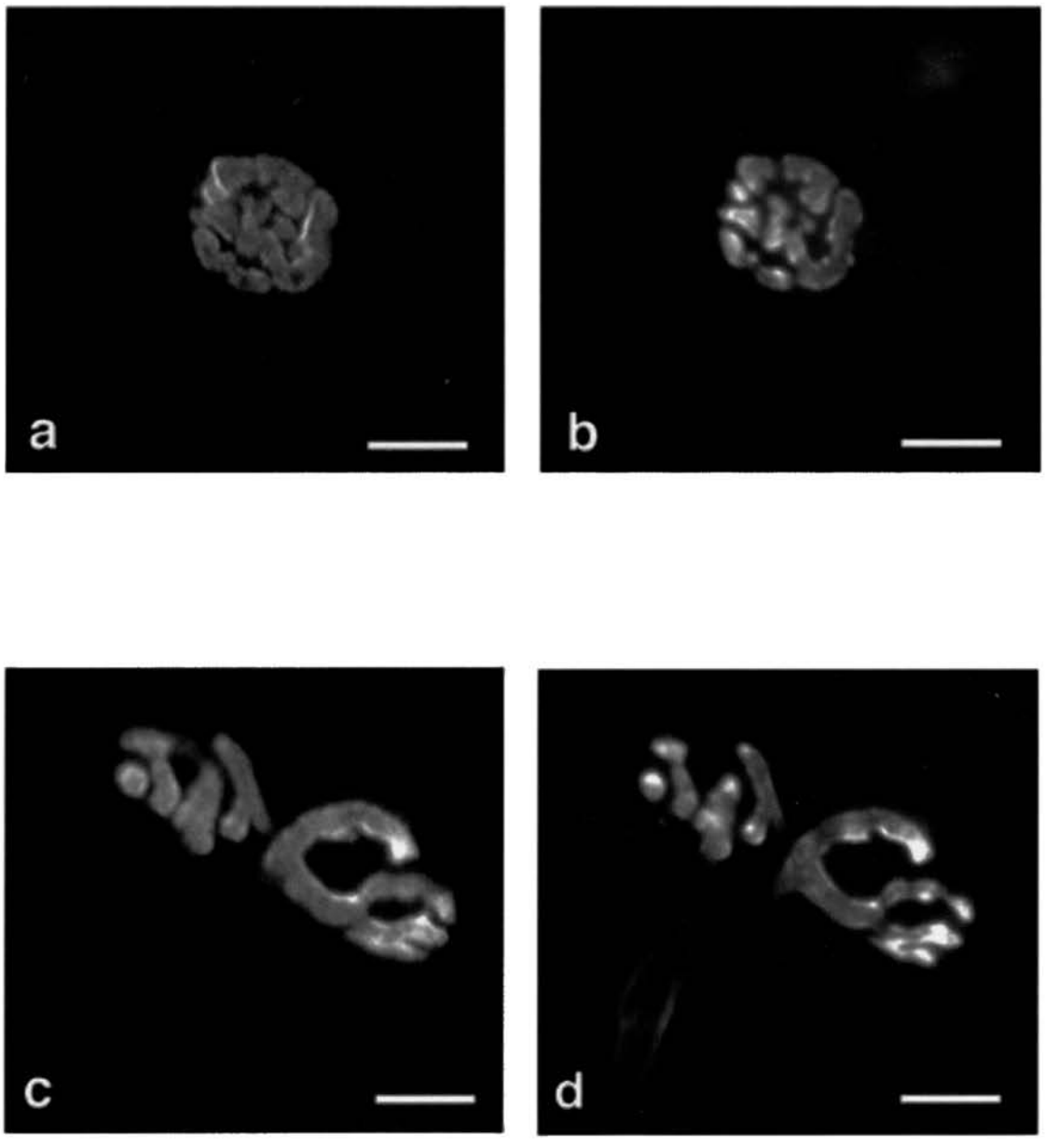
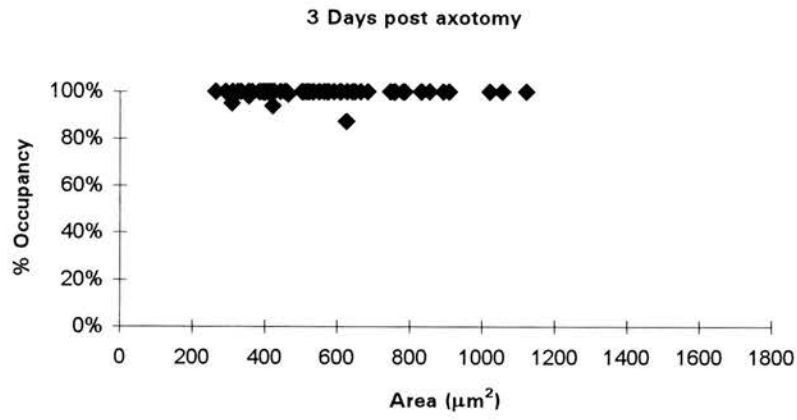

a
b
c
d

FIGURE 2.9: EFFECT OF ENDPLATE SIZE ON THE WITHDRAWAL OF AXOTOMIZED WLD^s NERVE TERMINALS.

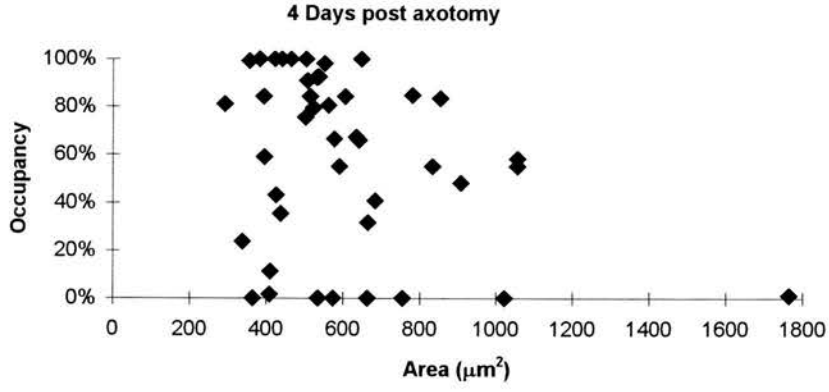
Graphs **A** to **H** (illustrating data from 3 to 10 days post axotomy) show the effect of the size of endplates on the withdrawal of axotomized Wld^s TA nerve terminals. Each point represents one endplate. Some points superimpose. These data are pooled from 948 endplates and 36 animals.

All graphs were statistically analysed using the non-parametric Spearman rank correlation to investigate the relationship between the size of a neuromuscular junction on the withdrawal of the nerve terminal from that junction. This analysis indicated that at 7 and 9 days (see **E** and **G**) post axotomy larger nerve terminals were significantly more predisposed to loss of FM1-43 staining than smaller ones (r = -0.243 and -0.352, p = for 7 and 9 days post axotomy respectively; see trend lines). At all other days there was no significant correlation between the size of an endplate and the withdrawal of the overlying nerve terminal.

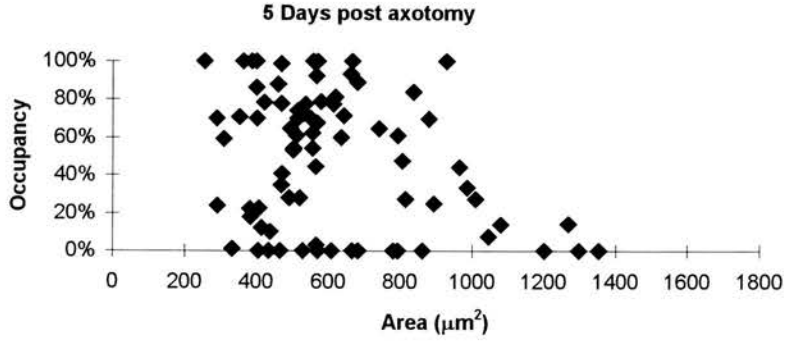
A.


3 Days post axotomy
% Occupancy
100%
80%
60%
40%
20%
0%
0
200
400
600
800
1000
1200
1400
1600
1800
Area (μm²)

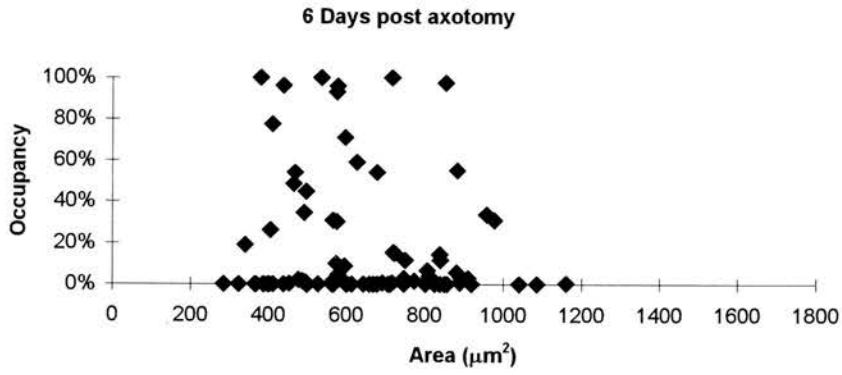
B.


4 Days post axotomy
Occupancy
100%
80%
60%
40%
20%
0%
0
200
400
600
800
1000
1200
1400
1600
1800
Area (μm²)

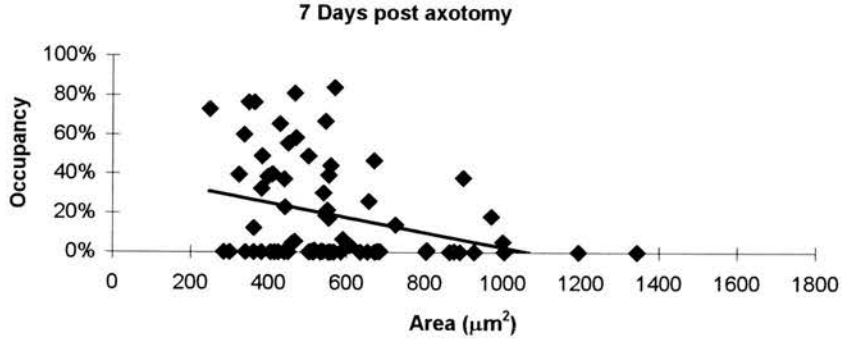
C.


5 Days post axotomy
Occupancy
100%
80%
60%
40%
20%
0%
0
200
400
600
800
1000
1200
1400
1600
1800
Area (μm²)

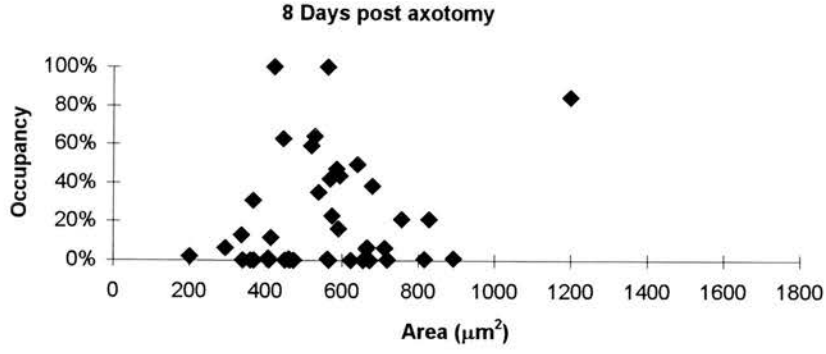
D.


6 Days post axotomy
Occupancy
100%
80%
60%
40%
20%
0%
0
200
400
600
800
1000
1200
1400
1600
1800
Area (μm²)

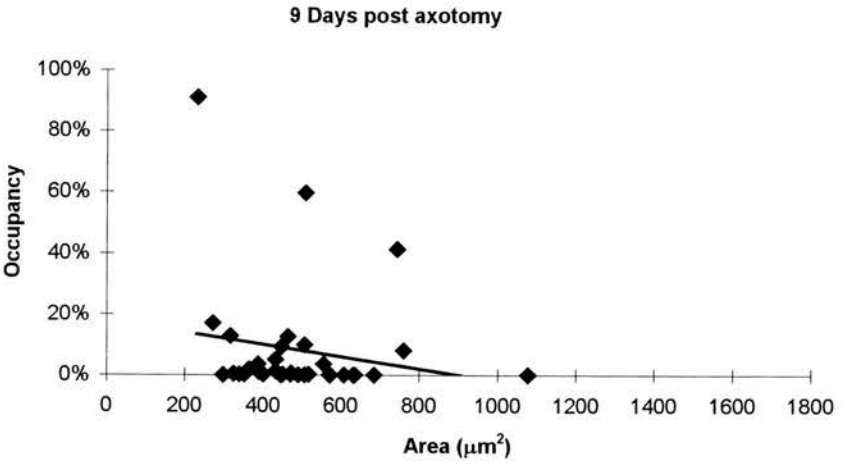
E.


7 Days post axotomy
Occupancy
100%
80%
60%
40%
20%
0%
0
200
400
600
800
1000
1200
1400
1600
1800
Area (μm²)

F.


8 Days post axotomy
Occupancy
100%
80%
60%
40%
20%
0%
0
200
400
600
800
1000
1200
1400
1600
1800
Area (μm²)

G.


9 Days post axotomy
100%
80%
60%
40%
20%
0%
Occupancy
0
200
400
600
800
1000
1200
1400
1600
1800
Area (μm²)

H.

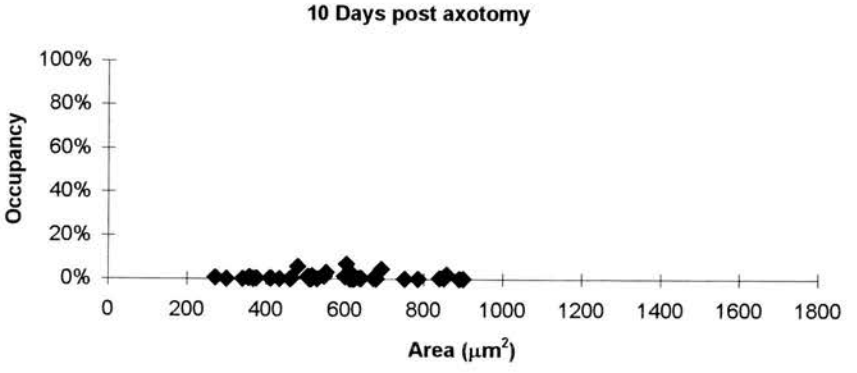

10 Days post axotomy
100%
80%
60%
40%
20%
0%
Occupancy
0
200
400
600
800
1000
1200
1400
1600
1800
Area (μm²)

FIGURE 2.10: EFFECT OF ENDPLATE SHAPE ON THE WITHDRAWAL OF AXOTOMIZED WLD^s NERVE TERMINALS.

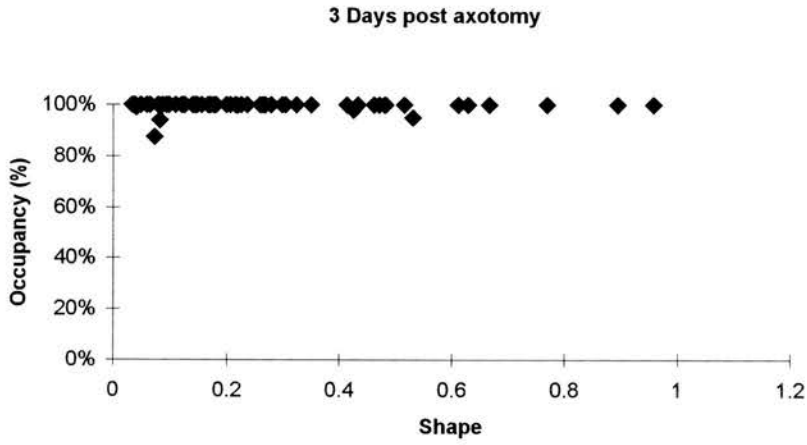
Graphs **A** to **H** (illustrating data from 3 to 10 days post axotomy) show the effect of the shape of endplates on the withdrawal of axotomized Wld^s TA nerve terminals. Each point represents one endplate. Some points superimpose. These data are pooled from 948 endplates and 36 animals.

The shape coefficient of an endplate was measured using the following formula:

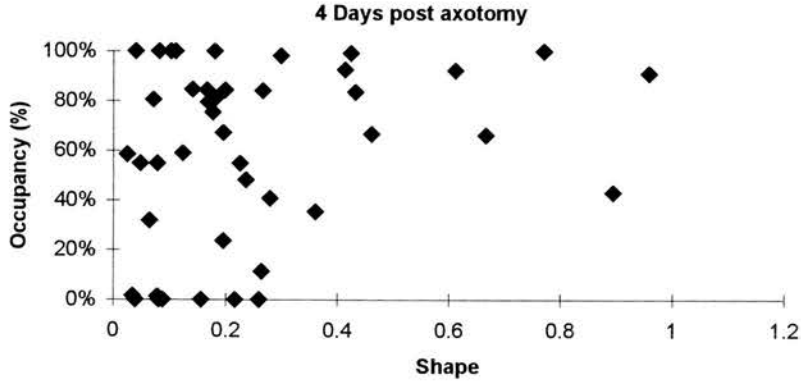
$4\ \pi$ Area/Perimeter^2, which returns a value of 1.0 for a perfect circle. Irregular shaped endplates have shape coefficients farther from 1.0.

These data were analysed using the non-parametric Spearman rank correlation to assess the influence of the shape of an endplate on the withdrawal of Wld^s nerve terminals following axotomy. At 6 days post axotomy (see **D**), irregularly-shaped endplates were significantly more persistent than more regular shaped endplates (r = -0.251, p =; see trend line). At 8 and 9 days (see **F** and **G**) more circular and regular shaped endplates were persistent (r = 0.345 and 0.479, p = for 8 and 9 days post axotomy respectively; see trend lines). At all other time points there was no significant relationship between the shape and probability of withdrawal of nerve terminals.

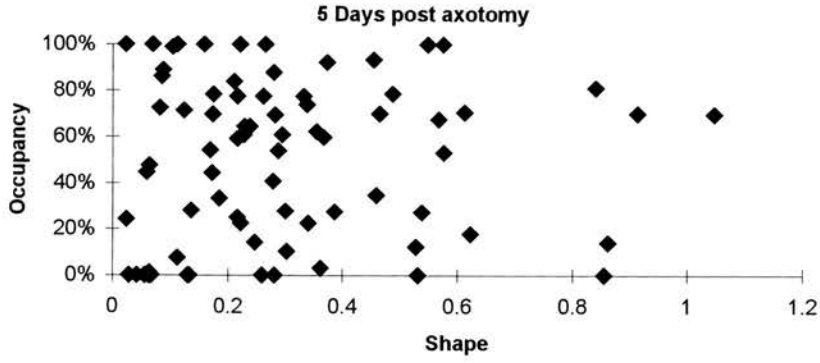
A.


3 Days post axotomy
Occupancy (%)
100%
80%
60%
40%
20%
0%
0
0.2
0.4
0.6
0.8
1
1.2
Shape

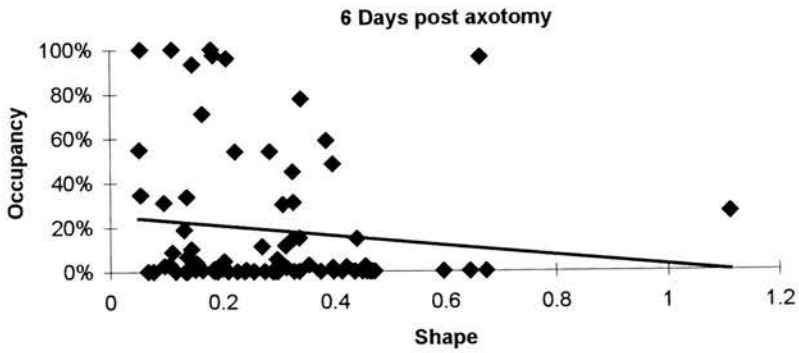
B.


4 Days post axotomy
Occupancy (%)
100%
80%
60%
40%
20%
0%
0
0.2
0.4
0.6
0.8
1
1.2
Shape

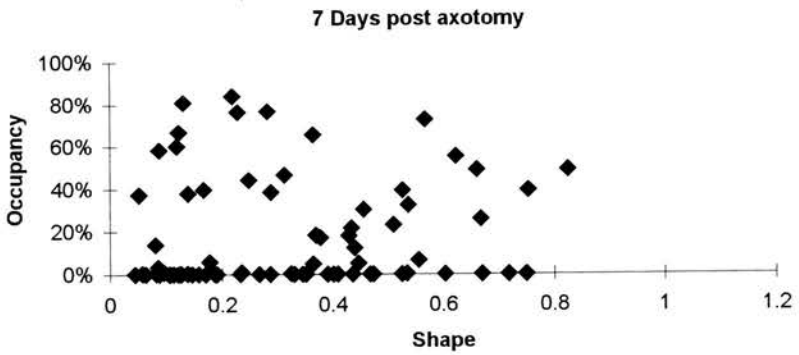
C.


5 Days post axotomy
Occupancy
100%
80%
60%
40%
20%
0%
0
0.2
0.4
0.6
0.8
1
1.2
Shape

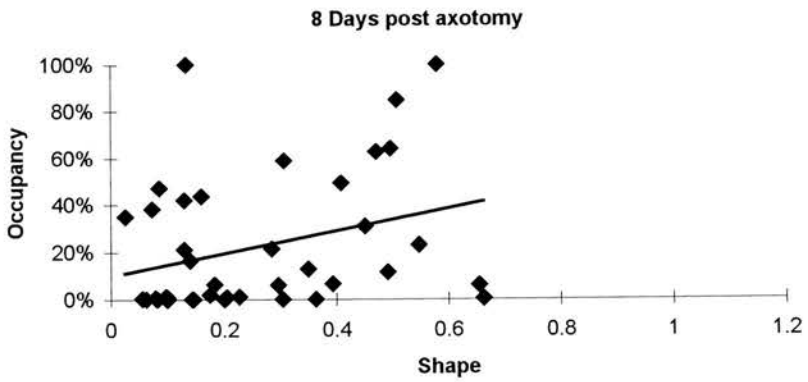
D.


6 Days post axotomy
Occupancy
100%
80%
60%
40%
20%
0%
0
0.2
0.4
0.6
0.8
1
1.2
Shape

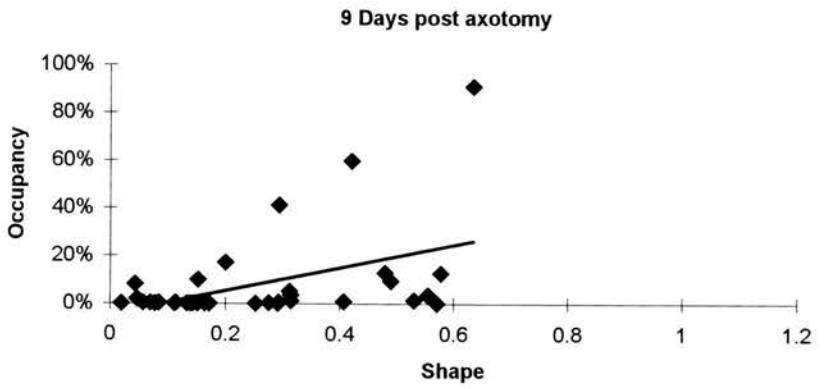
E.


7 Days post axotomy
Occupancy
100%
80%
60%
40%
20%
0%
0
0.2
0.4
0.6
0.8
1
1.2
Shape

F.


8 Days post axotomy
Occupancy
100%
80%
60%
40%
20%
0%
0
0.2
0.4
0.6
0.8
1
1.2
Shape

G.


9 Days post axotomy
100%
80%
60%
40%
20%
0%
Occupancy
0
0.2
0.4
0.6
0.8
1
1.2
Shape

H.

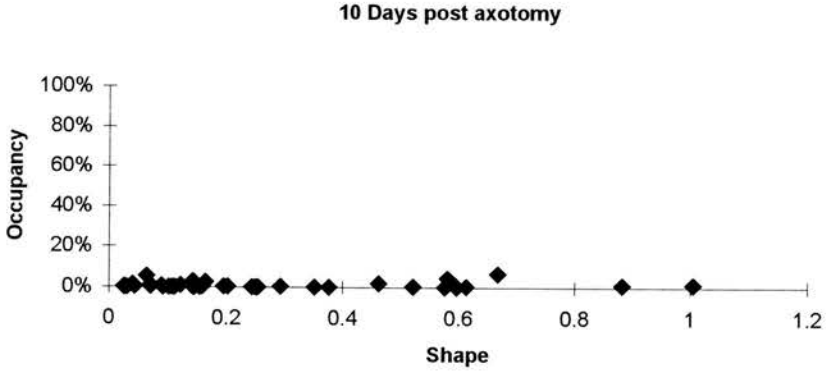
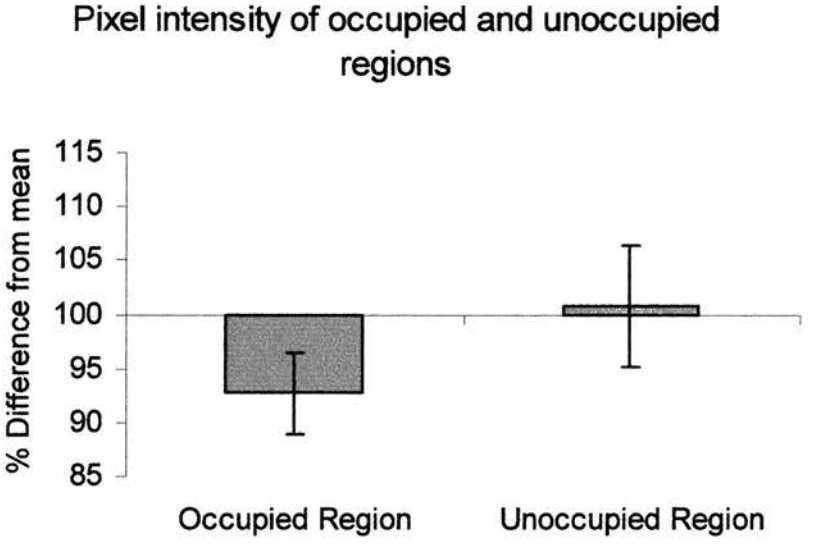

10 Days post axotomy
100%
80%
60%
40%
20%
0%
Occupancy
0
0.2
0.4
0.6
0.8
1
1.2
Shape

FIGURE 2.11: ACETYLCHOLINE RECEPTOR INTENSITY IN OCCUPIED AND UNOCCUPIED REGIONS OF PARTIALLY OCCUPIED ENDPLATES.

Acetylcholine receptor intensity measurements at TA neuromuscular junctions.

The pixel intensity of TRITC α-Bungarotoxin stained receptors was analysed in occupied and unoccupied regions of the same endplate. There was no significant difference (paired t-test, $p = 0.434$) in the pixel intensities of occupied and unoccupied areas of 30 endplates from 8 muscles, 3 to 10 days post axotomy.

Error bars are standard error of the mean.



2.3.1.2 Neurofilament/SV2 imaging

Motor nerve terminals of Wld^s mice 4, 6 and 10 days post axotomy were labelled with antibodies to neurofilament 200 protein (NF) and synaptic vesicle protein 2 (SV2). Acetylcholine receptors were labelled with TRITC conjugated α-Bungarotoxin.

In unoperated nerve terminals NF and SV2 labelled the preterminal axon, including the intramuscular nerve, and the nerve terminal itself. At the neuromuscular junction there was complete overlap between the nerve terminal and distribution of acetylcholine receptors labelled with α-Bungarotoxin (Figure 2.12, and Figure 2.17). This was designated as 100% occupancy, and appeared identical to the overlap between FM1-43 and α-Bungarotoxin staining patterns. The distribution of NF/SV2 labelling in unoperated Wld^s nerve terminals was identical to that observed in unoperated wild type terminals.

In accordance with the persistence of recycling synaptic vesicles, as assessed by FM1-43, NF/SV2 staining was observed in axotomized nerve terminals for up to 10 days post axotomy. The number of 100% occupied endplates gradually declined from 4 to 10 days (Figure 2.13). Correspondingly, the number of 0% occupied endplates increased during this time until at 10 days nearly all endplates examined showed no signs of NF/SV2 labelling. During this time, endplates that were partially occupied endplates transiently increased during this period (see Figure 2.14, and Figure 2.15).

These findings correlate with the data gathered using the FM1-43 staining protocol. A graphical comparison of FM1-43 and NF/SV2 staining procedures is presented in Figure 2.16. Unlike the loss of vesicle recycling, however, which was complete by 10 days post axotomy, some endplates did show signs of NF/SV2 labelling at this time, although it was impossible to distinguish whether this was attributable to labelling of NF or SV2 protein. It must be noted that the presence of NF/SV2 label in nerve terminals 10 days post axotomy was often difficult to discern from background levels. However, in these cases *any* staining pattern over the endplate area was considered to represent residues of the synaptic vesicles and quantified as such.

Similar to the observations of loss of FM1-43 staining, the loss of NF/SV2 proteins after axotomy is asynchronous both between different nerve terminals and within the

nerve terminal itself. Inter-terminal loss of NF/SV2 protein is shown in figure 2.15c, which illustrates fully occupied endplates adjacent to endplates which have no NF/SV2 labelling. Intra-terminal loss, which compares with images obtained using FM1-43 staining, is characterised by a piecemeal reduction in the number of boutons occupying the synaptic gutter (Figure 2.14, and Figure 2.15). Nerve terminals labelled with NF/SV2 were not fragmented, as would be expected if they were undergoing Wallerian degeneration, but rather appeared to be systematically withdrawing from the neuromuscular junction sequentially. An overview of a nerve muscle preparation 6 days after axotomy shows nerve terminals in varying stages of NF/SV2 loss (Figure 2.17). During the withdrawal period the parent axon always persisted, and eventually appeared to absorb its nerve terminal and recede. Thus various stages of withdrawal were apparent, ranging from an almost intact nerve terminal beginning to pull away from an outlying bouton to more a penultimate situation where only one or two boutons remain, connected to the preterminal axon by fine strands (Figure 2.18, and Figure 2.19). In cases where only a small portion of the nerve terminal remained, terminal boutons were always connected to the parent axon; isolated regions of nerve terminal were not observed at any of the 886 endplates assessed, consistent with the proposal that nerve terminal boutons undergo a withdrawal process. The final stage involved the withdrawal of all terminal ramifications into an oval swelling, or retraction bulb, near the margin of the endplate (Figure 2.18). Often one or more swellings were observed proximal to the terminal retraction bulb, resulting in a bead-like appearance of axonal staining. Terminal retraction bulbs and the more proximal swellings were usually oval in shape, although some were almost circular. Measurements of the shortest and longest cross-sectional diameters of 25 terminal retraction bulbs indicated that the average size was 5.4 μm x 9.9 μm (± 0.5 x 1.1 μm), whilst more proximal swellings were larger at 6.4 μm x 11.0 μm (± 1.1 x 1.2 μm). The largest terminal retraction bulbs were observed in preparations 10 days after axotomy. Retraction bulbs were first detected in nerve terminals in the final stages of withdrawing from the neuromuscular junction, from 4 days onwards. As time progressed, the incidence of retraction bulbs increased until at 10 days post axotomy nearly all axons were associated with a terminal retraction bulb. Initially most terminal swellings were

located on or close to the endplate zone. However, in the latter stages (6 days post axotomy onwards) retraction bulbs were most often observed nearby or within the intramuscular nerve. This is illustrated in Figure 2.19D. In sum, axotomized motor nerve terminals of C57BL/Wld^{s} mice withdraw from the neuromuscular junction after nerve section after a variable delay. This withdrawal is characterised by gradual loss of terminal boutons and formation of a retraction bulb, which then recedes from the endplate region into the intramuscular nerve. Withdrawal of the nerve terminal precedes any loss of preterminal axonal labelling.

FIGURE 2.12: NEUROFILAMENT AND SV2 STAINING AT WLD^s NEUROMUSCULAR JUNCTIONS.

NF/SV2 staining at unoperated control Wld^s TA neuromuscular junctions.

A shows acetylcholine receptors labelled with TRITC-conjugated α-Bungarotoxin.

B illustrates nerve terminal boutons and preterminal axons labelled with NF/SV2.

C depicts an image taken using a triple band pass filter block, which allows both red and green fluorescent wavelengths to be visualised simultaneously. Overlap between the green fluorescent nerve terminal and the red fluorescent acetylcholine receptors is visualised as yellow.

Note the precise overlap between the distribution of NF/SV2 stained nerve terminal ramifications and postsynaptic acetylcholine receptors. This is denoted as '100% occupancy'.

Scale bar = 20μm

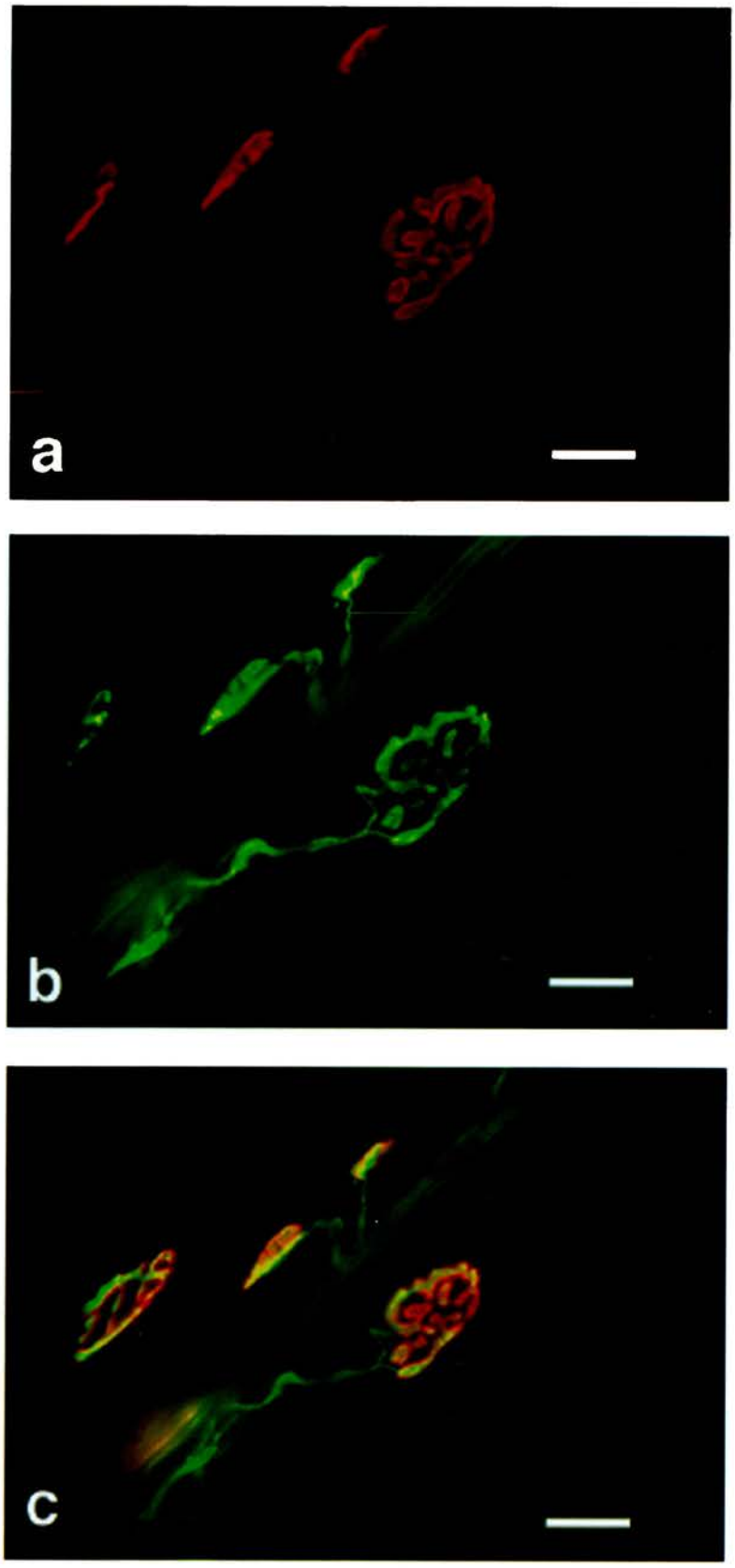

a
b
c

FIGURE 2.13: FRACTIONAL OCCUPANCY OF AXOTOMIZED WLD^s ENDPLATES AS ASSESSED BY NF/SV2 STAINING.

Loss of NF/SV2 labelling at axotomized TA neuromuscular junctions. Endplates from 886 endplates and 13 animals were morphometrically assessed for occupancy criteria, and placed into bins of 0%, 1-24%, 25-75%, 76-99%, and 100% occupancy. Each group is represented as a percentage of the total number of endplates analysed. The number of fully occupied (100%) endplates declines as the number of completely vacated endplates (0% occupied) increases.

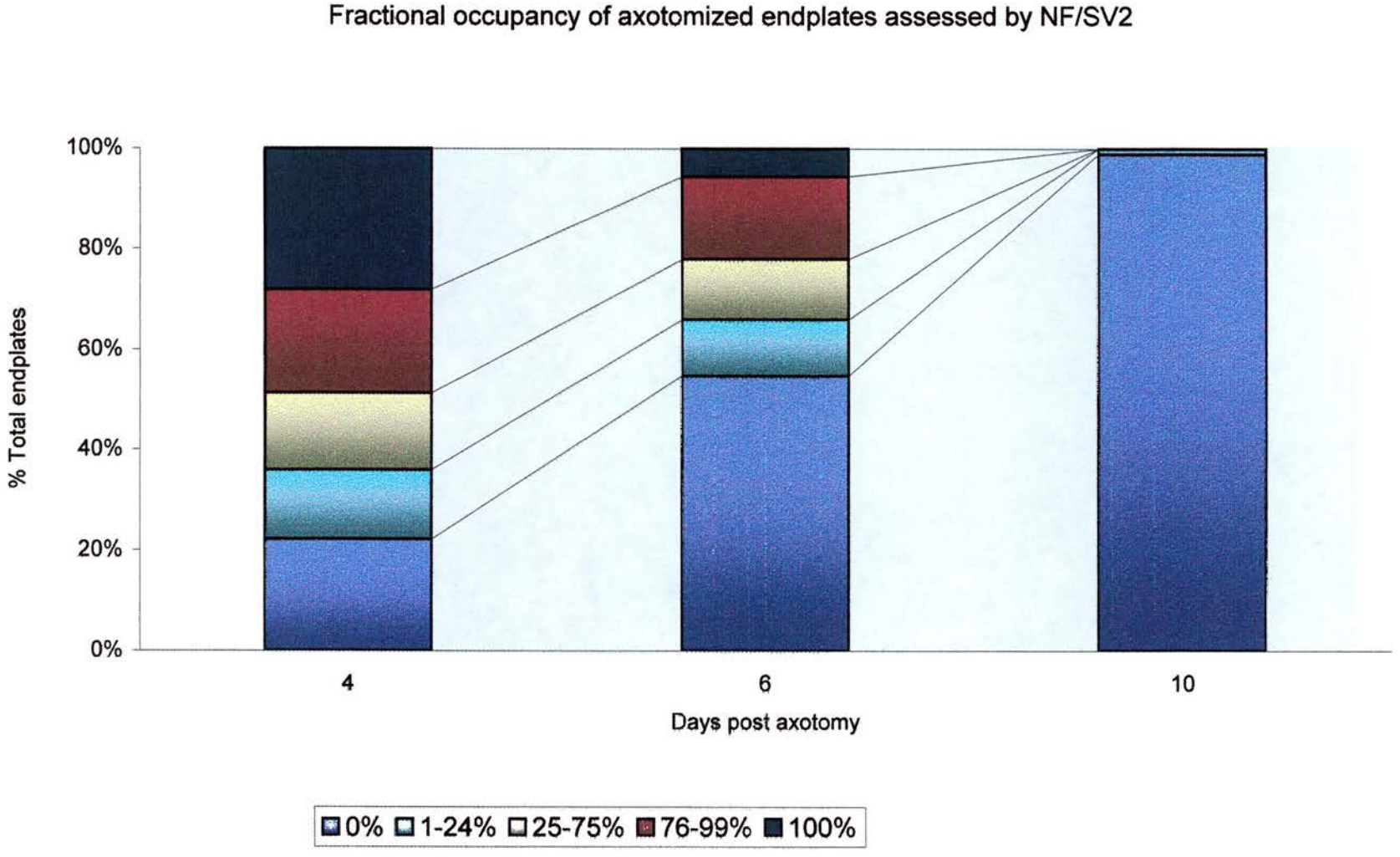

Fractional occupancy of axotomized endplates assessed by NF/SV2
100%
80%
60%
40%
20%
0%
% Total endplates
4
6
10
Days post axotomy
0%
1-24%
25-75%
76-99%
100%

FIGURE 2.14: WITHDRAWAL OF NERVE TERMINAL PROCESSES FROM AXOTOMIZED WLD^s NEUROMUSCULAR JUNCTIONS.

The loss of nerve terminal structure at axotomized Wld^s TA neuromuscular junctions results from the retraction of terminal ramifications from the endplate area.

A illustrates the withdrawal of nerve terminal boutons, leaving unoccupied regions of acetylcholine receptors. Fine strands connect retracting nerve terminal processes to the parent axon.

Scale bar = 20μm

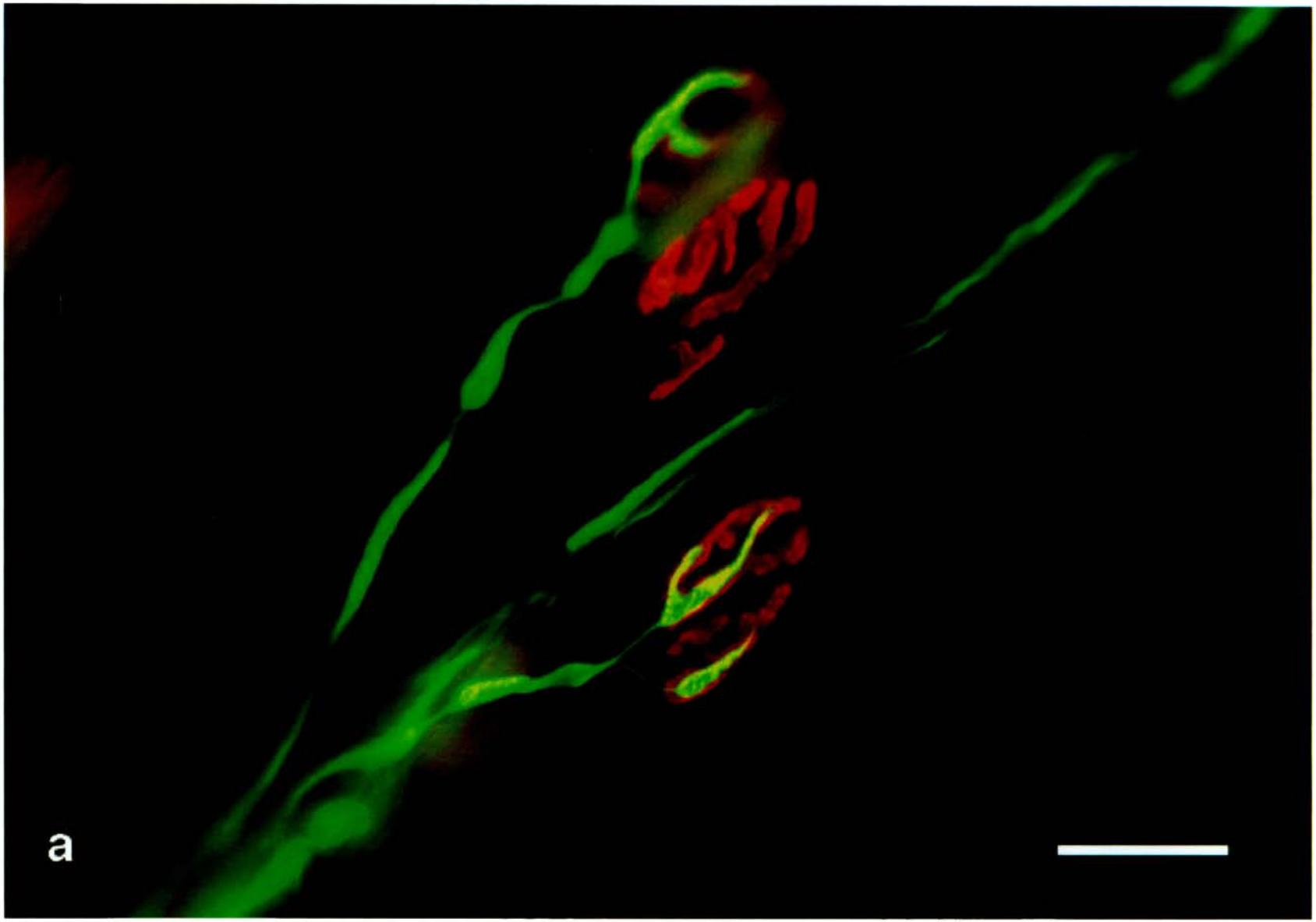

a

FIGURE 2.15: OCCUPIED AND UNOCCUPIED AXOTOMIZED WLD^s ENDPLATES.

Axotomized Wld^s TA neuromuscular junctions, in varying states of nerve terminal withdrawal.

A and **B** show nerve terminals which are in the process of retraction. Vacant regions of acetylcholine receptors, which have been denuded by the withdrawal of terminal boutons, are visualised as red.

C illustrates the inter-terminal variation between the retraction of nerve terminal boutons. Two almost fully occupied endplates lie adjacent to two endplates at which withdrawal is complete.

Scale bar = 20μm

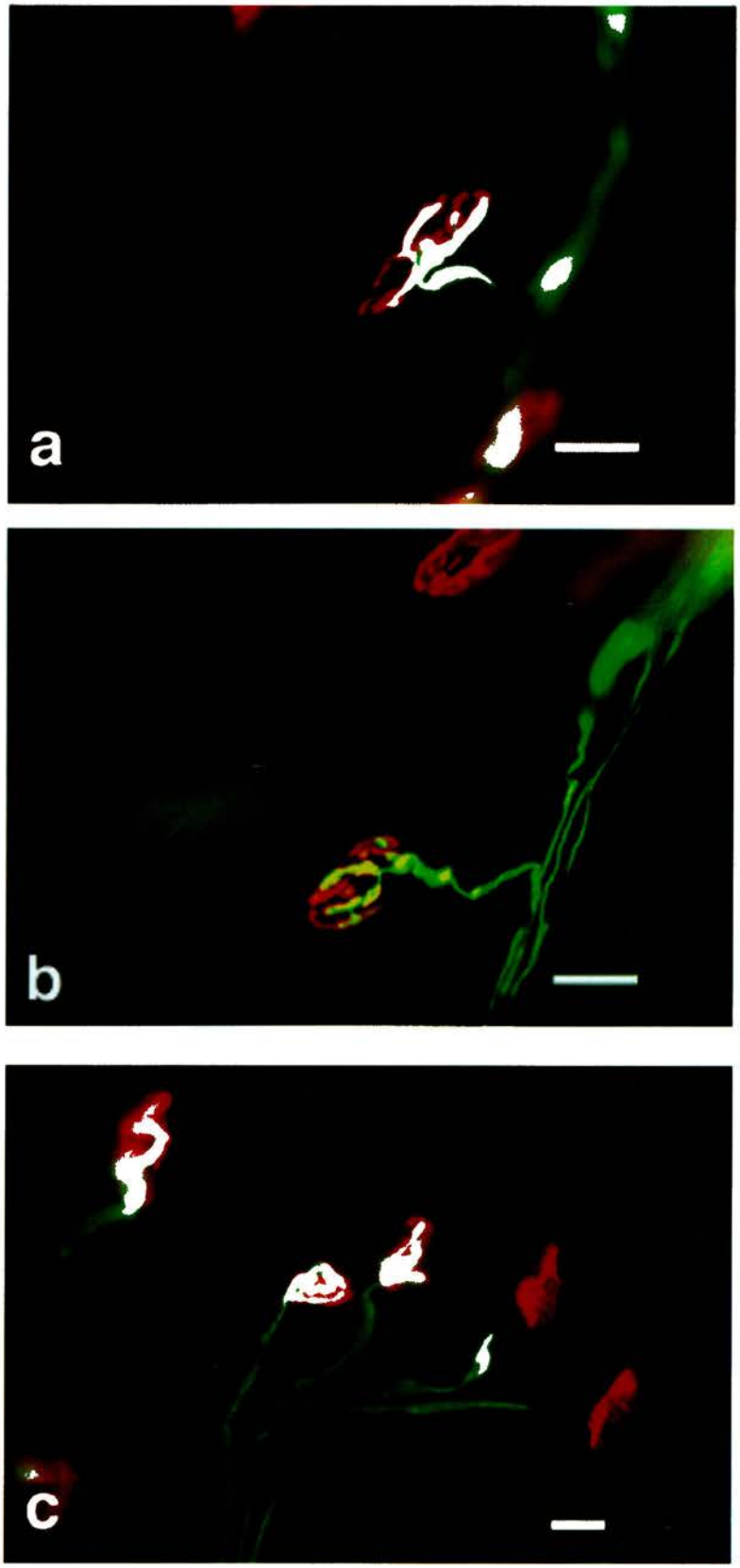

a
b
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FIGURE 2.16: COMPARISON OF FRACTIONAL OCCUPANCY OF AXOTOMIZED WLD^s ENDPLATES AS ASSESSED BY FM1-43 AND NF/SV2 STAINING.

Loss of FM1-43 staining and NF/SV2 labelling at axotomized TA neuromuscular junctions. Loss of NF/SV2 labelling at axotomized neuromuscular junctions.

Loss of actively recycling synaptic vesicles at axotomized Wld^s neuromuscular junctions was assessed at 189 endplates from 5 animals These endplates were measured and placed into bins of 0%, 1-24%, 25-75%, 76-99%, and 100% occupancy. Each group is represented as a percentage of the total number of endplates analysed.

NF/SV2 labelled endplates from 886 endplates and 13 animals were morphometrically assessed for occupancy criteria, and placed into bins of 0%, 1-24%, 25-75%, 76-99%, and 100% occupancy. Each group is represented as a percentage of the total number of endplates analysed.

These data indicate that there is a close correspondence between the two techniques used for labelling Wld^s nerve terminals following axotomy.

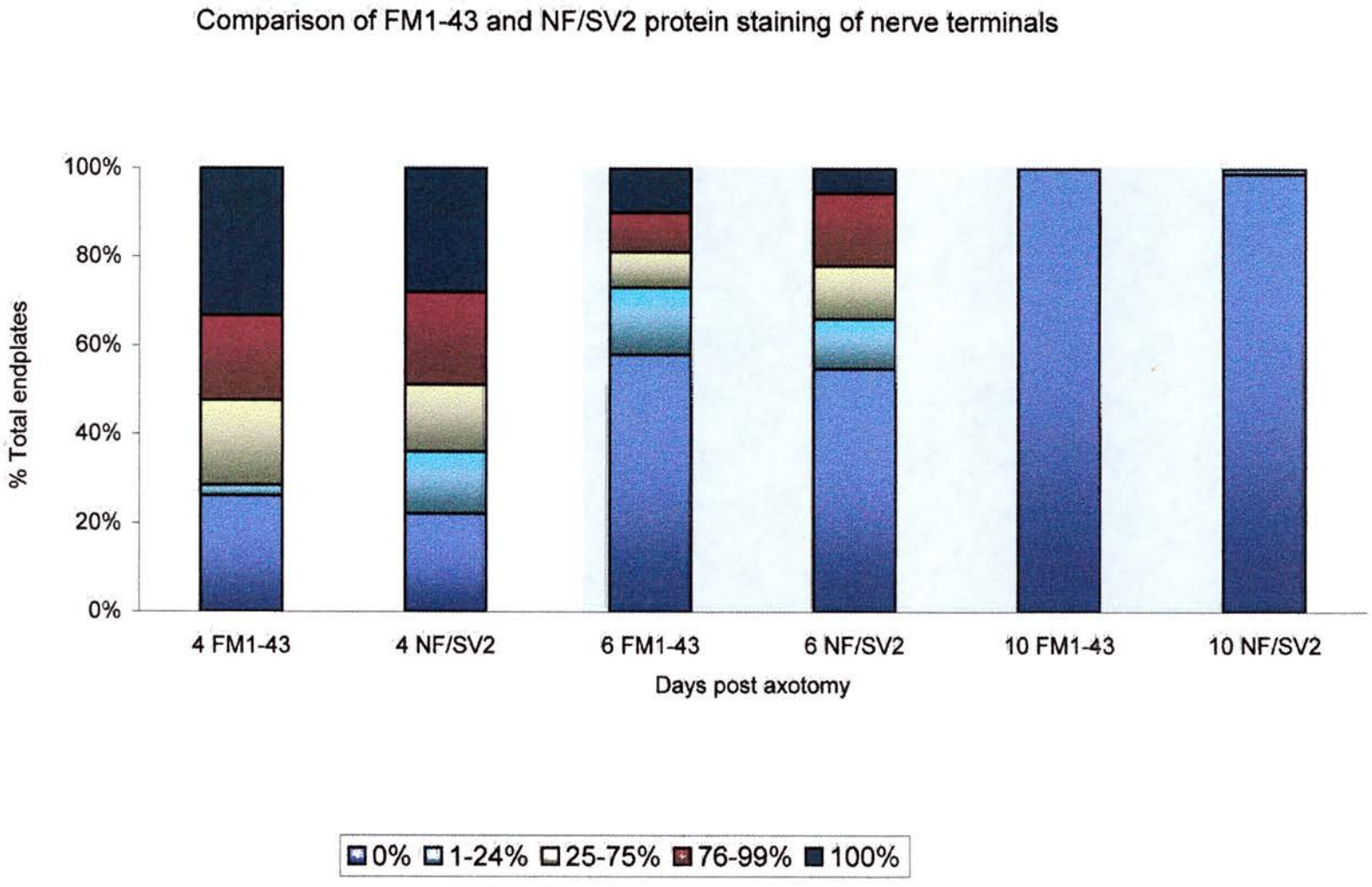
Comparison of FM1-43 and NF/SV2 protein staining of nerve terminals
% Total endplates
100%
80%
60%
40%
20%
0%
4 FM1-43
4 NF/SV2
6 FM1-43
6 NF/SV2
10 FM1-43
10 NF/SV2
Days post axotomy
0%
1-24%
25-75%
76-99%
100%

FIGURE 2.17: COMPARISON OF CONTRALATERAL CONTROL AND AXOTOMIZED WLD^s NEUROMUSCULAR JUNCTIONS.

Low power images of unoperated contralateral control and 6 day axotomized Wld^s TA neuromuscular junctions.

A shows x10 magnification of a control Wld^s TA muscle immunocytochemically labelled with antibodies to NF/SV2. All motor endplates are innervated by axons. Axons in intramuscular nerve bundles are clearly visible.

B shows an overview (x10 magnification) of a 6 day axotomized Wld^s TA muscle. Many endplates are unoccupied by nerve terminals. There is evidence of nerve terminal withdrawal at some endplates. Axons in the intramuscular nerve bundle are still visibly connected to retracting nerve terminal processes.

Scale bar = 100μm

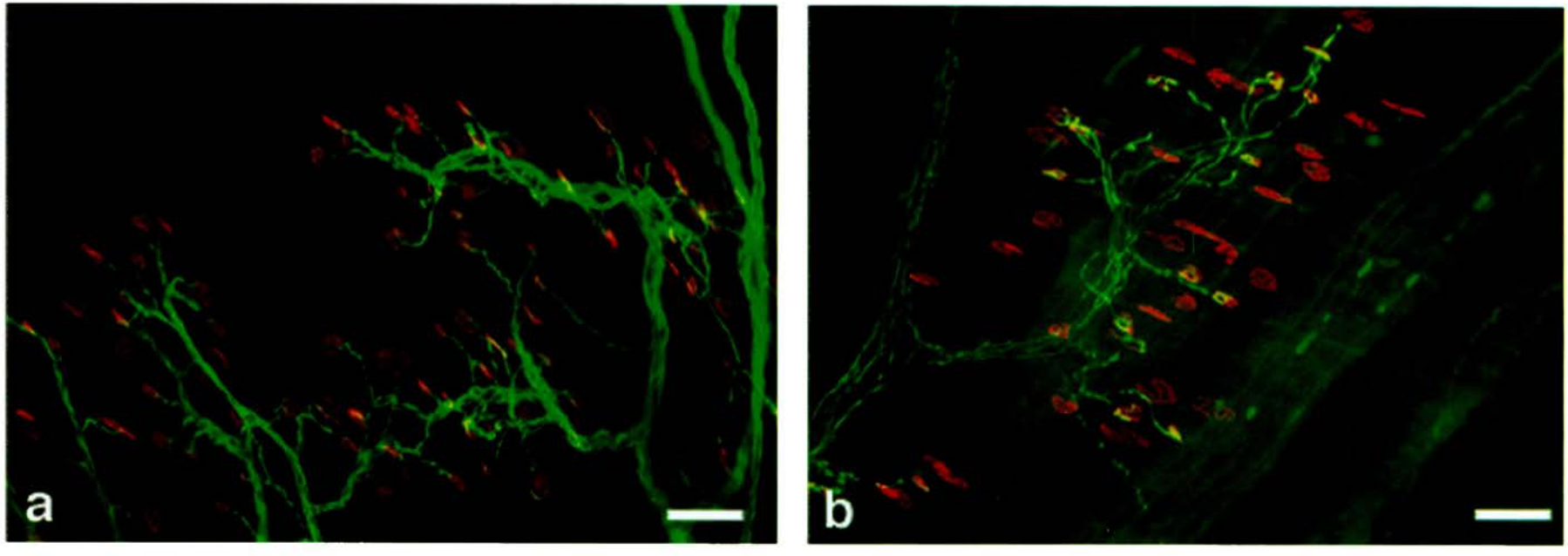
a
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FIGURE 2.18: WITHDRAWAL OF NERVE TERMINAL PROCESSES RESULTS IN THE FORMATION OF A RETRACTION BULB AT AXOTOMIZED WLD^S NEUROMUSCULAR JUNCTIONS.

The loss of nerve terminal structure at axotomized Wld^S TA neuromuscular junctions results in the formation of a perijunctional swelling known as a retraction bulb.

A illustrates a retraction bulb overlying a small area of acetylcholine receptors at a 4 day axotomized junction. The majority acetylcholine receptors are unoccupied, presumably reflecting the withdrawal of terminal boutons. A secondary, more proximal, swelling is also evident.

Scale bar = 20μm

a

FIGURE 2.19: RETRACTION BULBS AT AXOTOMIZED WLD^s NEUROMUSCULAR JUNCTIONS.

Retraction bulbs are present at various stages after nerve lesion, and eventually recede to the intramuscular nerve bundle.

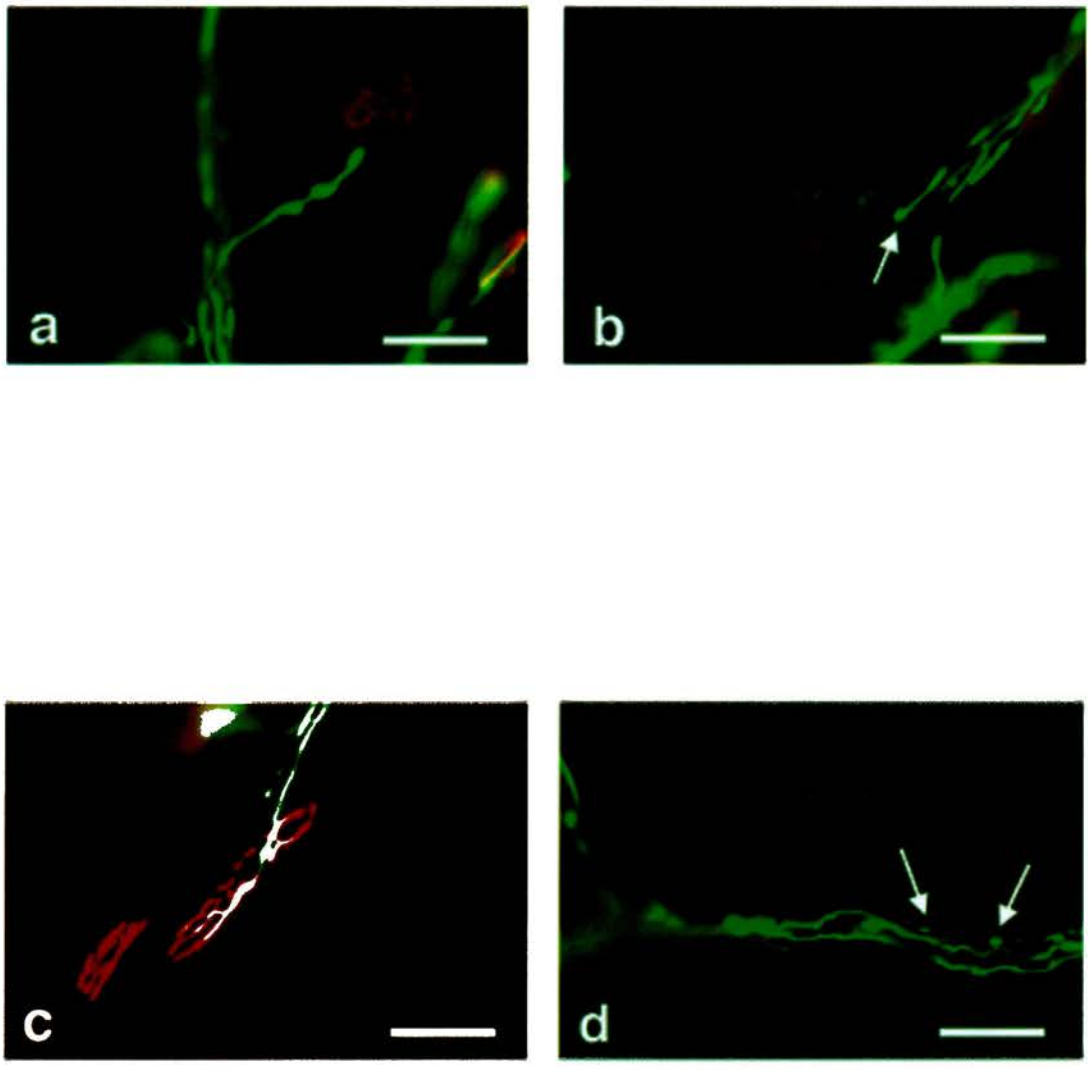
A to **D** depict retractions bulbs in axotomized Wld^s TA muscles.

A. Retraction bulb located close to a an endplate 4 days after nerve lesion. It is probable that this process originally innervated the endplate close to it.

B. and **C**. Progressive withdrawal of retraction bulbs (see arrow in **B**) from the endplate area in 4 and 6 day axotomized muscles. Retraction bulbs are located at distances further from the endplate region as time progresses.

D illustrates two retraction bulbs (see arrows) located within the intramuscular nerve bundle 6 days after axotomy.

Scale bar = 20μm


a
b
c
d

2.3.1.3 Repeated visualisation of axotomized Wld^S nerve muscle preparations

Motor nerve terminals of Wld^S mice were axotomized and after 4 days stained with the vital dye FM1-43 and TRITC α-Bungarotoxin and visualised over a period of 24 hours *ex vivo* in order to determine the rate of degeneration once initiated.

Individual endplates were chosen for repeated visualisation. It was not possible to distinguish any FM1-43 staining after 12 hours, so data was only obtained up to 9 hours *ex vivo*. In 3 muscles, several endplates were examined and 6 were selected for repeated visualisation. Of those, 3 endplates showed signs of loss of terminal staining over periods ranging from 1 to 7 hours *ex vivo*, and one of these endplates is illustrated in Figure 2.20. From these images, it appears that nerve terminals recede from the endplate region relatively swiftly once the withdrawal process has been initiated. These endplates were assessed for occupancy criteria and the data are represented in Figure 2.21. These observations confirm that the decline in synaptic staining takes place gradually and in a piecemeal manner rather than abruptly.

The decline in occupancy of endplates may merely be an effect of prolonged exposure to fluorescent light, and as such considered an artefactual finding. However, two observations suggest that this is not so. First, photobleaching, the reduction of fluorescence labelling by overexposure to fluorescent light, would presumably have a global effect on the nerve terminal being visualised, and as such result in the reduction of FM1-43 stain uniformly over the entire terminal. This did not occur in any instance. Second, phototoxicity (disruption of morphology resulting from toxic side effects of exposure to fluorescent light) or photobleaching would affect the entire region which was illuminated for that period, rather than selectively causing damage or bleaching to one nerve terminal and not another. Figure 2.22 shows examples of nerve terminals labelled with FM1-43 appear fully or nearly fully functional after 9 hours *ex vivo*. These terminals, although not directly repeatedly visualised for this period, were nevertheless exposed to fluorescent light for the same time as the endplates that were studied.

Alternatively, the decline in the occupancy may be a result of the trauma involved in dissection, and the conditions in which the preparation was placed. To counter this fresh physiological saline was routinely supplied to the preparation, and both the saline and the preparation were maintained at 37 °C at all times. Furthermore, as

previously mentioned, most endplates visualised appeared normal even after 9 hours *ex vivo* (Figure 2.22). Thus, not all nerve terminals underwent withdrawal, as would be expected at 4 days post axotomy. However, it would be imprudent to discount the possibility that some alterations in terminal morphology may have resulted from the constraints of the experimental protocol, and these results represent only one interpretation of the decline in FM1-43 staining. Further evidence is needed to consolidate these findings, although the technical difficulty of *ex vivo* labelling of living endplates precluded an in-depth study in this thesis.

Overall, it is apparent that nerve terminal withdrawal proceeds within a relatively short time span once initiated, over approximately 4 to 6 hours.

FIGURE 2.20: REPEATED VISUALISATION OF WITHDRAWING AXOTOMIZED WLD^S NERVE TERMINALS.

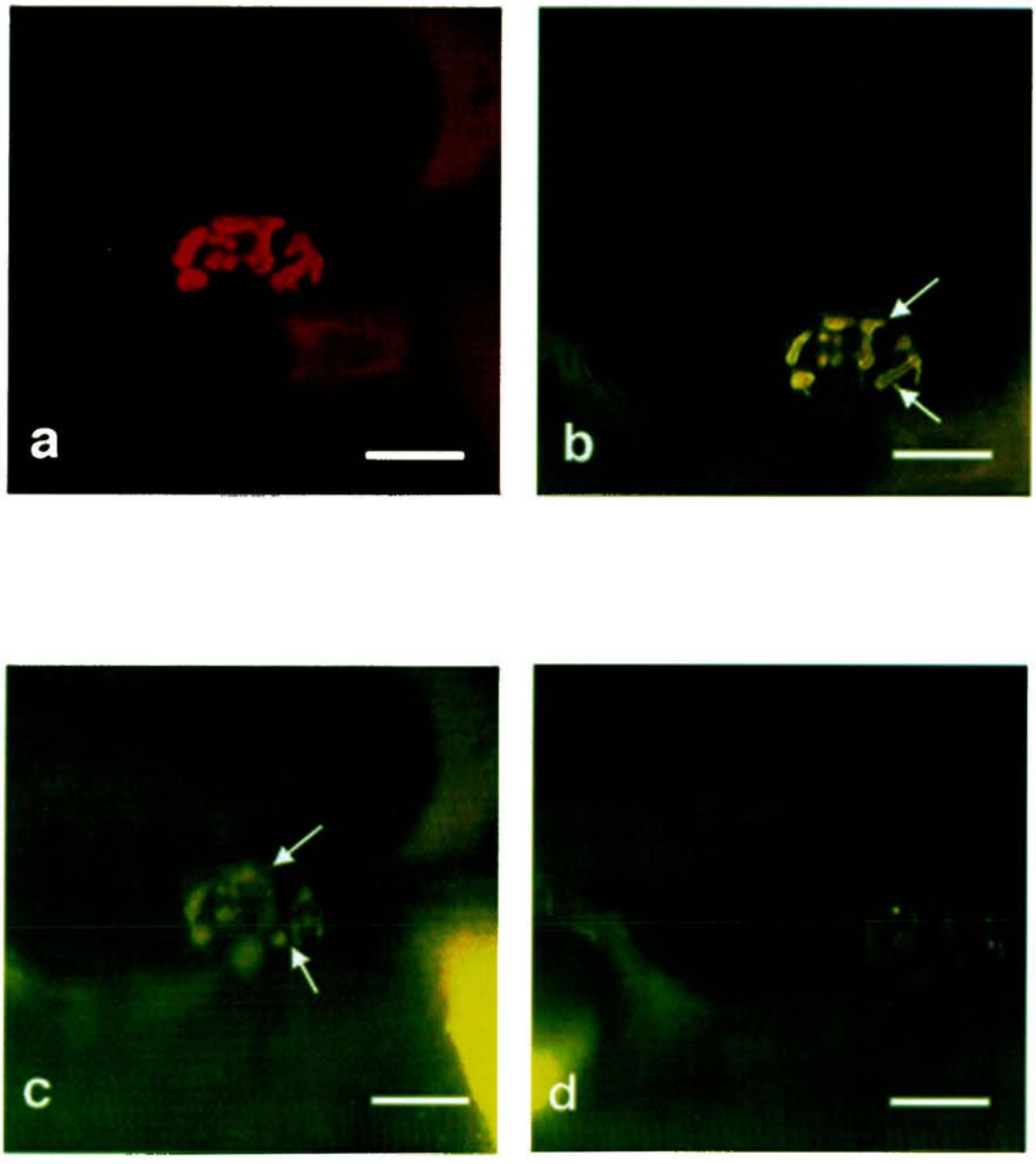
Nerve terminal withdrawal *ex vivo* in axotomized Wld^S TA muscles, visualised over 4 hours. These images depict the progressive retraction of terminal boutons at endplate 1 (see Figure 2.21).

A. and **B**. Acetylcholine receptors labelled with TRITC-conjugated α-Bungarotoxin (**A**), and FM1-43 staining (**B**) from the same neuromuscular junction 4 days after axotomy, *ex vivo*. These images were taken soon after dissection ($t = 0$).

C illustrates the decline in FM1-43 staining at some region of the nerve terminal (see arrows) 2 hours after first visualisation ($t = 2$). This may reflect the withdrawal of nerve terminal processes. Some regions appear more persistent than others.

D. Almost complete loss of FM1-43 staining over the endplate region, 4 hours after first visualisation ($t = 4$). Two bright spots remain.

Scale bar = 20μm


a
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d

FIGURE 2.21: REPEATED VISUALISATION OF WITHDRAWING WLD^s NERVE TERMINALS.

Repeated visualisation of Wld^s TA nerve terminals undergoing withdrawal. All data from 4 day axotomized TA endplates, 4 muscles.

Repeated visualisation of 3 endplates. Two endplates were visualised over a 6 hour period (endplates 2 and 3), and one endplate for 4 hours (endplate 1). Decline in occupancy was initiated at different times, but once begun proceeded to completion over approximately 4 hours. The dashed lines show projections of loss of occupancy.

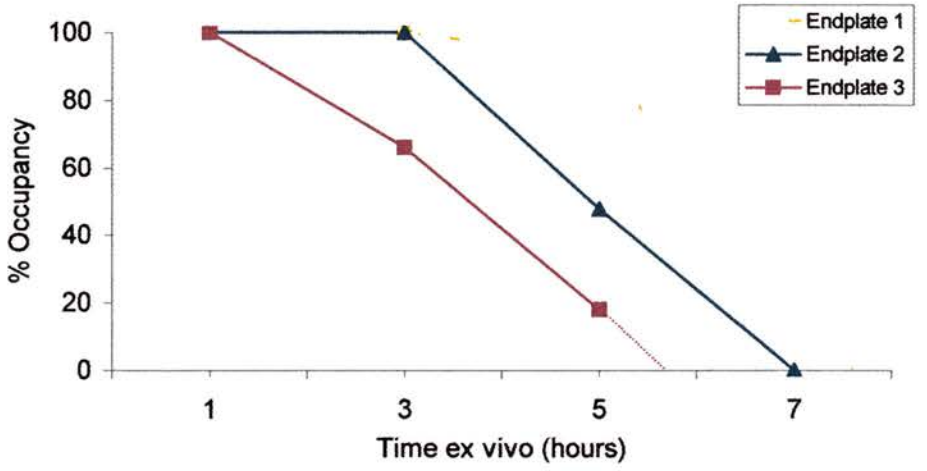


FIGURE 2.22: VISUALISATION OF AXOTOMIZED WLD^s NERVE TERMINALS 9 HOURS *EX VIVO*.

Persistent axotomized Wld^s TA nerve terminals are evident after 9 hours *ex vivo*.

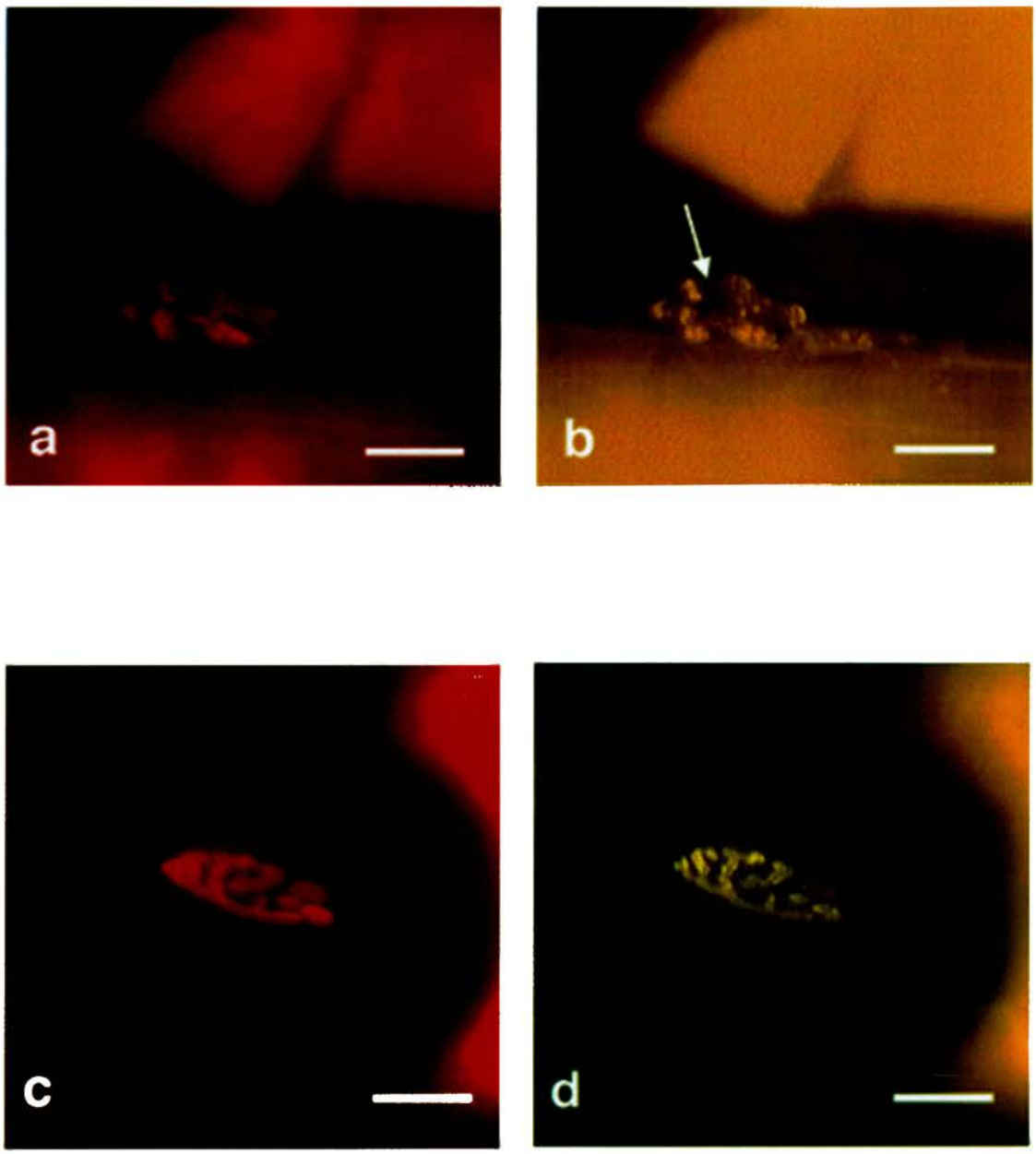
Images **A** and **C** show acetylcholine receptors labelled with TRITC-conjugated α-Bungarotoxin.

Images **B** and **D** show recycling synaptic vesicles loaded with the vital dye FM1-43.

A and **B**. Acetylcholine receptors (**A**) and presynaptic nerve terminal (**B**). A small region of the nerve terminal, stained by FM1-43, is absent.

C and **D**. 100% occupancy of the endplate by the nerve terminal. There appears to be complete overlap between the distribution of acetylcholine receptors (**C**) and recycling synaptic vesicles (**D**).

Scale bar = 20μm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2.3.2 Functional and morphological assessment of wild type nerve terminals after nerve section

Motor nerve terminals of C57BL/6J mice were denervated and nerve-muscle preparations stained with FM1-43 and TRITC α-Bungarotoxin 6, 12, 18 and 24 hours post axotomy. After visualisation using the vital dyes, preparations were fixed and labelled for NF/SV2. This procedure was problematic, as the process of labelling with FM1-43 appeared to impart a residual background fluorescence to the muscle, which made it difficult to immunocytochemically label junctions with NF/SV2. Furthermore, it proved extremely difficult to visualise a neuromuscular junction using the vital dyes and subsequently relocate and visualise the same junctions labelled with NF/SV2. However, sufficient results were obtained to conduct a broad survey of the degenerative changes that take place after the axotomy of wild type muscles.

Unoperated nerve-muscle preparations stained with the FM1-43 were identical in appearance to unoperated Wld^s preparations. There was complete overlap between the FM1-43 staining pattern and the distribution of the TRITC α-Bungarotoxin labelled acetylcholine receptors (Figure 2.23). Terminal morphology was assessed for occupancy criteria in the same way as Wld^s nerve terminals.

At 6 hours after denervation most endplates appeared fully occupied, with a few exceptions. At the majority of neuromuscular junctions FM1-43 staining revealed actively recycling vesicles similar to that of unoperated animals, and passive staining demonstrated that the preterminal axon appeared intact. However, some endplates shows signs of degeneration. In these cases, FM1-43 labelling appeared fragmented and diffuse, and these changes appeared to occur over the entire endplate region rather than localising to specific regions of the nerve terminal (Figure 2.24). NF/SV2 staining appeared normal in most cases, with the nerve terminal and innervating axon visibly labelled. However, some endplates were clearly undergoing degeneration. Degenerating endplates were characterised not only by a fragmented nerve terminal structure, but also by a reduction in the NF/SV2 labelling of the parent axon supplying the terminal. The staining pattern observed in these cases did not resemble that of axotomized Wld^s nerve terminals.

After 12 to 18 hours post axotomy, many endplates were abnormal and clearly undergoing degenerative changes (Figure 2.24). Some nerve terminals appeared to have completely degenerated at this stage, with only receptor staining visible at the endplate. Those terminals that had not entirely degenerated were diffusely stained with FM1-43, and appeared fragmented over the entire endplate region. At many degenerating nerve terminals FM1-43 staining appeared to be randomly clustered over various regions of the endplate, whilst other boutons were only slightly labelled see (Figure 2.25). NF/SV2 staining of these endplates confirmed that the terminal structure was fragmented, and suggested that synaptic vesicles were clustering in various areas of the nerve terminal (Figure 2.25, and Figure 2.26). Furthermore, preterminal axon staining was also extremely fragmented, and often apparently discontinuous with more proximal sections of the axon and the intramuscular nerve (Figure 2.26). In some cases the preterminal axon persisted beyond the in situ degeneration of its associated nerve terminal (Figure 2.26).

Motor nerve terminal degeneration was largely complete by 24 hours post denervation. Some isolated incidences of FM1-43 staining were observed, but in every case appeared severely fragmented.

At all the denervated endplates examined (437 endplates in 11 muscles), there was no evidence of piecemeal withdrawal, sequestration of the nerve terminal into the parent axon, formation of terminal retraction bulbs, or withdrawal from the endplate region. In wild type preparations degeneration clearly took place in situ, with the nerve terminal disassembling at the neuromuscular junction, followed by the loss of the parent axon at various points. This was confirmed by both vital FM1-43 staining and NF/SV2 labelling of fixed preparations.

FIGURE 2.23: FM1-43 STAINING AT WILD TYPE MOUSE NEUROMUSCULAR JUNCTIONS.

FM1-43 staining at unoperated control C57BL/6J mouse neuromuscular junctions in TA muscles.

A illustrates acetylcholine receptors labelled with TRITC-conjugated α-Bungarotoxin.

B shows recycling synaptic vesicles loaded with the vital dye FM1-43. Preterminal staining is evident in this image.

Note the precise overlap between the distribution of FM1-43 stained synaptic vesicles and postsynaptic acetylcholine receptors. This is denoted as '100% occupancy'.

Scale bar = 20μm

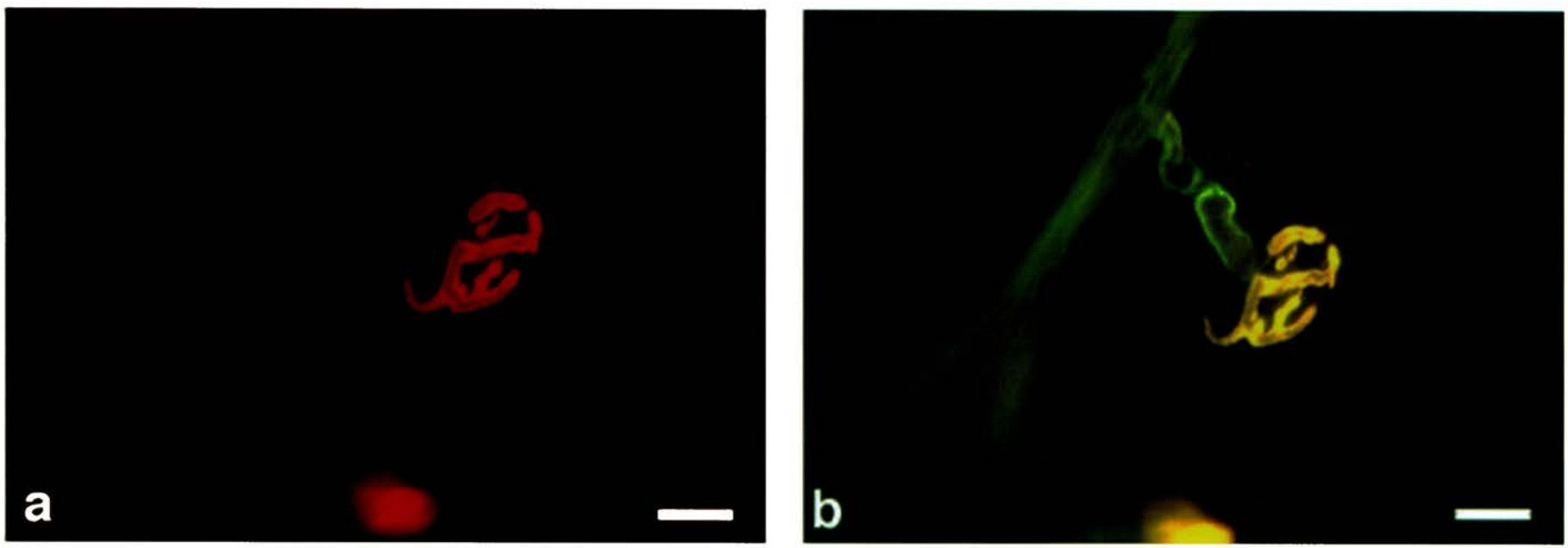

a
b

FIGURE 2.24: FRAGMENTATION OF FM1-43 STAINING AT DENERVATED WILD TYPE NEUROMUSCULAR JUNCTIONS.

FM1-43 staining at denervated C57BL/6J mouse TA neuromuscular junctions appeared fragmentary and disorganised.

Acetylcholine receptors labelled with TRITC-conjugated α-Bungarotoxin (**A**, **C** and **E**), and FM1-43 staining (**B**, **D** and **F**) from the same neuromuscular junction.

Images **A** and **B** show that the distribution of recycling synaptic vesicles 6 hours after nerve section appears disrupted. FM1-43 staining is extremely fragmented and irregular, and there is no clear pattern of FM1-43 loss. Brightly stained regions are evident.

C and **D**. At 12 hours post denervation, two boutons appear heavily loaded with FM1-43 dye, whilst the rest of the nerve terminal labelling is scattered, and reduced to background levels in most areas.

E and **F**. At 18 hours after nerve section, very few recycling synaptic vesicles remain. Two heavily stained regions remain. Some areas of brightly stained preterminal axon are visible.

Scale bar = 20μm

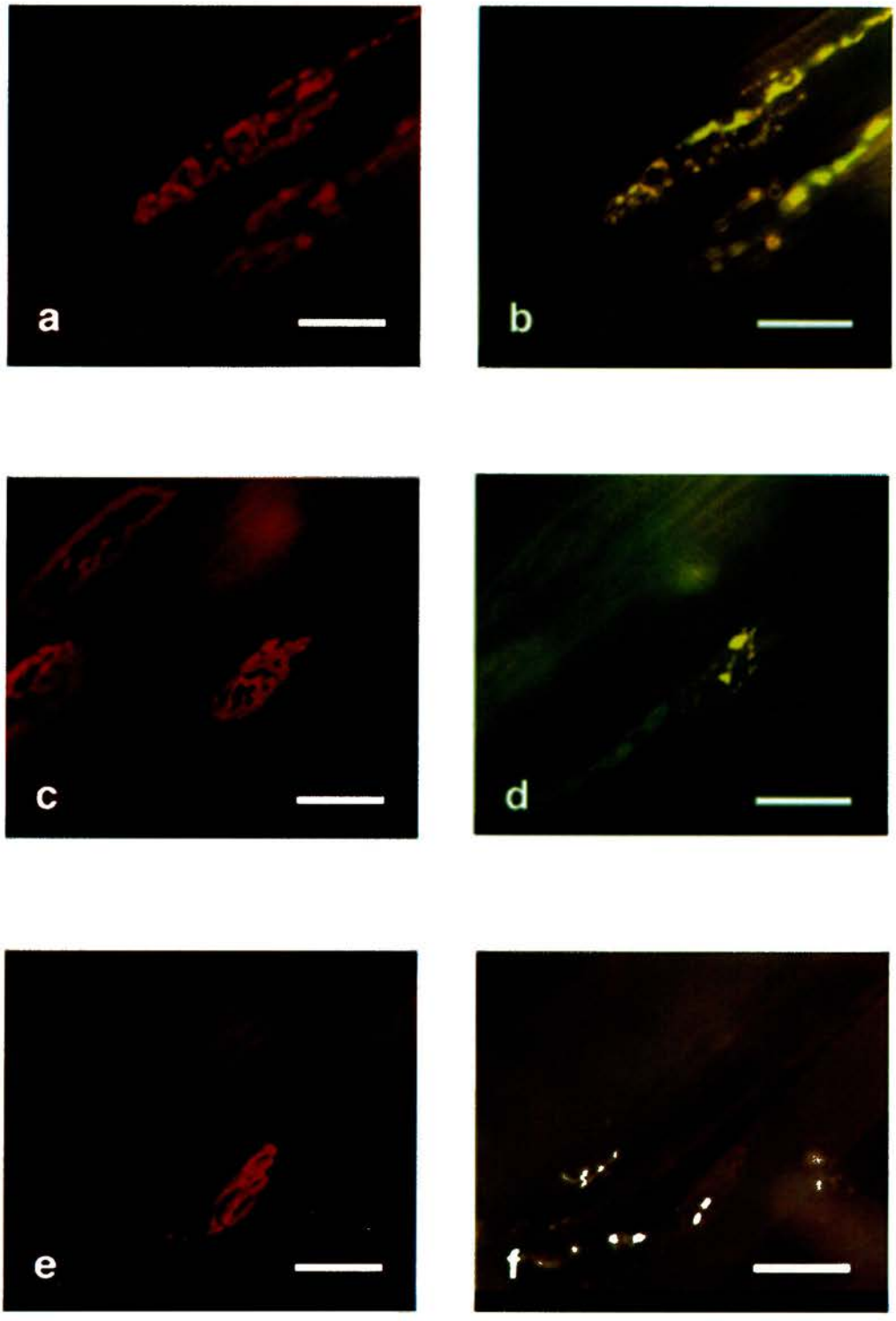

a
b
c
d
e
f

FIGURE 2.25: FM1-43/NF/SV2 STAINING AT DENERVATED WILD TYPE NEUROMUSCULAR JUNCTIONS.

FM1-43 visualisation at denervated C57BL/6J mouse TA neuromuscular junctions was followed by labelling with antibodies to NF and SV2 verified loss of recycling vesicles was coincident with a disruption in nerve terminal structure (**A** to **C**).

Acetylcholine receptors labelled with TRITC-conjugated α-Bungarotoxin (**A**), FM1-43 staining (**B**), and NF/SV2 labelling (**C**) from the same neuromuscular junction 18 hours after denervation.

B shows irregular and fragmentary FM1-43 staining.

C illustrates the concurrent morphological disruption of NF/SV2 protein distribution. Note that the preterminal axon appears to be discontinuous with the nerve terminal.

Scale bar = 20μm

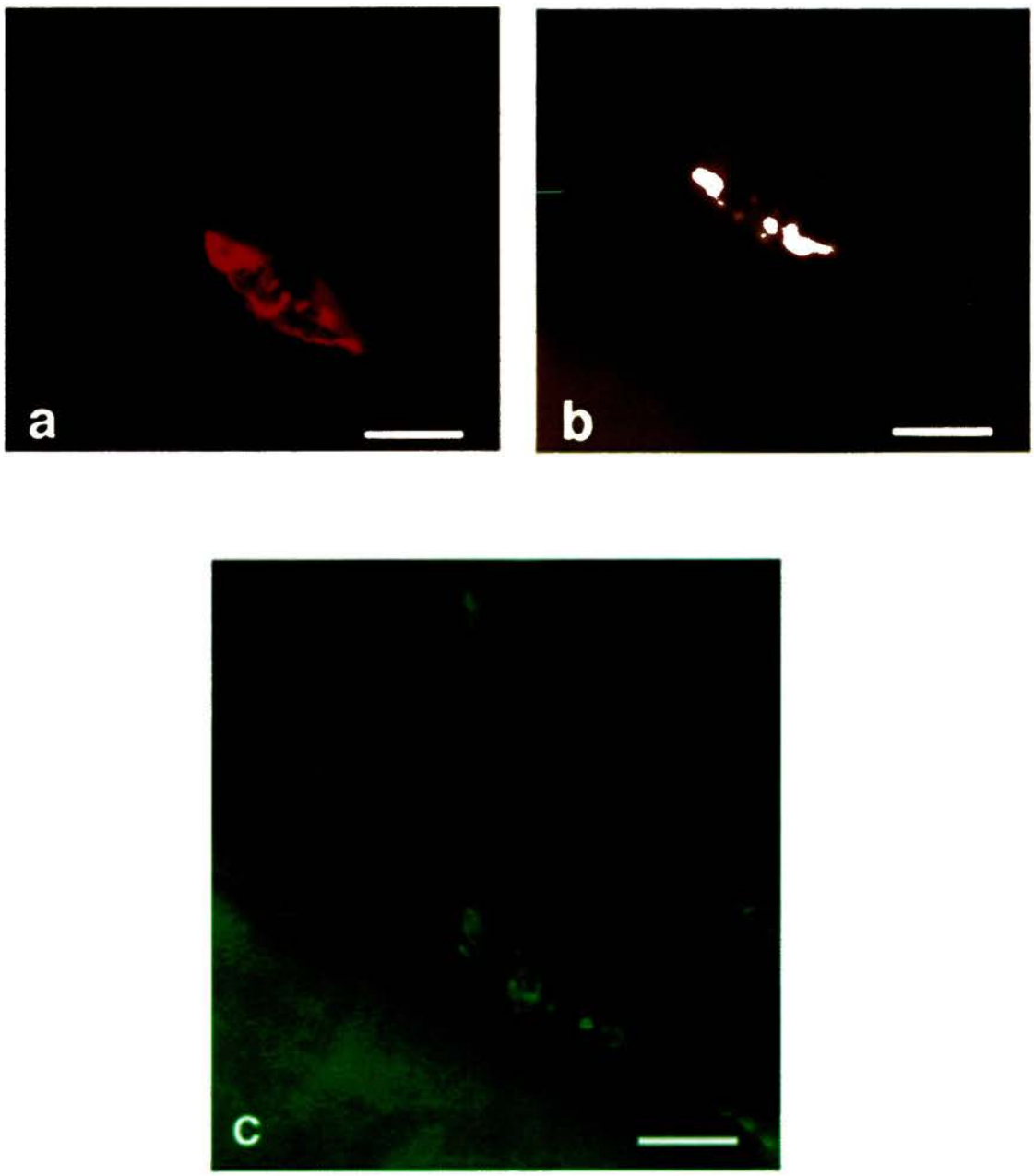

a
b
c

FIGURE 2.26: NF/SV2 STAINING AT DENERVATED WILD TYPE NEUROMUSCULAR JUNCTIONS.

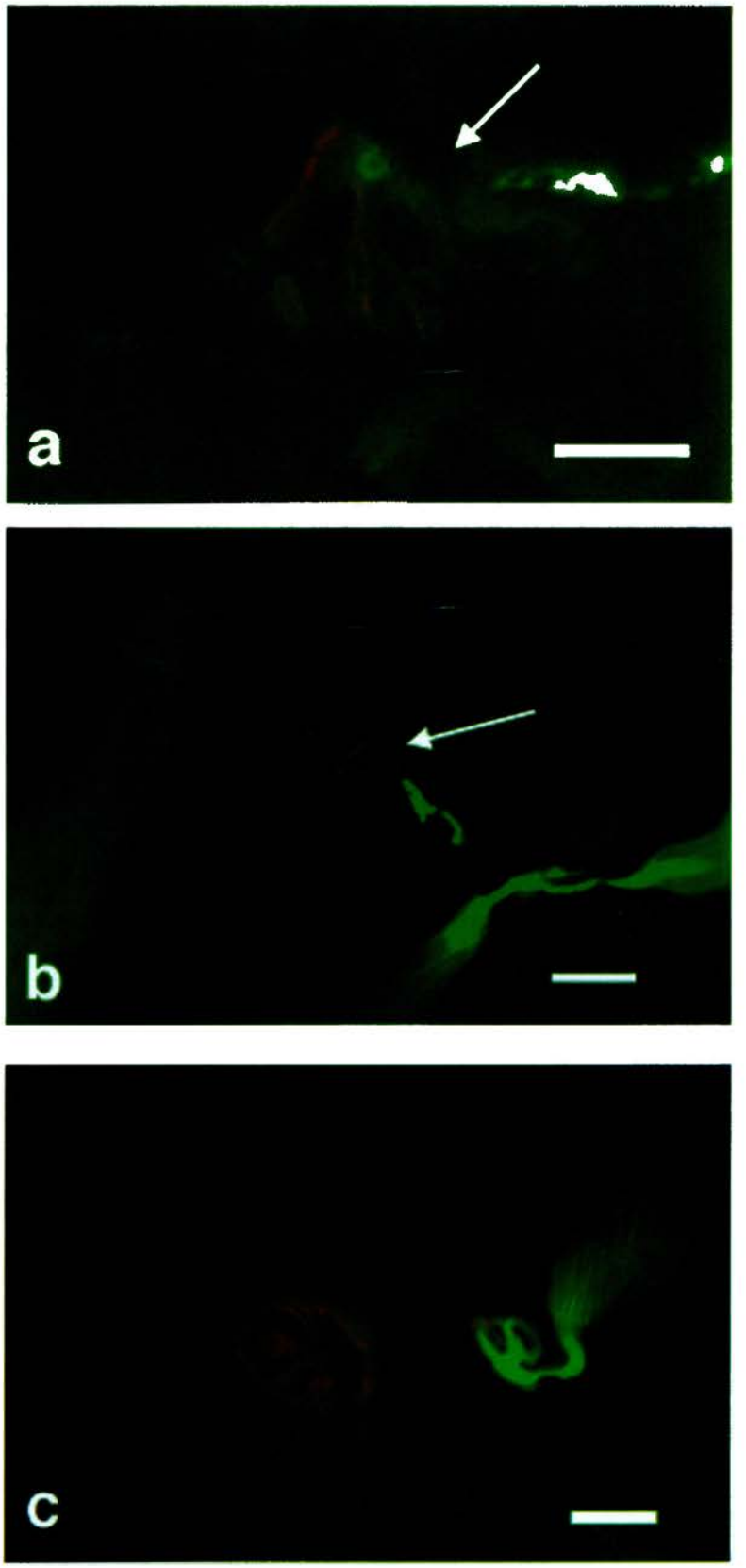
NF/SV2 visualisation of degenerating nerve terminals at denervated C57BL/6J mouse TA neuromuscular junctions.

A depicts a neuromuscular junction occupied by one small NF/SV2 region 18 hours after nerve section. The preterminal axon appears fragmented and is not connected with the remaining nerve terminal process (arrow).

B Illustrates a largely intact nerve terminal overlying acetylcholine receptors 18 hours after axotomy. The nerve terminal does not appear to be connected to the parent axon (see arrow).

C. A completely denervated endplate lies adjacent to an innervated endplate 12 hours after nerve section, illustrating the variation of Wallerian degeneration between nerve terminals.

Scale bar = 20μm


a
b
c

2.3.3 Electrophysiological assessment of nerve terminal withdrawal in C57BL/Wld^s mice after axotomy.

Standard electrophysiological techniques were employed to assess functional alterations at Wld^s neuromuscular junctions after nerve lesion.

Inspection of the distal nerve stump up to 2 weeks after axotomy revealed the characteristic banded appearance of intact nerves, confirming previous reports that the main nerve trunk remains structurally intact for prolonged periods. Freshly dissected muscles often had signs of muscle fibre fibrillation, probably caused by spontaneous contractile activity (Ribchester *et al.*, 1995). Intracellular recordings were performed on muscles 4, 5, 6, 7, 8, 12 and 13 days axotomized. In unoperated control muscles, electrical stimulation of the distal nerve stump invariably resulted in either an action potential or an EPP. At 4 days post lesion, the majority of muscles fibres responded to nerve stimulation (39 out of 40). The number of innervated fibres gradually declined from 4 days post axotomy, until at 12 days no fibres responded to maximal nerve stimulation (Figure 2.27). During this time MEPPs were recorded, and in many cases MEPPs were registered in fibres that were otherwise unresponsive to nerve stimulation, confirming previous reports (Ribchester *et al.*, 1995). Some muscle fibres showed signs of deterioration of transmitter release (low EPPs, sporadic failure to respond to stimulation) and in these fibres MEPPs were often unusually large, and were sometimes of frequencies of more than 10 MEPPs per 10 second sweep. However, from 6 to 13 days post axotomy lower frequencies of MEPPs were recorded. This is represented in Figure 2.28. Unoperated control Wld^s muscles had MEPP frequencies distributed around 1 MEPP/ 10 second sweep, or 0.1 Hz. At 4 days post axotomy, this distribution was similarly represented. From 6 days post axotomy most endplates had unusually low MEPP frequencies, which resulted in a skewed distribution. This is illustrated in the histograms by an asymmetric tail extending towards the lower frequencies. Thus, the number of innervated muscle fibres and the MEPP rate at those endplates declines during the withdrawal of Wld^s nerve terminals after axotomy.

In some cases repetitive stimulation of a muscle fibre resulted in intermittent failure of synaptic transmission. This suggests that there may have been a decline in transmitter release at some endplates. Therefore, a more detailed study was

undertaken to examine transmitter release (quantal content) at endplates that remain functionally innervated, and responded to nerve stimulation. In order to determine the quantal content of nerve terminals, preparations were first treated to abolish muscle action potentials so that EPPs could be analysed. Nerve-muscle preparations were bathed either in physiological saline containing lowered levels of calcium and elevated levels of magnesium, or μ-conotoxin was added to the bathing medium. Elevating the magnesium concentration has the effect of suppressing the overall quantal content of nerve terminals, which abolishes action potentials. μ-conotoxin blocks sodium channels on the muscle membrane which removes the possibility of muscle action potentials without affecting the overall quantal content.

FIGURE 2.27: INTRACELLULAR RECORDINGS FROM AXOTOMIZED WLD^s ENDPLATES.

Timecourse of loss of synaptic transmission after axotomy in FDB muscles.

Incidence of functionally innervated fibres per 20 impaled, from 15 muscles. Each point represents fibres sampled from one muscle. Some points superimpose.

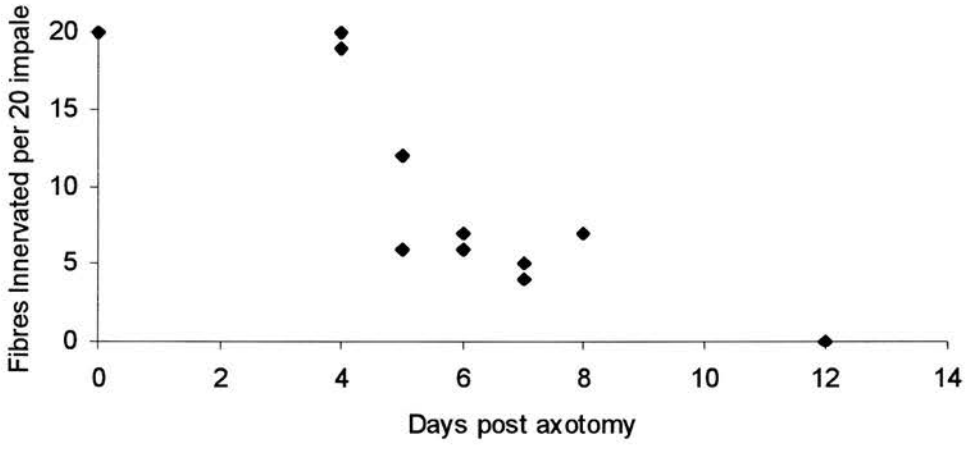
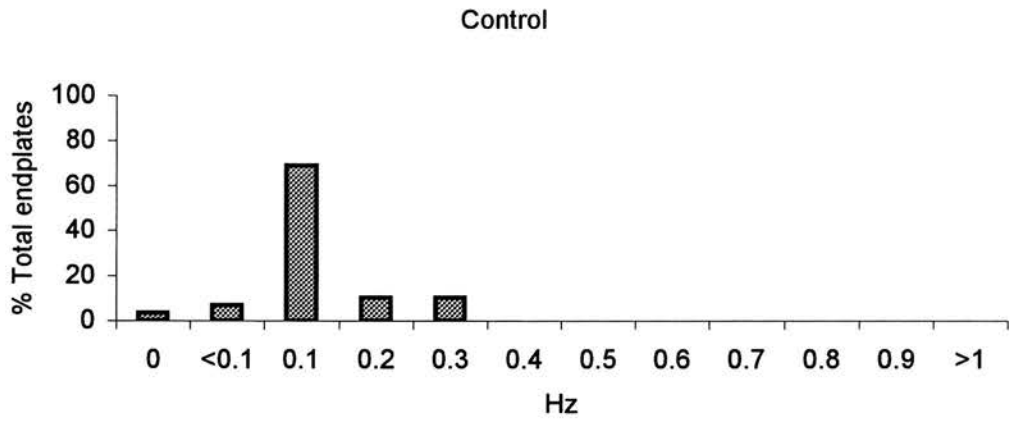


FIGURE 2.28: MINIATURE ENDPLATE POTENTIAL FREQUENCIES FOLLOWING AXOTOMY OF WLD^s ENDPLATES.

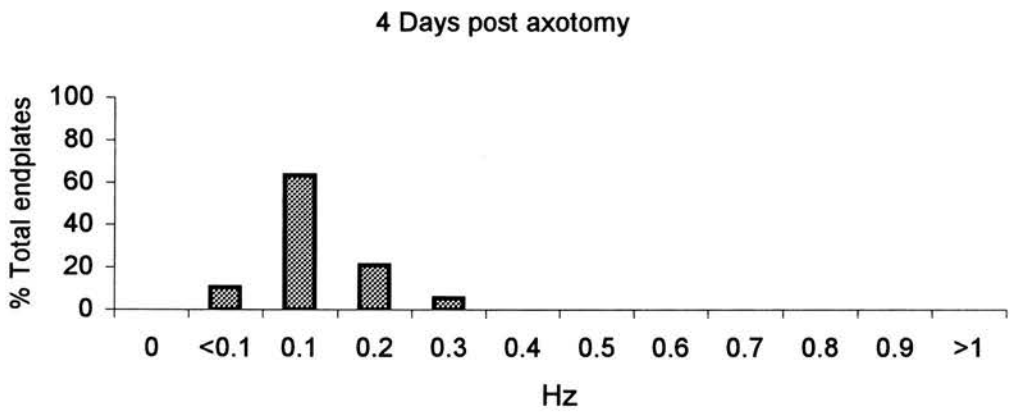
MEPP rates decline following axotomy of Wld^s FDB muscles.

Graphs **A** to **H** (illustrating data from control, 4, 5, 6, 7, 8, 12 and 13 days post axotomy) show the change in MEPP rate at axotomized Wld^s motor endplates. These data are pooled from 323 endplates and 15 muscles. MEPPs were sampled over a period of 10 seconds. These data are represented as the number of MEPPs per 10 second sweep, placed into bins, plotted against the percentage of the total number of endplates.

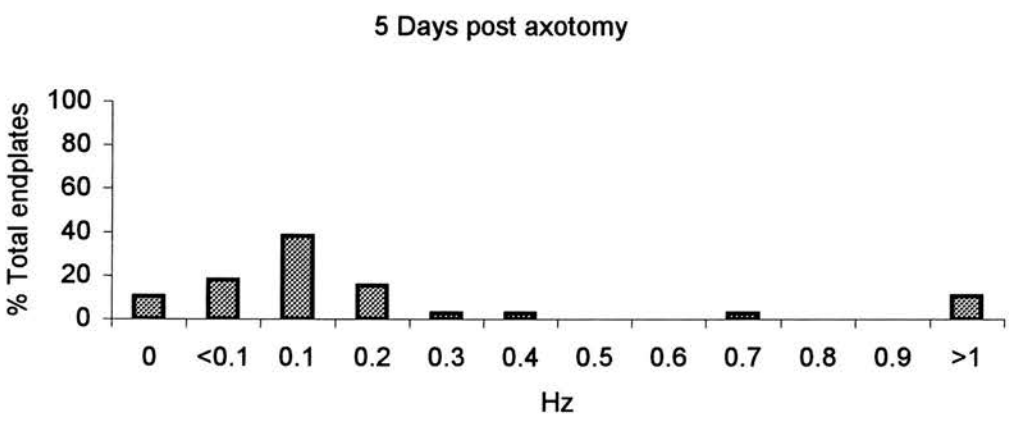
A.


Control
% Total endplates
100
80
60
40
20
0
0
<0.1
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
>1
Hz

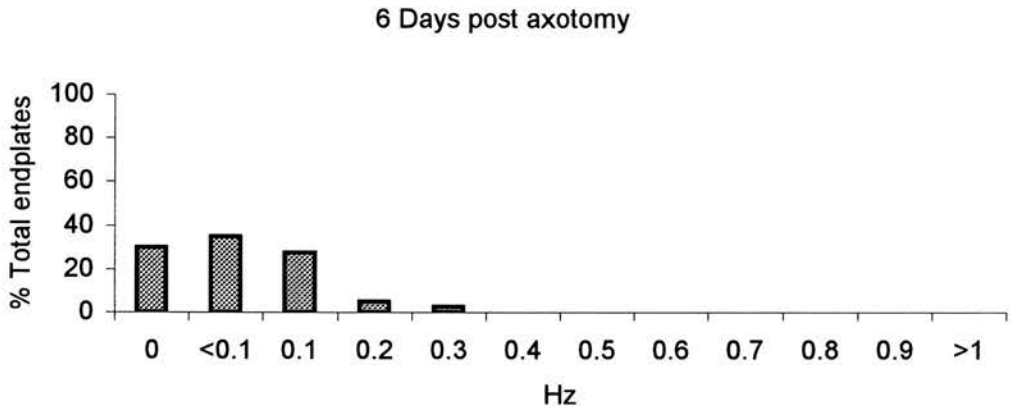
B.


4 Days post axotomy
% Total endplates
100
80
60
40
20
0
0
<0.1
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
>1
Hz

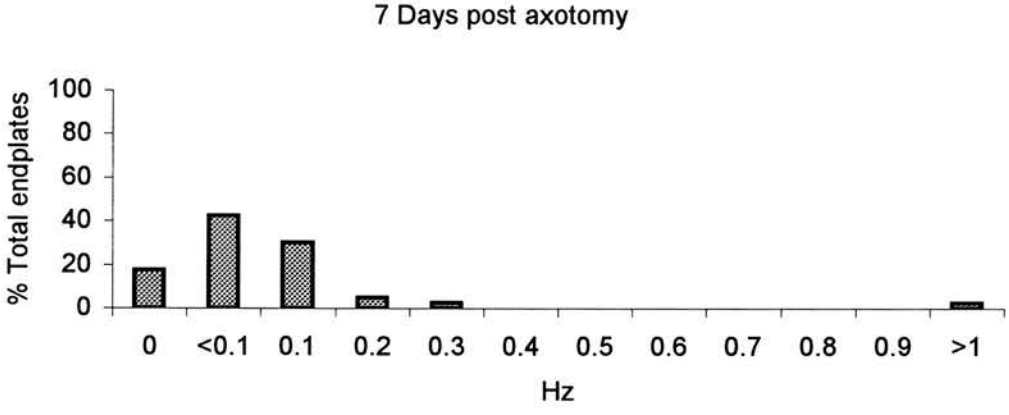
C.


5 Days post axotomy
% Total endplates
100
80
60
40
20
0
0
<0.1
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
>1
Hz

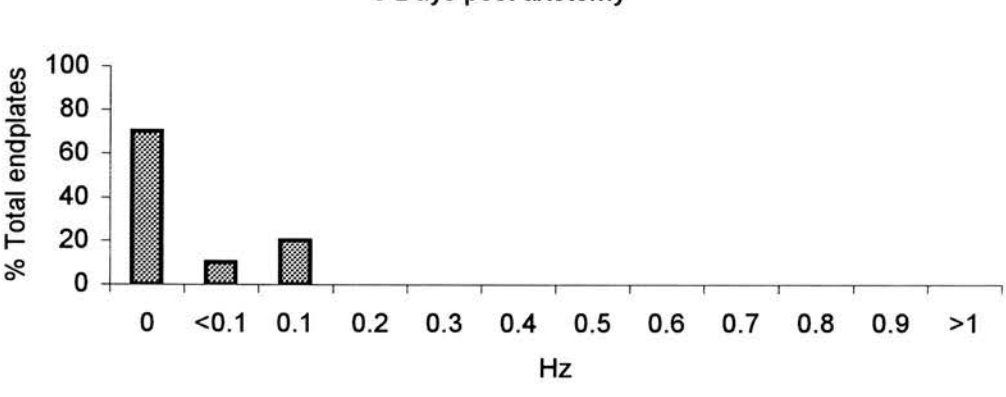
D.


6 Days post axotomy
% Total endplates
100
80
60
40
20
0
0
<0.1
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
>1
Hz

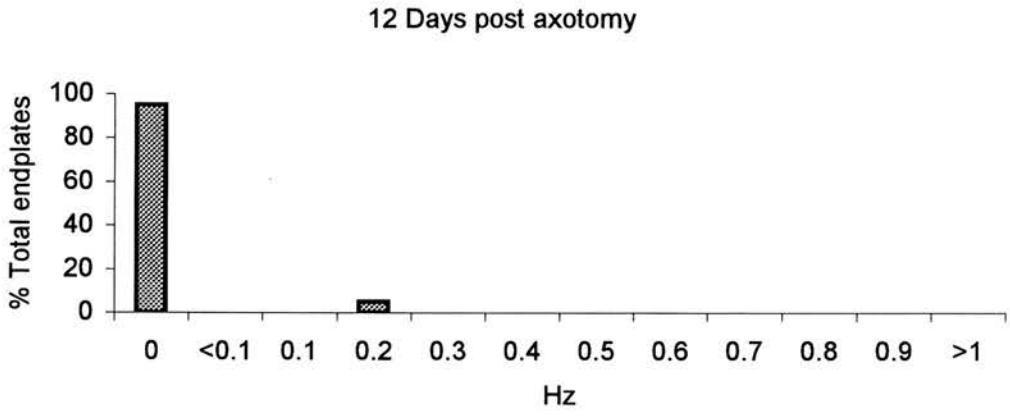
E.


7 Days post axotomy
% Total endplates
100
80
60
40
20
0
0
<0.1
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
>1
Hz

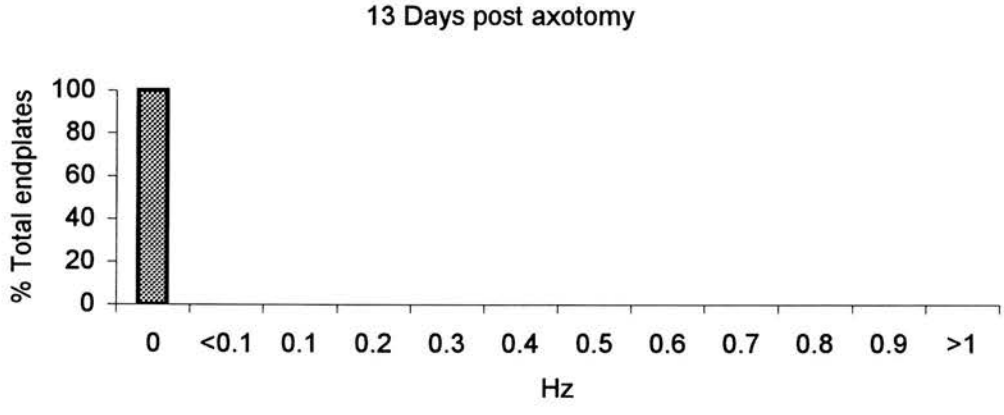
F.


8 Days post axotomy
% Total endplates
100
80
60
40
20
0
0
<0.1
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
>1
Hz

G.


12 Days post axotomy
% Total endplates
100
80
60
40
20
0
0
<0.1
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
>1
Hz

H.


13 Days post axotomy
% Total endplates
100
80
60
40
20
0
0
<0.1
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
>1
Hz

2.3.3.1 Quantal analysis using elevated levels of magnesium

Nerve-muscle preparations were first tested in normal physiological saline, then incubated in physiological saline incorporating elevated levels of magnesium, and lowered levels of calcium (designated as high magnesium saline).

Muscles that were 12 and 13 days axotomized did not respond to nerve stimulation in normal saline, and were discounted for further analysis. Intracellular recordings were performed on 8 axotomized muscles, ranging from 4 to 8 days post axotomy inclusive, and 8 contralateral muscles. Several fibres in each muscle were impaled and those that responded to stimulation were further investigated. Functionally transmitting muscle fibres were repetitively stimulated (over 100 times), and the resulting EPPs analysed using the 'failures' method for quantal content.

The quantal contents of nerve terminals that responded to nerve stimulation are presented in Figure 2.29. Intracellular recordings from contralateral muscles bathed in high magnesium saline varied considerably at each data point. This was probably due to the differential effect of high magnesium saline on each set of muscles. Therefore, comparisons were only made between an axotomized muscle and its contralateral control, rather than between days post axotomy. In this way, experimental and contralateral muscles were from the same animal, subjected to identical environmental conditions, and bathed in the same high magnesium solution. The results indicate that at 4 days post axotomy, axotomized and contralateral muscle fibres did not have significantly different quantal content. Thus, in muscle fibres 4 days axotomized there was no difference in the amount of transmitter release at neuromuscular junctions which exhibited EPPs or action potentials. In this case, the majority of neuromuscular junctions responded to nerve stimulation (see Figure 2.27) and therefore synaptic transmission remained normal in nearly all junctions. From 5 days post axotomy onwards, the mean quantal content was significantly different from that of nerve terminals from the contralateral muscles. This clearly represented a deterioration of synaptic transmission at endplates that were still able to respond to nerve stimulation. Therefore, rather than an all or none response synaptic transmission in these muscles appears to decline gradually. In these muscles the quantal content of individual nerve terminals varied greatly between muscle fibres: repetitive stimulation of some fibres resulted in very few failures, or none at all,

whilst in neighbouring adjacent fibres many failures were recorded with only a few weak EPPs. In some muscle fibres nerve stimulation produced varying responses from the nerve terminal: some EPPs were relatively large, whilst another stimulation would produce a very low amplitude EPP. This variability of response is illustrated in (Figure 2.30). The average standard deviation of EPP amplitude, pooled from individual muscle fibres, was considerably greater from 5 to 7 days post axotomy than that of the amplitude from the contralateral muscles. These data indicate that at 5, 6, and 7 days post axotomy Wld^s nerve terminals produce erratic EPPs after nerve stimulation in comparison with controls. At 4 and 8 days, the variability of EPP amplitude was comparable with control. There may be two explanations for this. First, at 4 days post axotomy the quantal content was similar to that of unoperated controls, and most muscle fibres responded to nerve stimulation. Therefore, it is reasonable to propose that the variability of EPPs would not differ from that of normal muscles. At 8 days post axotomy the quantal content was significantly lower than that of the contralateral, and very few muscles fibres responded to nerve stimulation. In these cases, the number of endplates that remain functionally persistent is relatively small, and therefore the sample number was accordingly low (7 out of 35 muscle fibres responded to stimulation). In addition, the mean amplitude was *consistently* low when compared to EPPs from the contralateral muscle, which would lower the variability of the response.

Thus, EPPs recorded from muscle fibres 8 days post axotomy probably represent nerve terminals in the final stages of withdrawal, which results in low quantal contents, low amplitude, and a decline in variability. Nerve terminals 5 to 8 days axotomized have significantly lower quantal contents in comparison to contralateral controls. Furthermore, intracellular recordings from these muscles indicate highly variable response between endplates, and also high variability of responses within endplates. At 4 days post axotomy nearly all endplates appeared functionally identical to those of unoperated contralateral controls.

FIGURE 2.29: QUANTAL ANALYSIS OF AXOTOMIZED WLD^s ENDPLATES.

Quantal content (transmitter release) of axotomized FDB endplates bathed in high magnesium saline.

A. Intracellular recording in elevated magnesium saline from contralateral control muscle fibres (100 traces overlaid). There were few incidences of failures at this endplate, and the mean EPP amplitude was relatively high at 5.7mV.

B. Intracellular recordings from repetitively stimulated 7 day axotomized FDB neuromuscular junction (100 traces overlaid). Note the large number of failures, and the mean amplitude of EPP was low at 2.1mV.

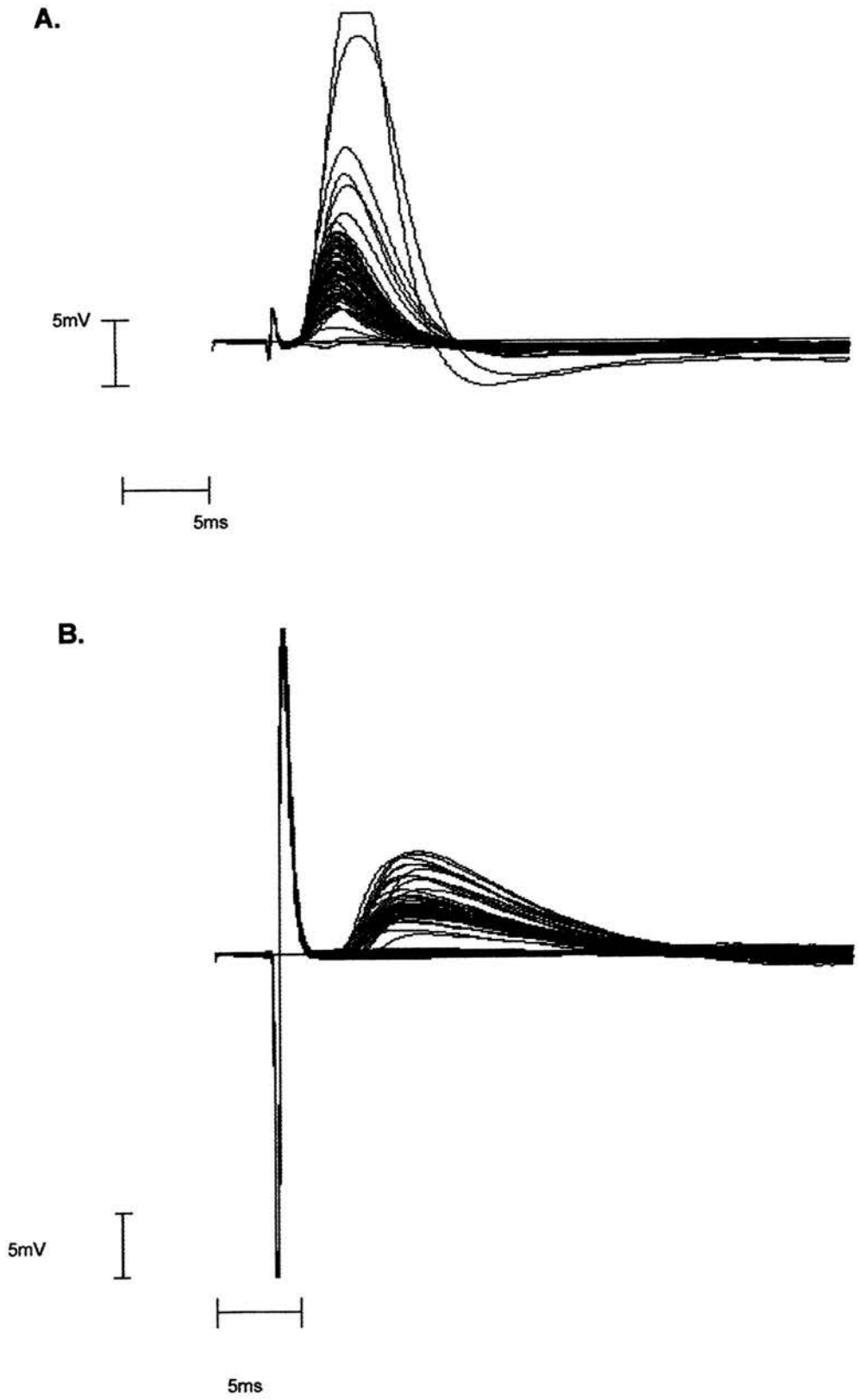
C. Average quantal content of axotomized and contralateral endplates, data pooled from 217 fibres and 19 muscles. Error bars represent the standard error of the mean. Significances were tested using Students' paired t-test.

* denotes $p < 0.05$

** denotes $p < 0.001$

*** denotes $p < 0.0001$

ns denotes not significant


A.
5mV
5ms
B.
5mV
5ms

C.

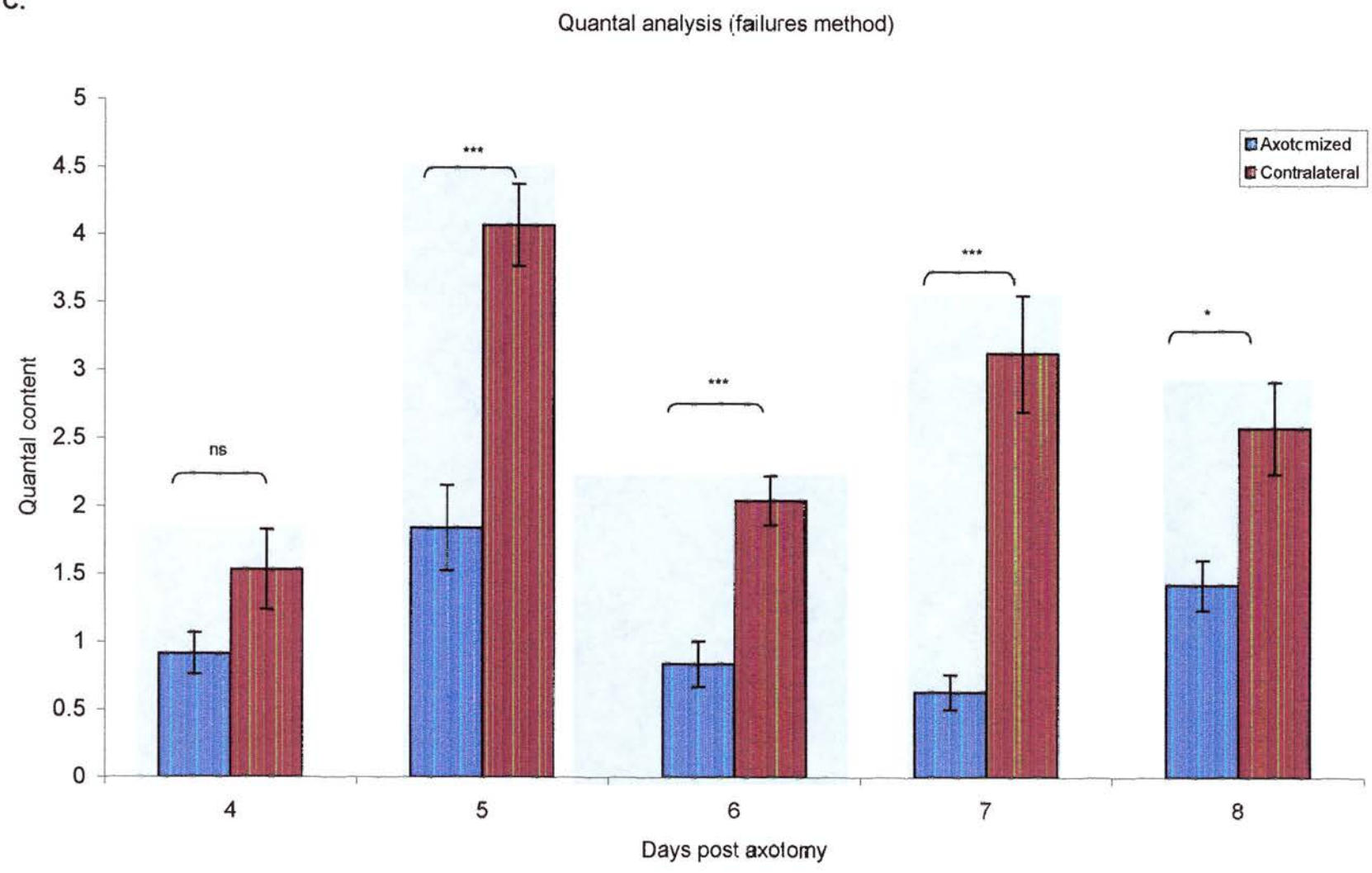
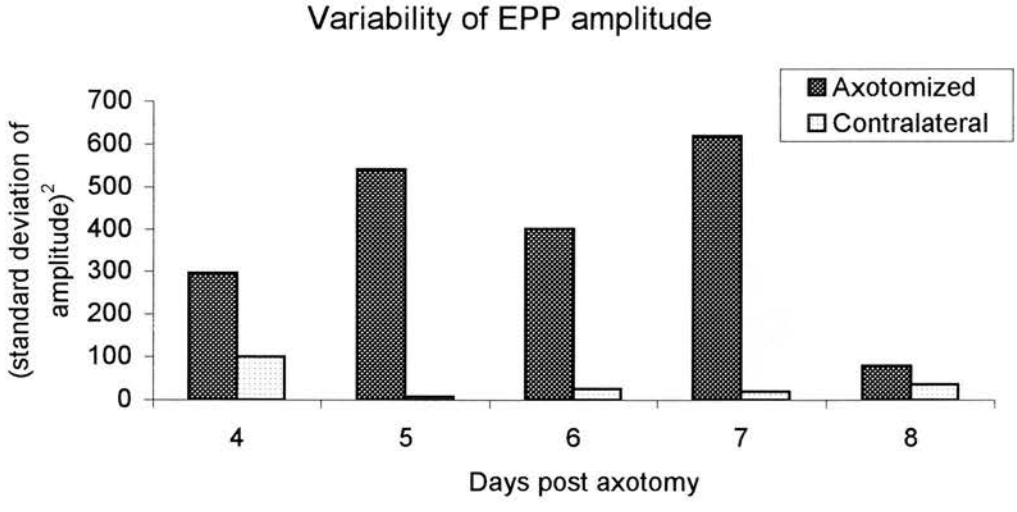

Quantal analysis (failures method)
Axotomized
Contralateral
Quantal content
Days post axotomy
ns
***
***
***
*
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
4
5
6
7
8

FIGURE 2.30: VARIABILITY OF RESPONSE OF AXOTOMIZED WLD^s ENDPLATES TO NERVE STIMULATION.

The variation in EPP amplitude increased in FDB muscle fibres that remained functionally innervated. These data represent the average standard deviation of EPP amplitude, pooled from individual muscle fibres. These data are pooled from 217 fibres and 19 FDB muscles. Muscles were bathed in high magnesium saline.



2.3.3.2 Endplate potential analysis using μ-conotoxin

Nerve-muscle preparations were bathed in μ-conotoxin (2μM) to abolish muscle action potentials, enabling an accurate analysis of EPPs at Wld^s neuromuscular junctions after axotomy. Although this concentration was sufficient to abolish muscle action potentials in wild-type muscles for long periods of time, in axotomized Wld^s muscles the effect of μ-conotoxin appeared to wear off relatively rapidly. The cause of this effect is unknown, but may as a result of a change in sodium channel isoform to a μ-conotoxin-resistant form, perhaps induced by denervation changes in the muscle fibre due to absence of evoked activity. This made it difficult to perform electrophysiological techniques for the necessary period of time. Nonetheless, sufficient data were gathered to make the following observations.

Intracellular recordings were performed on muscles 3 to 10 days post axotomy. Muscle fibres that responded to nerve stimulation were further analysed. At 3 days post axotomy repeated stimulation did not result in any cases of failures (100 repetitions or more). In every case an EPP was elicited upon nerve stimulation. From 4 days post axotomy onwards the number of muscle fibres that displayed no failures declined until at 10 days post axotomy all muscle fibres sampled had some failures (Figure 2.31). The number of muscle fibres that showed 1-99% failures, such that repeated nerve stimulation only erratically induced EPPs, transiently increased from 3 to 5 days post axotomy declining thereafter. Concurrently, the number of fibres in which no EPP was elicited after repetitive stimulation linearly increased from 3 days to 10 days after nerve lesion, at which time 95% of all fibres failed to respond to stimulation.

Recordings from contralateral controls indicated that the majority of muscle fibres invariably responded to nerve stimulation (see graph 0% failures). In contralateral muscles to 6 day axotomized preparations 12.5% of muscle fibres intermittently failed to respond to nerve stimulation (see graph 1-99% failures). However, at no time did nerve stimulation entirely fail to produce an EPP after 100 stimulations in contralateral control preparations.

In sum, reliable (100% probability) synaptic transmission at Wld^s nerve terminals that are still able to induce EPPs deteriorates from 3 to 10 days post axotomy. After 10 days post axotomy neuromuscular transmission is almost entirely abolished at all

endplates. Thus, decline in the transmission of nerve impulses at a neuromuscular junction appears to take place gradually, rather than precipitously. These data correlate strongly with observations of nerve terminal withdrawal and loss of endplate occupancy, as visualised by FM1-43 (Figure 2.13B,C).

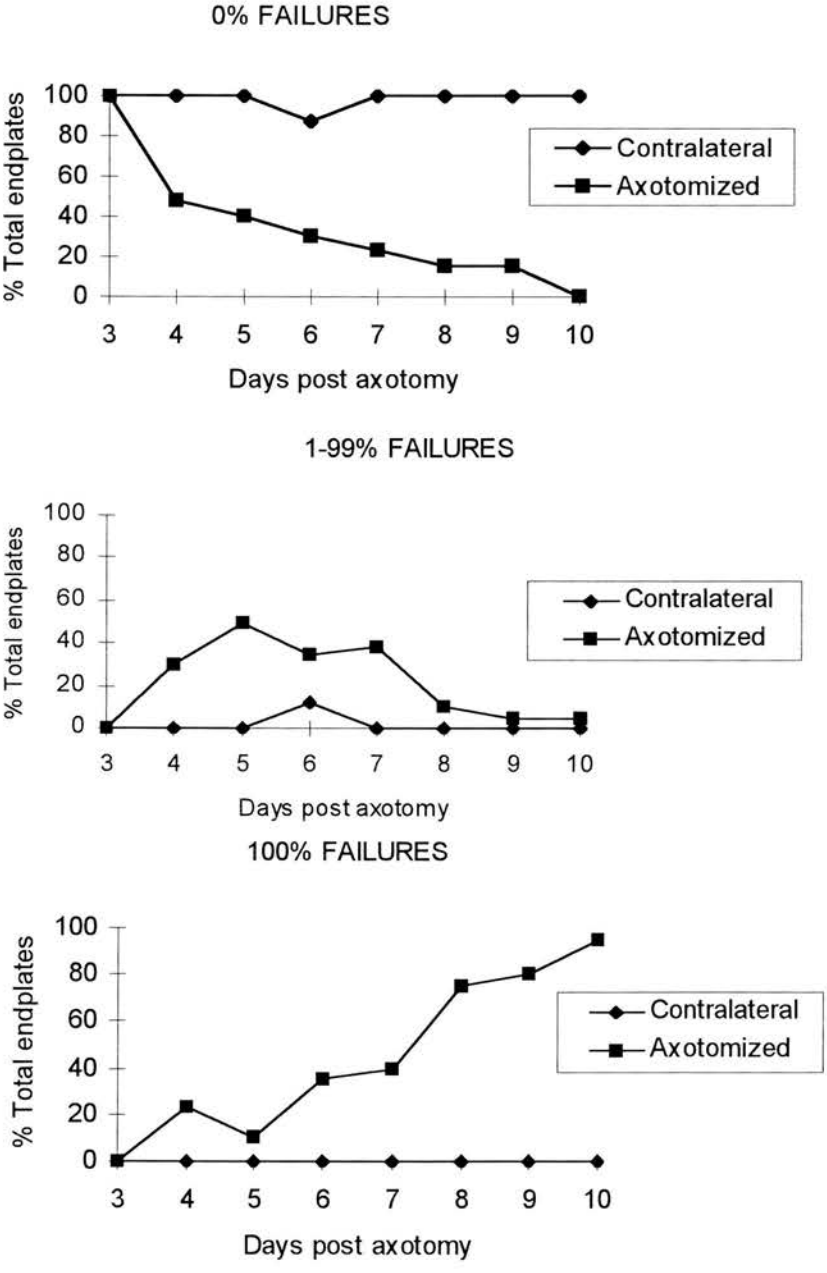
FIGURE 2.31: QUANTAL ANALYSIS OF FAILURES AT AXOTOMIZED WLD^s ENDPLATES.

A. The number of failures to respond to nerve stimulation increased at Wld^s axotomized FDB endplates bathed in saline containing μ-conotoxin. Data pooled from 280 muscle fibres from 14 axotomized muscles, and 240 muscle fibres from 12 contralateral muscles.

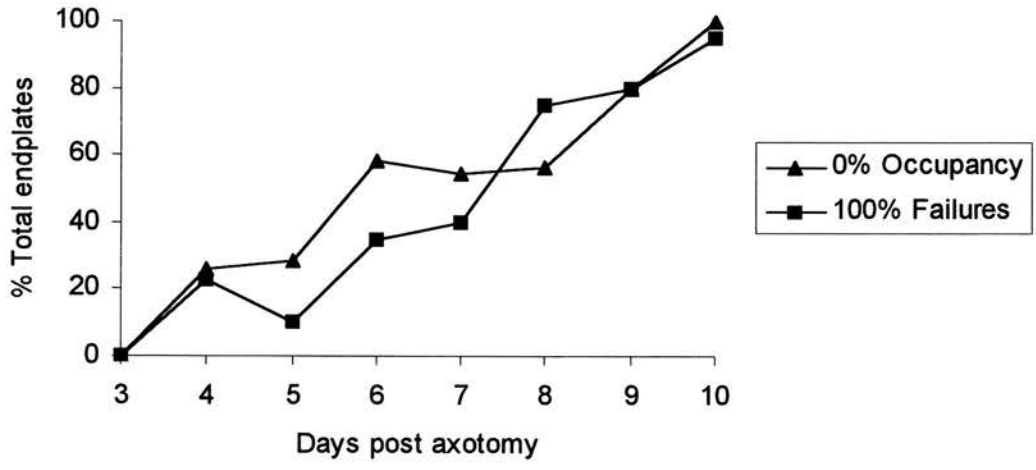
B. Correlation between loss of endplate occupancy as assessed by FM1-43 (0% occupancy) and loss of neuromuscular transmission (100% failures; in μ-conotoxin saline).

C. Correlation between onset of nerve terminal withdrawal as assessed by FM1-43 (<100% occupancy) and onset of failure of transmission (<0% failures; in μ-conotoxin saline).

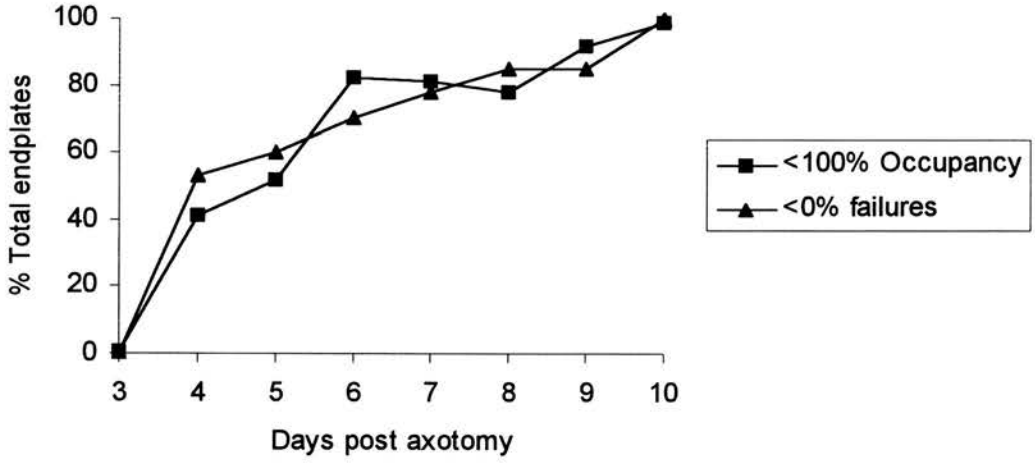
A.



B.


100
80
60
40
20
0
% Total endplates
3
4
5
6
7
8
9
10
Days post axotomy
0% Occupancy
100% Failures

C.


100
80
60
40
20
0
% Total endplates
3
4
5
6
7
8
9
10
Days post axotomy
<100% Occupancy
<0% failures

2.3.3.3 Intracellular recording from identified junctions

In order to correlate morphological observations to the electrophysiological findings, a study was undertaken to directly determine the relationship between structure and function at axotomized Wld^s endplates. Electrophysiological recordings were made on junctions that had been stained with the vital dye FM1-43 4 days post nerve lesion. Independent experiments have established that the FM1-43 loading protocol did not affect the recovery of EPP amplitude or quantal content (Betz, Ridge and Bewick, 1993; E. Costanzo, personal communication). μ-conotoxin was used to suppress the muscle action potential, but, as noted above, the effectiveness of this procedure was limited at axotomized Wld^s junctions. This procedure was technically challenging, and the majority of attempts at recording from identified junctions were unsuccessful. This was reflected in the low sample number of this study.

Recordings were made from 8 muscle fibres in 4 muscles, and the data represented in Figure 2.32. Overall, the number of failures increased as the nerve terminal withdrew (expressed as a decline in the % occupancy). This change was not statistically significant. Analysis of the quantal content of identified endplates indicated that there was no change in the amount of transmitter released per μm^2 from withdrawing nerve terminals (see Figure 2.32).

Thus, from the current data, it appears that the number of failures may increase during the period of withdrawal. This does not appear to be attributable to a weakening of the transmitter release per unit area, but as a consequence of the decline in the area of the nerve terminal occupying the endplate region.

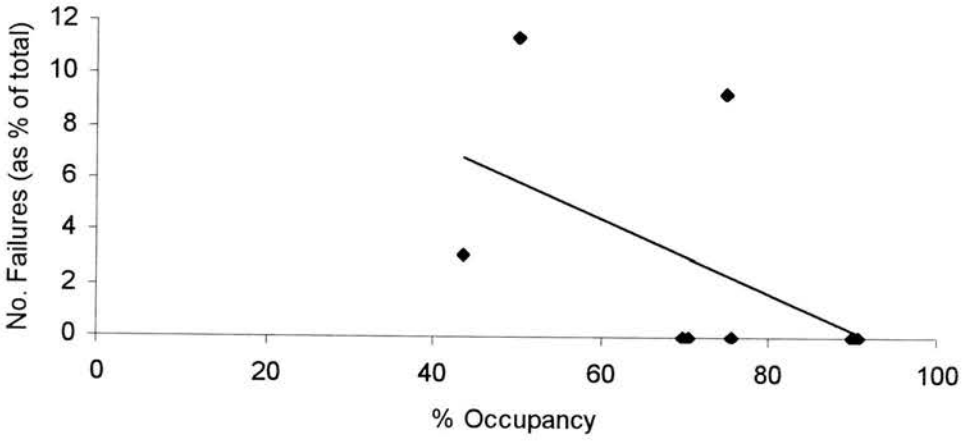
FIGURE 2.32: QUANTAL ANALYSIS OF FAILURES AT IDENTIFIED AXOTOMIZED WLD^S ENDPLATES.

Analysis of quantal efficacy at 4 day axotomized Wld^S TA neuromuscular junctions. Data from 8 muscle fibres from 4 muscles, 4 days after nerve lesion. Muscles were bathed in saline containing μ-conotoxin.

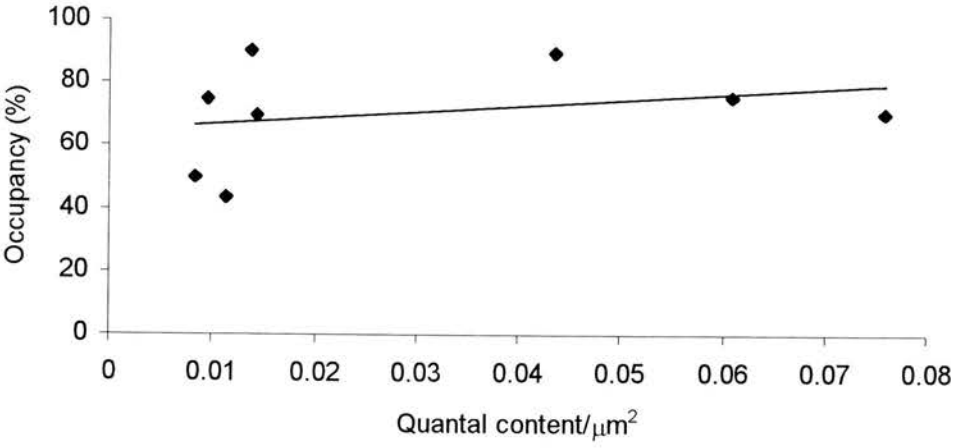
A. Graph to show that the number of failures increased at Wld^S axotomized endplates as nerve terminal processes retracted. This change was not significant (Spearman rank correlation: r = -0.5728, two tailed p = 0.1511).

B. Graph to show the relationship between the quantal content per unit area and the % occupancy. There is no significant change in quantal content per unit area as overall endplate occupancy decreased (Spearman rank correlation: r = 0.381, two tailed p = 0.3599).

A.



B.



2.4 DISCUSSION

The main findings presented in this chapter are as follows. At Wld^s neuromuscular junctions, the majority of nerve terminals remain morphologically and functionally normal for at least 3 days after a nerve lesion. From 3 days onwards there is a systematic decline in the number of intact nerve terminals, coinciding with a decline in transmitter release at these junctions. Loss of the nerve terminal did not occur abruptly over the entire neuromuscular junction, but was manifested by a gradual piecemeal reduction of terminal boutons. Fine strands connecting the remaining regions of nerve terminal with the parent axon were evident (Figures 2.14, 2.15). Eventually, the loss of terminal ramifications was accompanied by the formation of a perijunctional retraction bulb (Figure 2.18). Spatio-temporal examination of retraction bulbs indicated that these structures withdrew from the neuromuscular junction, eventually receding to the intramuscular nerve: at 4 to 6 days retraction bulbs were located close to neuromuscular junctions, whilst at later stages the majority of retraction bulbs were located in or near to the intramuscular nerve bundle (Figure 2.19). At 10 days post axotomy no structural remains of nerve terminals could be detected at the neuromuscular junction. By 12 days after nerve lesion stimulation of the severed distal nerve segment did not in any case elicit any transmitter release at axotomized motor endplates (Figure 2.27).

In comparison, wild-type terminals assessed by the same morphological criteria were in some cases visibly degraded at 6 hours after axotomy, and nearly all terminals were completely degenerated by 24 hours post axotomy (Figures 2.24, 2.25, and 2.26).

Wld^s nerve terminals proceed from a morphologically intact and functionally replete state to a situation where there is a decline in the coverage of the endplate by the nerve terminal, coinciding with a decrease in transmitter release and transmission probability. This study provides evidence to support the hypothesis that axotomy of Wld^s nerve terminals results in withdrawal of axonal processes from the neuromuscular junction rather than degeneration *in situ* (see discussion below, and Fig 2.33: schematic of withdrawing synapse). Withdrawal is by nature progressive, initiated at the endplate and resulting in retraction from the neuromuscular junction. The alterations to Wld^s nerve terminals are progressive in two ways. First, some

nerve terminals withdraw earlier than others. Thus, in any one particular area, a structurally intact endplate may neighbour another endplate in which withdrawal is complete. Second, withdrawal is progressive within each endplate. FM1-43 labelling indicates that at 3 days post axotomy nearly all nerve terminals were comparable to unoperated terminals (Figure 2.3). As time progressed an increasing number of endplates showed partial loss of synaptic staining over the endplate, until at 10 days post axotomy all nerve terminals had retracted from the neuromuscular junction (Figure 2.5). Thus, nerve terminal withdrawal is asynchronous on two levels: inter- and intra-terminal.

The size and shape of nerve terminals undergoing withdrawal was analysed to determine whether these factors influenced the rate of nerve terminal retraction (Figures 2.9, 2.10). Overall, synapse withdrawal at Wld^s terminals was not influenced by either the shape or the size of the junction. However, at 7 and 9 days post axotomy smaller endplates were significantly more persistent than larger ones. This may have a bearing on the nature of withdrawal at Wld^s junctions, when contemplating the motor unit as a whole. Consider one motor unit and its collaterals, terminating at endplates of large and small sizes. Perhaps larger nerve terminals are more likely to retract at earlier stages due to the constraints and demands of their size on the parent axon. Parent axons may retract larger endplates sooner due to metabolic demands and overstretching of resources, before beginning to withdraw smaller endplates. Elaborating on this hypothesis, presumably endplates more distant from the intramuscular nerve would also be more likely to withdraw sooner.

These speculations are further substantiated by the finding that at 8 and 9 days post axotomy regularly shaped (circular) endplates were more likely to have persistent nerve terminals than irregularly shaped ones. Presumably retraction of a nerve terminal would begin at the periphery of the junction and proceed back towards a central point before reabsorption into the parent axon collateral. Large and irregular shaped endplates would more likely show signs of withdrawal, therefore, than small circular shaped endplates.

Intracellular recordings from axotomized Wld^s fibres indicated that the number of functionally innervated endplates declined from 4 to 13 days post axotomy (Figure 2.27). At those endplates that remained functionally innervated during this period,

quantal content was significantly lower than that of contralateral controls (Figure 2.29). Repeated stimulation of axotomized but still innervated junctions resulted in a higher probability of failure as time post axotomy progressed (Figure 2.30). Fibres in which no EPP/action potential could be elicited still had spontaneous MEPPs, demonstrating that axotomized Wld^s nerve terminals spontaneously release transmitter even after loss of evoked activity. The overall MEPP frequency declined with time (Figure 2.28), but occasionally fibres with 'bursting activity', comprising high MEPP rates (>10/10 second), were observed. These data indicate that there is a gradual decline in the functional innervation of an axotomized muscle. In parallel to the morphological observations, this takes place asynchronously between muscle fibres. In addition, nerve terminals proceed through a transitional weakened state of low quantal release, and MEPP frequency decreases.

It seems likely that the decline in function at Wld^s junctions can be attributed to the decline in the occupancy of the endplate. This proposition is supported by the strong correlation between the incidence of 0% occupancy and 100% failures, as shown in Figure 2.31B and C. Intracellular recordings from identified junctions indicated that the number of failures appeared to increase at Wld^s axotomized endplates as nerve terminal processes retracted, although this change was not significant (Figure 2.32A). Thus, it is likely that endplates that are only partly occupied by the nerve terminal have lowered quantal responses, as would be expected from the reduced number of boutons overlying acetylcholine receptors. Reduction of nerve terminal area and volume would probably result in a reduction in the number of active zones, and therefore the number of synaptic vesicles available for docking. This would reduce the safety factor of neuromuscular transmission, which would result in a higher probability of failure of transmission, and a lower MEPP rate. Indeed, this is observed in muscles in the latter stages of withdrawal. Furthermore, the spontaneous bursting activity observed in some fibres may be an indicator of the crowding of synaptic vesicles into a more confined space, as would presumably occur during withdrawal. Overcrowding of synaptic vesicles may increase the number of synaptic vesicles in the vicinity of an active zone, and thus increase the probability of spontaneous quantal release, resulting in very high frequency MEPP rates. There were morphological indicators for this hypothesis: occasionally, FM1-43 labelled

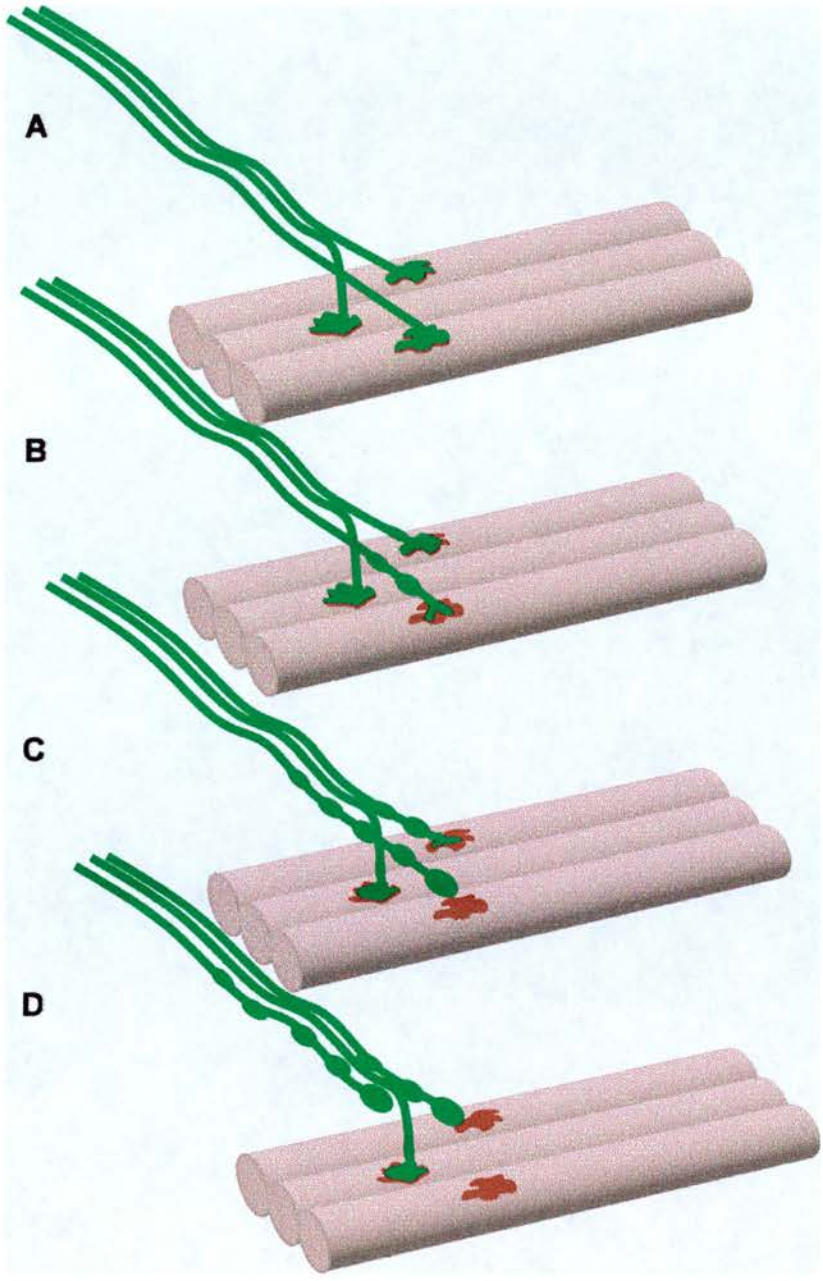
nerve terminals had a punctate staining pattern in some areas, which implied clustering of synaptic vesicles.

FIGURE 2.33: WITHDRAWAL OF NERVE TERMINAL BOUTONS AT AXOTOMIZED WLD^s NEUROMUSCULAR JUNCTIONS.

A schematic to illustrate the putative phenomenon of nerve terminal withdrawal following nerve damage in the C57BL/Wld^s mutant mouse.

A. Morphologically intact nerve terminals (green) closely juxtapositioned to acetylcholine receptors (red) on the postsynaptic muscle fibre.
B. Retraction of nerve terminal boutons at axotomized Wld^s neuromuscular junctions. The onset of withdrawal varies between endplates.
C. Complete withdrawal of nerve terminal processes is accompanied by the formation of a perisynaptic retraction bulb, and several more proximal swellings. Other nerve terminals initiate withdrawal of nerve terminal boutons.
D. Withdrawal of axonal processes into the parent axon in the intramuscular nerve bundle. Adjacent nerve terminals also form retraction bulbs.

Withdrawal of axotomized Wld^{S} nerve terminals



2.4.1 Nerve terminal morphology following axotomy

Nerve terminal structure was visualised using FM1-43 and NF/SV2. These techniques enabled the observation nerve terminal morphology following axotomy. FM1-43 labels actively recycling vesicles at the neuromuscular junction. Therefore, it is possible that the reduction in vesicular staining may not represent an alteration in the overall structure of the nerve terminal. In order to determine this, FM1-43 staining was validated in several ways. First, FM1-43 is a lipophilic dye that labels not only synaptic vesicles, but also passively labels the nerve terminal membrane and preterminal axon (Figures 2.2 and 2.23). Alterations in the distribution and quantity of vesicles would lead to a reduction in staining, but if the nerve terminal membrane remained this would be detected as a passively stained object. This did not occur. Second, NF/SV2 labelling of fixed preparations, which yielded similar results, appeared identical to FM1-43 staining in every instance. Third, the patterning of FM1-43 and NF/SV2 labelling appears identical to that of zinc iodide osmium staining (Ribchester *et al.*, 1995; personal observations) and silver cholinesterase staining (Crawford *et al.*, 1995). Therefore, loss of FM1-43 staining or NF/SV2 labelling is assumed to represent a reduction in the size of the nerve terminal overlying the muscle endplate.

2.4.2 Synapse withdrawal vs. degeneration

Synapse withdrawal rather than Wallerian degeneration is indicated at axotomized Wld^s neuromuscular junctions in several ways.

First, changes in the structure of axotomized nerve terminals took place in a gradual bouton-by-bouton manner, as indicated by serial staining of preparations using FM1-43 and NF/SV2, and by repeated visualisation. This was unlike the staining pattern of denervated wild-type nerve-muscle preparations. At wild-type neuromuscular junctions, this study indicated that nerve terminals undergo an abrupt and global fragmentation resulting in the disintegration of the terminal and its parent axon. Degeneration at wild-type junctions took place *in situ*, without any withdrawal or gradual loss of synaptic staining. These findings are in agreement with other morphological and ultrastructural investigations. Experiments which employed silver stain techniques to label denervated nerve terminals (Tello, 1907; Korneliussen and

Jansen, 1976; Kawabuchi *et al.*, 1991) indicated that terminal structure becomes fragmented and vacuolated, and the preterminal axon disintegrates along its length. Numerous ultrastructural studies demonstrate that nerve terminals disintegrate in situ, characterised by loss of synaptic vesicles, formation of vesicular aggregates, disruption of mitochondria and eventual fragmentation and degeneration of terminal structure resulting ultimately in Schwann cell phagocytosis (Birks, Katz and Miledi, 1960; Miledi and Slater, 1970; Manolov, 1974; Winlow and Usherwood, 1975; Riley, 1981). FM1-43/NF/SV2 staining of wild-type terminals display many parallels of these findings: reduction of FM1-43 staining over the entire junction coincident with regions of intense staining (indicating a reduction of synaptic vesicles and formation of vesicular aggregates), diffuse staining of the terminal by NF/SV2 (indicating fragmentation and loss of nerve terminal proteins), and discontinuous loss of preterminal axonal labelling (disintegration of the intramuscular axon). None of these structural alterations were observed at axotomized Wld^s terminals. A study of the physiological properties of axotomized Wld^s muscles indicated that nerve terminals proceed through a transitional weakened state of low quantal release, and MEPP frequency decreases. These data differ in several ways from other reports of functional decline in denervated wild-type muscles. Miledi and Slater (1970) demonstrated that the ability to produce an EPP following distal nerve stimulation declined with time after denervation. However, although the time course of degeneration varied from fibre to fibre, the loss of neuromuscular transmission at an individual muscle fibre occurred abruptly, without passing through an intermediary stage. Thus, an all or none response was obtained after nerve stimulation. This would be expected, as morphological degeneration occurs over the entire junction almost simultaneously. Furthermore, this study reported that failure of the ability to transmit action potentials was accompanied by cessation of spontaneous MEPPs. In Wld^s mice, MEPPs persist beyond the loss of neuromuscular transmission, and spontaneous bursting activity occurs in some fibres. Loss of functionality occurs gradually, with a gradual decline in quantal release. These differences highlight the fact that Wld^s nerve terminals proceed through an intermediate stage before complete loss of neuromuscular transmission. This is unlike the decline in function in

denervated wild-type muscles. To conclude, Wld^s motor nerve terminals appear not to be removed via a delayed Wallerian degeneration mechanism.

Although it is evident that severed Wld^s axons do eventually degenerate, various studies have provided evidence that the changes that occur after axotomy in Wld^s mice are dissimilar to those that occur during Wallerian degeneration. Isolated wild type axon segments placed into culture undergo Wallerian degeneration at the same rate as *in vivo*. However, axotomized Wld^s axons placed into culture medium appear to degenerate faster than *in vivo* (Buckmaster *et al.*, 1995, Parson *et al.*, 1997). Also, the rate of axotomy-induced changes increases in ageing Wld^s mice (older than 3 months), whereas Wallerian degeneration is slower in older wild type mice. The wave of mitotic division of Schwann cells, observed after nerve section in wild type mice, occurs in Wld^s mice but the total number of Schwann cells never reaches that observed in wild type mice (Brown *et al.*, 1991b). If Wallerian degeneration was merely delayed in Wld^s mice, then one would expect identical phenomena to take place after nerve section, although imposed on a slower timecourse. Clearly, Schwann cells not only have a delayed reaction, but also a *reduced* reaction to axotomy in Wld^s mice. The rate of degeneration in Wld^s mice should increase with age, similar to normal mice, but the opposite takes place. The reaction of axotomized Wld^s axons in culture systems should reflect that of normal axons, but with a delay: in fact, Wld^s degenerate more rapidly. These observations indicate that axotomized Wld^s axons may utilise a separate and distinct mechanism than classical Wallerian degeneration. Thus, it is not known at present whether the actual process of axon destruction observed in the distal stump of axotomized Wld^s is mediated by Wallerian degeneration (Brown, Lunn and Perry, 1992).

Thus, it seems reasonable to propose that the reduction in motor nerve terminal staining at the neuromuscular junction and the presence of retraction bulbs, combined with a concurrent decline in quantal release, are as a result of the withdrawal of Wld^s nerve terminals following nerve lesion.

At face value, this seems to be a strange proposition when one would expect degeneration of nerve terminals and axons after nerve damage. However, the mutation inherent to the C57BL/Wld^s mouse may convey a complete abolishment of classical Wallerian degeneration rather than merely a delay, as was previously

assumed. In the absence of rapid degeneration, nerve terminals may default to a different and underlying mechanism involving retraction and resorption.

Cajal (1928) described terminal retraction bulbs in regenerating peripheral nerves. These terminal bulbs were 'sterile axon tips' belonging to axons that had failed to establish peripheral contacts, perhaps going awry during their growth phase, and were destined to be reabsorbed. Recent evidence suggests that such aberrant growth cones may have retracted due to the influence of repulsive cues (Pini, 1993; Fan and Raper, 1995; Messersmith *et al.*, 1995; Püschel, Adams, and Betz, 1995). These repulsive cues have two mechanisms of action. First, by *contact inhibition* whereby axons that come into direct contact with substrate adherent molecules collapse and retract. The second mechanism involves the diffusion of soluble factors that, upon contact with receptors on the growth cone, induce retraction. These are termed *chemorepellants*. Both these processes inhibit axonal growth in the 'wrong' direction by collapsing growth cones entering inappropriate areas. This collapse does not result in the death of the axon, but rather encourages axonal elongation in the correct direction. Perhaps these mechanisms effect the retraction of axotomized nerve terminals at the neuromuscular junction. The cue for retraction could arise from other cell types at the neuromuscular junction, such as Schwann cells, or the muscle fibre. The initiating factor may be that the nerve terminal is no longer wholly functional, and thus receives a signal for retraction in the form of repelling molecules from other cell types. This will be proposal will be explored further in the discussion to follow.

It is possible that withdrawal in Wld^s nerve terminals represents a recapitulation of other fundamental withdrawal mechanisms. For instance, withdrawal of synapses takes place both in the developing CNS and PNS as part of the refinement of connections that leads to the establishment of mature neuronal networks. Thus, in the visual system, auditory system, cerebellum, autonomic ganglia and skeletal muscle the number of axons that form synapses on individual postsynaptic cells decreases in the perinatal period (Purves and Lichtman, 1980, 1985). In these cases, loss of some synaptic inputs during development leads to the strengthening of the other inputs, which ultimately results in the efficacious innervation of the target cells by relatively few (sometimes only one) axons. What causes withdrawal in these paradigms is still subject to speculation. However, one system that has been intensively studied in

recent years is that of *synapse elimination* at the neuromuscular junction. The exact role of synapse elimination during development is not definitively known. In broad terms, the process can be defined as follows. In developing mammalian muscle, many axons converge on an individual muscle fibre, which results in the multiple innervation of that fibre (Cajal, 1909; Redfern, 1970; Bennett and Pettigrew, 1974). This is termed *polyneuronal innervation*. However, these supernumerary connections are reduced during the first two to three weeks after birth until each muscle fibre is innervated by only one axon. This reduction is not caused by a decrease in the number of motor neurons innervating each muscle, but instead results from a decline in the number of muscle fibres each motor neuron innervates (Brown, Jansen and van Essen, 1976; Balice-Gordon and Thompson, 1988). This process results in a one-to-one relationship between muscle fibre and axon, which is thought to aid gradation of tension of the muscle by sequential motor unit recruitment (Lichtman 1995). The loss of surplus synapses takes place via the phenomenon known as synapse elimination. During elimination, it is known that axons that are not ultimately destined to innervate a fibre are removed through a gradual process of withdrawal (Korneliussen and Jansen, 1976; Riley, 1977, 1981; Bixby, 1981; Gorio *et al.*, 1983; Balice-Gordon *et al.*, 1993). Riley (1977) first coined the phrase 'retraction bulb', whilst describing the loss of excess inputs during synapse elimination. Riley (1977, 1981) described the withdrawal of terminal boutons from the endplate region, which resulted in the formation of a vesicle-laden retraction bulb. This retraction bulb then receded from the junction and was eventually reabsorbed into the parent axon. Such observations were later reaffirmed using a technique that selectively labels convergent inputs with lipophilic dye isoforms called DiI and DiA, which fluoresce red and green respectively (Balice-Gordon *et al.*, 1993). This study revealed that one input selectively lost synaptic territory whilst the other input consolidated synaptic area. This eventually resulted in the complete removal of all nerve terminal boutons from one input and the formation of a retraction bulb. Interestingly, the silver stained images of Riley's and the fluorescently labelled endplates of Balice-Gordon and colleagues, illustrating retracting axons undergoing synapse elimination, are extremely similar to the images of withdrawal of Wld^S nerve terminals following axotomy. These images are compared with the withdrawal and retraction of

axotomized Wld^s nerve terminals in Figure 2.34. Such similarities may reflect an underlying homology between these phenomena.

FIGURE 2.34: COMPARISON OF MOTOR AXON RETRACTION BULBS.

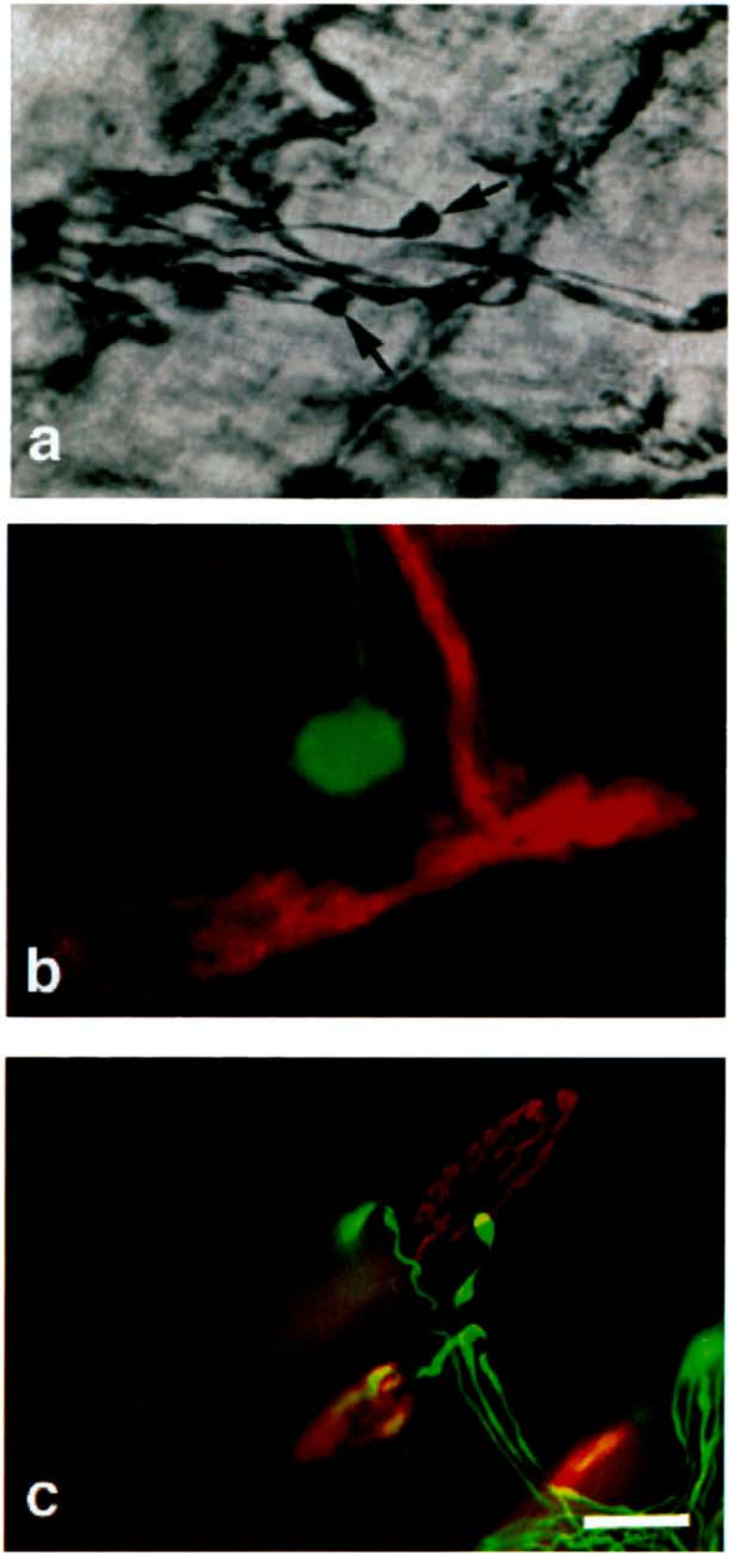
Retraction bulbs arise from the withdrawal of motor axon processes during synapse elimination and after axotomy in Wld^s TA muscles.

A. Silver impregnated axons 14 days after birth. Arrows point to retraction bulbs, that form as a result of axon collateral retraction during synapse elimination. Image taken from Riley (1977).

B. DiI (red) and DiA (green) labelled axon arbors at postnatal day 10. The image shows a mouse neuromuscular junction that is innervated by one axon (labelled red), with a perijunctional retraction bulb (green). The authors proposed that the retraction bulb arose as a consequence of synapse elimination. Image taken from Balice-Gordon *et al.*, 1993.

C. Perijunctional retraction bulb at a 4 day axotomized Wld^s TA neuromuscular junction (see Figure 2.18).

Scale bar = 20μm


a
b
c

One obvious difference between the two processes is that synapse elimination involves the retraction of a nerve terminal from a multiply innervated junction, whilst the withdrawal of axotomized Wld^s terminals entails the loss of only one input from a singly innervated junction. Synapse elimination is thought to be driven by competition between the multiple axons resulting in 'losers', which retract, and a 'winner', which becomes firmly established. This kind of competitive interaction does not seem possible in Wld^s muscles because only one input resides at the neuromuscular junction. However, it is far from clear how multiply innervating axons compete at developing neuromuscular junction. From a theoretical standpoint competitive interaction could take place in one of two ways: *direct competition* and *indirect competition*.

Direct competition would be manifested by the successful establishment of one input via the demise of the other inputs. In this paradigm, a conflict between inputs ensues, after which one nerve terminal directly weakens the other nerve terminals, resulting in their removal. For instance, direct competition could be mediated by activity-dependent release of a protease from the nerve terminal, which would detach the competitors from the synapse. This hypothesis was supported by the findings of Vrbová and co-workers, who demonstrated that stimulation of muscles *in vitro* resulted in the release of proteolytic enzymes (O'Brien, Ostberg and Vrbová, 1978). In addition, activity induced loss of nerve terminals was reduced by the application of broad-spectrum protease inhibitors (O'Brien, Ostberg and Vrbová, 1984; Connold, Evers and Vrbová, 1986; Vrbová and Fisher, 1989). Further experiments suggested that calcium modulation within the nerve terminal may be the cue for release of endogenous neutral protease release (Connold, Evers and Vrbová, 1986; Swanson and Vrbová, 1987; Tyc and Vrbová, 1995), supporting the theory that surplus synapses are directly removed by the nerve terminal that is destined to remain.

Indirect competition would involve the loss of one input through no direct fault of the other inputs. Thus, the competitors do not directly affect each other, but may be competing for a limited resource or affected in an independent way by a third factor. In this case, the conflict is resolved via the actions of a third factor. One such determinant of synapse elimination may be the muscle fibre. The muscle fibre may 'choose' one axon over another axon, and effect the withdrawal of the losing axons.

It is possible that synapse elimination is resolved by a combination of both direct and indirect competition. However, there is accumulating evidence to suggest that a more passive mechanism for the removal of superfluous axons, such as that of indirect competition, may operate during synapse elimination. Various reports have suggested that the postsynaptic cell acts as the arbiter in deciding which inputs are removed. Developmental studies of multiply innervated ganglion cells show that these cells also undergo a reduction in the number of innervating axons (Lichtman, 1977; Lichtman and Purves, 1980). These studies demonstrated that there is a remarkable correlation between the number of dendrites on the postsynaptic neuron and the number of axons the neuron is ultimately innervated by (Hume and Purves, 1981; Purves and Lichtman, 1985). Furthermore, ganglion cells that eventually become singly innervated often have no dendrites (Lichtman, 1977, 1980; Lichtman and Purves, 1981). These reports raise the possibility that withdrawal of axonal inputs may be determined by the postsynaptic cell. At the neuromuscular junction there is some evidence to suggest that the muscle fibre plays a role in synapse elimination. *In situ* studies of developing muscles demonstrated that there was a removal of postsynaptic acetylcholine receptors before terminal retraction (Balice-Gordon and Lichtman, 1993). This precocious loss of receptor staining implies that the muscle fibre is in some way initiating the withdrawal of the nerve terminal. Although this does not occur at axotomized Wld^s junctions (there was no significant difference between receptors underlying persistent and withdrawn nerve terminals) perhaps another mechanism, also mediated by the muscle fibre, induces the withdrawal of axotomized Wld^s nerve terminals. For example, in a modification of the protease hypothesis, Nelson and colleagues provided evidence that muscle fibres release thrombin or thrombin-like proteases into the synaptic cleft (Nelson *et al.*, 1993; Liu *et al.*, 1994a; Liu *et al.*, 1994b). Both prothrombin, the precursor to thrombin, and thrombin itself are present in muscle fibres during early postnatal life (Zoubine *et al.*, 1996), and thrombin induces neurite retraction via thrombin receptors *in vitro* (Suidan *et al.*, 1992). Recent experiments demonstrated that prothrombin and the thrombin receptor are maximally expressed at birth, and decrease 10-fold by postnatal day 20 (Kim, Buonanno and Nelson, 1999). Indiscriminate release of proteases would presumably effect the withdrawal of all

synapses simultaneously. However, it is thought that presynaptic activity protects the more active input from elimination. Protease nexin 1 and hirudin are specific thrombin inhibitors, and application of these substances delays synapse elimination (Liu *et al.*, 1994b). Protease nexin 1 is expressed by glia and muscle fibres, and is localised to the neuromuscular junction (Gloor et al., 1986; Festoff, Rao and Hantai, 1991). In addition, protease nexin 1 is downregulated 4 to 5-fold at postnatal days 10 and 15, during the most intense period of synapse elimination (Kim, Buonanno and Nelson, 1999). Therefore, release of thrombin from muscle fibres may result in the elimination of synapses, whilst more active synapses are protected by the presence of protease inhibitors. It is conceivable that withdrawal of axotomized Wld^s nerve terminals could be executed by such a mechanism. The release of a protease from the muscle fibre could act as a repelling cue for the withdrawal of the nerve terminal in a manner similar to that of a chemorepellant.

Another putative mechanism for indirect competition during synapse elimination involves the release of a maintenance factor from the muscle fibre. In this paradigm, the muscle fibre releases limiting amounts of a trophic factor which the more competitive (active) nerve terminal is better able to utilise. This in turn strengthens the more active nerve terminal whilst at the same time weakens the less active terminals. Several factors produced by muscle fibres are capable of retarding synapse elimination, although none as yet have entirely prevented elimination. The putative trophic factors that delay elimination include androgenic steriods, brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), fibroblast growth factor 2 (FGF-2), glial derived neurotrophic factor (GDNF), insulin-like growth factor 1 (IGF-1) and leukaemia inhibitory factor (LIF) (androgenic steriods: Jordan *et al.*, 1995; BDNF: Kwon and Gurney, 1996; CNTF: Jordan, 1996; CNTF, FGF-2: English and Schwartz 1995; GDNF: Nguyen *et al.*, 1998; IGF-1: Caroni and Becker, 1992; LIF: Kwon *et al.*, 1995). However, the interpretation of many of these studies is problematic. For instance, some of these factors induce axonal sprouting (CNTF, FGF: Gurney, Yamamoto, and Gurney, 1992; CNTF: Kwon and Gurney, 1994), and CNTF may cause a delay in synapse elimination by delaying muscle fibre maturation (Martin *et al.*, 1996). This issue is further complicated by the fact that trophic factors often act in concert: there is usually inherent redundancy in trophic systems, whereby

the removal of one factor is compensated by the increase in other factor(s). Therefore, the experimental removal or addition of a single factor may not provide startling results.

The withdrawal of trophic support could account for the retraction of nerve terminals from Wld^s neuromuscular junctions. Wld^s nerve terminals undergo a decline in quantal efficacy and spontaneous activity, which may give rise to a decline in the release of a factor necessary for synaptic stability. Evidence from other systems suggests that a decline in trophic factor support can induce axonal withdrawal. Atrophy of axon arborisations has been induced by NGF withdrawal in sympathetic neurons (Campenot, 1982a, b). Application of BDNF and neurotrophin-4 (NT-4) prevent the formation of ocular dominance columns, suggesting that these factors stabilise geniculate axon synapses that would otherwise be removed during the segregation of axons into eye-specific regions (Cabelli, Hohn, and Schatz, 1995). Increasing levels of activity, by adding of potassium chloride or lowering glutamate concentration, dramatically increases BDNF synthesis by cortical neurons *in vitro* (Ghosh, Carnahan, and Greenberg, 1994). In addition, the survival rate of retinal ganglion cells is higher when cultured in the presence of neurotrophins and potassium chloride, as opposed to neurotrophins alone (Meyer-Franke *et al.*, 1995). These reports suggest that neurotrophin release can be stimulated by activity, and that the ability of axons to respond to trophic factors increases when they themselves are active. At axotomized Wld^s junctions, only spontaneous activity remains. Loss of evoked activity may induce a reduction in the level of trophic support and thereby induce nerve terminal withdrawal.

An alternate mechanism for synapse elimination involves the removal of a nerve terminal without any competitive interactions. This theory, known as *intrinsic withdrawal*, was first proposed in the light of an experiment by Brown and colleagues (1976). In this study, neonatal rat soleus muscles were partially denervated to the extent that the number of axons was drastically diminished to only one or two. Therefore, some muscle fibres were only contacted by one axon collateral. The authors predicted that synapse elimination would be prevented in the absence of competitive interaction with other axons. However, this experiment resulted in a surprising finding – the motor unit size, as estimated by tension

measurements, still decreased. In a fully innervated muscle, the motor unit size (the number of muscle fibres contacted by an individual neuron) declines during the period of synapse elimination. This is as a consequence of the retraction and reabsorption of some axon collaterals whilst the consolidation of other collaterals ensues. As previously discussed, the retraction of axons from some endplates may be due to competition with other axons at that neuromuscular junction. However, the experiment of Brown and co-workers suggested that axons withdrew from some junctions even though there were no other innervating nerve terminals present. There are some technical criticisms of this study (see Lichtman, 1995), namely that tension measurement can often be misleading because subthreshold responses are not detected. More recently, Fladby and Jansen (1987) investigated this phenomenon in the mouse soleus muscle using intracellular recording techniques in addition to tension measurements to verify the innervation state (mono- or polyneuronally innervated) of endplates after synapse elimination had ended, and reported a similar result. Again, partial denervation of neonatal muscle, such that only one or two axons remain, resulted in the reduction of average motor unit size. The remaining junctions were tested using intracellular methods and all junctions were found to be mono-neuronally innervated. Thus, some elimination of inputs must have occurred in the absence of competitors. On the basis of these findings, axons are apparently able to withdraw from neuromuscular junctions without any impetus from co-innervating axons. The mechanism for this withdrawal remains subject to speculation. The nerve terminal could self-activate a pre-programmed withdrawal mechanism after a critical situation has been reached, resulting in the retraction of its terminal boutons from that particular endplate. 'Inappropriate' innervation of muscle fibre may initiate this withdrawal program. For instance, there is some evidence that adult innervation patterns are derived via the pairing of appropriate motor units with a particular muscle fibre type. Before synapse elimination, nerve terminals from a motor unit often innervate both fast and slow muscle fibre types. However, the preferential segregation of synaptic inputs through synapse elimination results in the homogenous innervation of *either* fast *or* slow muscle fibres by a particular motor unit (Thompson, Sutton and Riley, 1984; Fladby and Jansen, 1990; Gates and Ridge, 1992). This is known as mismatch withdrawal: fast fibres are paired with

appropriately matched axons, and slow axons are similarly paired. Loss of inappropriate inputs may occur via intrinsic withdrawal. Such a phenomenon may apply to the withdrawal of axotomized Wld^s axons. These axons are no longer appropriately innervating the axotomized muscle fibres, and are therefore withdrawn. Perhaps this is mediated via an alteration of the adhesive attachments at the junction, resulting in stepwise retraction of terminal boutons.

Thus, there are many similarities between the naturally occurring developmental phenomenon of synapse elimination and the withdrawal of axons from Wld^s neuromuscular junctions after nerve damage. There are numerous morphological correlates between these process – both involve the retraction of terminal ramifications from the endplate, eventually resulting in the formation of a retraction bulb which withdraws from the endplate region. Both types of withdrawal appear to proceed to completion once initiated within 1-2 days. There are also physiological similarities between withdrawal of Wld^s nerve terminals after axotomy and synapse elimination. During synapse elimination, the quantal content and efficacy of the axon destined to withdraw declines as time progresses (Dunia and Herrera, 1993; Coleman, Nabekura, and Lichtman, 1997). Likewise, the quantal efficacy of axotomized Wld^s nerve terminals declined as the nerve terminal withdrew from the endplate region. Both processes are gradual and progressive, in terms of loss of function and loss of morphological territory, at inter- and intra-endplate levels. This is evidenced by the asynchronous loss of nerve terminals between endplates and within endplates.

After consideration of all these factors, synapse elimination and nerve terminal withdrawal in the C57BL/Wld^s mouse after axotomy may indeed be related phenomena. If these processes are the same, then it should be possible to manipulate the withdrawal of Wld^s nerve terminals using experimental perturbations that affect synapse elimination. For instance, it has previously been shown that synapse elimination can be prevented by abolishing transmitter release at the neuromuscular junction (see Thompson, 1985). If the same treatment were applied to Wld^s nerve terminals one would expect the same result. This hypothesis will be explored further in the following chapter (Chapter 3).

In sum, the findings of this chapter are that Wld^s motor nerve terminals undergo retraction and withdrawal following axotomy, concurrent with a decline in quantal release.

CHAPTER 3

EFFECT OF ACTIVITY ON NERVE TERMINAL WITHDRAWAL FOLLOWING AXOTOMY IN C57BL/WLD^S MICE.

CHAPTER 3: THE ROLE OF ACTIVITY IN NERVE TERMINAL WITHDRAWAL FOLLOWING AXOTOMY IN C57BL/WLD^S MICE.

3.1 INTRODUCTION

The previous chapter demonstrated that Wld^S nerve terminals appear to withdraw from the neuromuscular junction rather than degenerate after nerve damage. The nature of this withdrawal is not known, but the discussion accompanying Chapter 2 alluded to the similarities between the withdrawal of nerve terminals that occurs during developmental synapse elimination and the withdrawal that takes place after axotomy at Wld^S neuromuscular junctions. Abolishing activity during synapse elimination has the effect of preventing nerve terminal withdrawal (see below, 3.12). This chapter comprises a study of the consequences of abolishing activity at withdrawing Wld^S motor nerve terminals. Axotomized Wld^S nerve terminals do not sustain evoked activity, in the form of action potentials conducted from the cell body to the neuromuscular junction, because nerve conduction is interrupted by the axotomy. However, Wld^S nerve terminals disconnected from their cell bodies maintain robust spontaneous activity, and remain able to release neurotransmitter after stimulation of the severed distal nerve for considerable periods of time after axotomy (see 2.3.3). Therefore, it is proposed that if synapse elimination and nerve terminal withdrawal of severed Wld^S motor axons share similar mechanisms, then removal of activity will prevent or delay withdrawal.

3.1.1 Hypotheses

Hypothesis: Activity regulates motor nerve terminal withdrawal.

Three eventualities may occur as a of result abolishing transmitter release at withdrawing Wld^S junctions, and these can be summarised as follows:

1. Nerve terminal withdrawal is abolished.
2. Nerve terminal withdrawal is delayed, but not prevented.
3. Nerve terminal withdrawal is accelerated.

If axotomy-induced withdrawal of Wld^s nerve terminals is identical to the withdrawal that takes place during synapse elimination, then abolishing activity should have the effect of preventing nerve terminal withdrawal.

3.1.2 The role of activity in synapse elimination

Numerous studies have demonstrated that alterations in the level of activity can profoundly influence synapse elimination. For instance, the removal of activity from developing neuromuscular junctions prevents or delays synapse elimination. Experimental inhibition of activity has been carried out in several ways. Muscle tenotomy, deafferentation, and spinal cord section all delay synapse elimination at the neuromuscular junction (Benoit and Changeux, 1975; Pestronk, Drachman and Griffin, 1976; Caldwell and Ridge, 1983). Pharmacological manipulation of neuromuscular activity has been carried out in three ways: via tetrodotoxin (TTX), botulinum toxin, or bungarotoxin administration. Local TTX infusion of the entire nerve supply, which prevents the conduction of nerve action potentials via sodium channel blockade, effectively blocks synapse elimination (Thompson, Kuffler and Jansen, 1979; Taxt, 1983), as does the inhibition of neurotransmitter release by botulinum toxin (Brown, Hopkins and Keynes, 1982). Postsynaptic inhibition of activity, using bungarotoxin to prevent the binding of acetylcholine to its receptors, also blocks synapse elimination (Duxson, 1982; Callaway and Van Essen, 1989). Thus, it appears that synapse elimination is inhibited or retarded at inactive nerve terminals, and at paralysed neuromuscular junctions.

The conduction of an action potential into a nerve terminal results in the simultaneous and uniform release of neurotransmitter from all the boutons of the affected nerve terminal (Betz, Mao and Bewick, 1992; Ribchester, Mao and Betz, 1994). Thus, at the level of an individual neuromuscular junction, it is probable that activity-dependent synapse elimination is regulated by the relative levels of activity of each nerve terminal, rather than each bouton. Current paradigms favour the proposal that the more active nerve terminal has a competitive advantage over less active nerve terminals. This hypothesis is corroborated by *in vitro* experiments which indicate that in dually innervated frog myotubes selective stimulation of one input results in the rapid and long-lasting suppression of the less active co-innervating

axon (Lo and Poo, 1991). Simultaneous stimulation of both axons in most cases prevented alterations in the synaptic strengths of the axons (Dan and Poo, 1992), consistent with the theory that competition is mediated by contrasting levels of activity. Selective stimulation of motor units during synapse elimination appears to convey a competitive advantage to the more active inputs: the stimulated motor units remain expanded in comparison with unstimulated axons (Ridge and Betz, 1984). Similarly, regenerating axons that are active reinnervate more muscle fibres than axons in which evoked activity has been prevented, by infusion of TTX (Ribchester and Taxt, 1983, 1984; Ribchester, 1988), although these experiments are subject to interpretation (Callaway, Soha, and Van Essen, 1987, 1989). Furthermore, during synapse elimination withdrawing axons, although still capable of transmitter release after some terminal boutons have been retracted, appear to have lowered quantal efficacy (Dunia and Herrera, 1993; Coleman *et al.*, 1997).

Overall, it is apparent that disparate levels of activity of each innervating axon influence synapse elimination. Thus, different levels of activity at multiply innervated neuromuscular junctions appears to effect the retraction of terminal boutons belonging to less active terminals. On the other hand inhibition of activity, resulting in inactive nerve terminals results in the stable coexistence of multiple nerve terminals, which do not undergo synapse withdrawal. Furthermore, mature synapses in mammalian muscles are generally extremely efficaceous, with a high safety factor of transmission and large quantal contents (Wood and Slater, 1997). Thus, it appears that disparate levels of activity, and the relative strength of transmission, are major determinants of synapse elimination.

However, conflicting results indicate that increasing neuromuscular activity, which simultaneously raises the level of activity at all nerve terminals, accelerates synapse elimination: chronic nerve stimulation precipitates synapse elimination (O'Brien, Østberg and Vrbová, 1978). This treatment is more effective at high frequency bursts than with equal numbers of stimuli at 1 Hz (Thompson, 1983). There is some difficulty in interpreting these experiments. Chronic stimulation of a neonatal nerve may elicit different levels of activity at each neuromuscular junction, both due to the loss of nerve conduction at weak branch points, and due to relatively lower transmitter release at endplates occupied by smaller nerve terminals (in comparison

to their neighbours), resulting in subthreshold responses. Further, the chronic synchronous activity imposed on each junction by the experimental paradigm may merely superimpose the endogenous asynchronous activity of each input.

These apparently conflicting results may reflect the subtlety of the process. In sum, although inhibition of activity has a profound effect on axon withdrawal during synapse elimination, the role of dissimilar levels of activity at polyneuronally innervated neuromuscular junctions remains unclear.

3.1.3 Botulinum Toxin

Botulinum toxins are produced by the bacterium *Clostridium botulinum*. Their mode of action is confined to the peripheral nervous system, where they block presynaptic release of neurotransmitters into the synaptic cleft. They exist in several different serotypes, which share structural homology: all botulinum toxins are composed of two disulfide-linked polypeptide chains. The larger subunit is responsible for neurospecific binding and cell penetration. Reduction releases the smaller chain into the axonal cytoplasm, which cleaves specific protein components of the neuroexocytosis apparatus via zinc-endopeptidase activity. Botulinum A and E specifically cleave synaptosome-associated protein of 25kDa (SNAP-25) (Blasi *et al.*, 1993a; Schiavo *et al.*, 1993a; Binz *et al.*, 1994), whilst botulinum C cleaves syntaxin (Blasi *et al.*, 1993b; Schiavo *et al.*, 1995). Both SNAP-25 and syntaxin are proteins specific to the presynaptic plasma membrane. Botulinum toxins B, D, F and G specifically recognise vesicle-associated membrane protein (VAMP/synaptobrevin) (Schiavo *et al.*, 1992, 1993c,d, 1994; Yamasaki *et al.*, 1994a,b). This is an integral protein of the synaptic vesicle membrane, and is cleaved at single peptide bonds which differ for each serotype. SNAP-25 and syntaxin on the presynaptic membrane, and VAMP/synaptobrevin on vesicles, interact by binding identified amino acid domains (Hayashi *et al.*, 1994; Chapman *et al.*, 1995; Pellegrini *et al.*, 1995) and are thought to facilitate vesicle docking at active zones, sites at which neurotransmitter is released from vesicles into the synaptic cleft (Söllner *et al.*, 1993; Pevsner *et al.*, 1994). At a physiological level, Botulinum toxin A prevents all evoked and spontaneous presynaptic release from motor nerve

terminals, thereby preventing neuromuscular conduction of action potentials/EPPs and MEPPs.

The administration of botulinum toxin to intact adult neuromuscular junctions results in the formation of fine nerve processes known as sprouts, which elongate to form an intricate network across the muscle (Duchen and Strich, 1968; for review see Brown, Holland and Hopkins, 1981). In neonatal muscles botulinum toxin has been shown to prevent nerve terminal withdrawal during developmental synapse elimination (Brown, Hopkins and Keynes, 1982).

3.2 MATERIALS AND METHODS

Animal care and surgery were as detailed in chapter 2 (2.2.1, 2.2.2), except where otherwise described here. One month old female C57BL/Wld^s mice were used for this study. The left transversus abdominus (TA) muscle was axotomized by unilateral section of the left second to eighth intercostal nerves. At this time, botulinum toxin was applied to the endplate region of the left TA muscle. A small section of the superficial internal oblique muscle was carefully dissected away to expose the endplate region of the underlying TA muscle. A Gilson micropipette was used to administer 0.15ng of botulinum toxin serotype A in 5 µl of buffer (see solutions – appendix). This buffer solution was used by Thompson and colleagues, in a study of the effect of botulinum toxin on Schwann cell sprouting, and was shown to have minimal effects on the muscle (Son and Thompson, 1995a). During this procedure the muscle was not touched by the tip of the pipette, in order to minimise the risk of muscular damage due to the operation. The muscle was then left for 5 minutes to allow dispersion of the toxin over the muscle. The region was then liberally washed with buffer solution (without botulinum toxin), and carefully resealed using 7/0 Mersilk suture. Although the dosage appeared to be correct in terms of neuromuscular transmission blockade, a small number of animals became systemically affected by the toxin, and did not survive the experiment. All animals were carefully monitored, and affected animals were immediately sacrificed. Negative controls for botulinum toxin administration were performed, using the same procedure as above but omitting botulinum toxin from the buffer solution.

Alternatively, as another form of control, the left TA muscle was axotomized by unilateral section of the second to eighth intercostal nerves, but without botulinum or buffer administration directly to the TA muscle.

TA nerve-muscle preparations were dissected, attached to the base of a Slygard-lined recording chamber using fine steel pins, and bathed with aerated normal physiological saline. Neuromuscular transmission was tested in botulinum and buffer-alone treated muscles. The axotomized intercostal nerves were carefully pared away from the intercostal muscles to expose a 5 mm length of nerve. The muscle nerve was then electrically stimulated using a suction electrode with pulses up to 100

V in amplitude. TA muscle fibres were carefully examined for signs of contraction, as evidence of neuromuscular transmission. This procedure was repeated on all axotomized intercostal nerves, and for all (unoperated) contralateral control intercostal nerves. Neuromuscular conduction was blocked in the vast majority of preparations. Only one preparation displayed any signs of activity, and was discarded.

Wholemount nerve-muscle preparations were fixed, the axon/nerve terminal labelled with antibodies to NF and SV2, and acetylcholine receptors stained with TRITC-conjugated α-Bungarotoxin as previously described (see 2.2.4). The slides were analysed for morphological criteria (endplate occupancy) as described previously (see 2.3.1.2).

3.3 RESULTS

A morphological study was carried out to determine the effect of presynaptic inhibition of synaptic vesicle fusion on synapse withdrawal of Wld^s nerve terminals 4, 6 and 10 days after nerve lesion. Nerve terminal and preterminal axonal morphology was assessed in fixed preparations using immunocytochemical techniques to stain NF and SV2 proteins. Postsynaptic acetylcholine receptors were irreversibly labelled using TRITC-conjugated α-Bungarotoxin. Morphometric assessment of endplate occupancy by the nerve terminal was identical to that of the morphological studies detailed in Chapter 2: namely that the overlap between the staining pattern of the nerve terminal boutons and the distribution of acetylcholine receptors was quantitatively compared. Complete correspondence between the staining patterns was denoted 100% occupancy. Terminals that were in various stages of withdrawal were placed into 'bins' of 0%, 1-24%, 25-75%, and 76-99% occupancy.

Unoperated motor nerve terminals appeared identical to those of wild-type terminals. All unoperated terminals observed had complete overlap between the NF/SV2 and α-Bungarotoxin staining patterns (100% occupancy).

Muscles that had been axotomized, but not subjected to botulinum toxin, had similar withdrawal of endplates in comparison to FM1-43 terminal staining at the same stages. Axotomized muscles treated with buffer alone displayed similar levels of nerve terminal withdrawal to that of axotomized-alone preparations (Figure 3.1) indicating that the buffer solution had no inherent effect on Wld^s nerve terminal withdrawal after axotomy.

Nerve-muscle preparations superfused with 0.15 ng botulinum toxin were clearly labelled by antibodies to NF/SV2 proteins, and α-Bungarotoxin. Although there was evidence of some intact nerve terminals, particularly at 4 and 6 days, nerve terminal withdrawal was not prevented. Terminals in varying stages of withdrawal are represented in Figures 3.2 and 3.3. Thus, Wld^s nerve terminal withdrawal was not entirely abolished with removal of activity. Nerve terminals withdrew from axotomized neuromuscular junctions in the same way as axotomized-alone terminals, with gradual retraction of nerve terminal processes and manifestation of a

perijunctional retraction bulb. Changes in terminal morphology leading to the formation of retraction bulbs appeared similar to that of axotomized-alone nerve terminals (Figure 3.4). However, morphometric analysis of nerve terminal occupancies revealed that nerve terminal withdrawal was delayed by the removal of activity (Figure 3.5). In axotomized but untreated muscles, 22% of nerve terminals had completely withdrawn (0% occupancy) by 4 days post axotomy, increasing to 55% at 6 days and 99% at 10 days post axotomy. In botulinum treated Wld^s muscles, there was a delay in the withdrawal of motor nerve terminals following axotomy: at 4 days only 6% had withdrawn, increasing to 18% at 6 days and 91% at 10 days. This delay was most striking at 6 days post axotomy, where 36% more endplates remained occupied by Wld^s nerve terminals (significantly different; paired t-test, p = 0.0071). A low magnification comparison of these preparations is presented in Figure 3.3.

The rate of withdrawal was clearly affected by the addition of botulinum toxin to the muscle. However, the difference between axotomized botulinum treated and axotomized-alone muscles at 10 days post axotomy, although significant (paired t-test, p = 0.0008), was small – only 7%. Thus, although the inhibition of activity delayed withdrawal at 4 and 6 days, it appears that the rate of withdrawal closely resembled that of untreated axotomized muscles by 10 days. This suggests that the effect of botulinum toxin lessened during the latter stages of withdrawal in axotomized Wld^s muscles. However, whilst the majority of nerve terminals had withdrawn at 10 days post axotomy in botulinum treated muscles, persistent nerve terminals appeared to be localised to relatively specific regions of the muscle, demonstrating that nerve terminals in some areas of the muscle were more resistant to withdrawal than those in other areas (Figure 3.3). This did not appear to be the case in axotomized –alone muscles.

Analysis of the linear regression using the Spearman rank correlation indicated that the numbers of endplates that become completely vacated (0% occupancy) increases linearly for both axotomized-alone and botulinum treated muscles (r = 0.9461 and 0.9066 respectively, two-tailed p value <0.0001 for both correlations; see graph X). The rate of nerve withdrawal in botulinum treated muscles, estimated from the slope of the linear regression, was 14.9% in comparison to 12.6% for axotomized alone

muscles. This suggests that nerve terminal withdrawal proceeds faster in botulinum treated muscles, once initiated. However, there appears to be a delay in the initiation of nerve terminal withdrawal in muscles treated with botulinum toxin. Although the linear regression lines converge, an estimate of the overall delay can be made by taking the difference between the linear regressions, taken at the midpoint at 7 days post axotomy, and calculating the delay over the entire period of the experiment (Figure 3.6). Overall, nerve terminal withdrawal in botulinum treated muscles was delayed by approximately 1.14 days following the inhibition of activity, caused by the topical administration of botulinum.

In addition to the withdrawal of nerve terminals, muscles treated with botulinum toxin also exhibited a small number of nerve terminal sprouts (Figure 3.7). These sprouts were NF/SV2 stained filamentous processes originating from axotomized nerve terminals. They were of small diameter (no larger than 5 micrometer in diameter) and sometimes spanned some distance from the neuromuscular junction. Motor nerve terminal sprouts were not observed at any of the 812 axotomized-alone endplates. This demonstrates that axotomized Wld^s nerve terminals treated with botulinum toxin have the ability to sprout even after isolation from their cell body.

FIGURE 3.1: WITHDRAWAL OF NERVE TERMINAL PROCESSES FROM AXOTOMIZED WLD^s NEUROMUSCULAR JUNCTIONS FOLLOWING BOTULINUM BUFFER ADMINISTRATION.

The superfusion of botulinum buffer solution does not affect the rate of withdrawal of Wld^s TA nerve terminal processes from the endplate following nerve lesion. The histogram shows a comparison of occupancies at 6 days post axotomy with and without botulinum buffer administration. There was no significant difference within these groups (paired t-test).

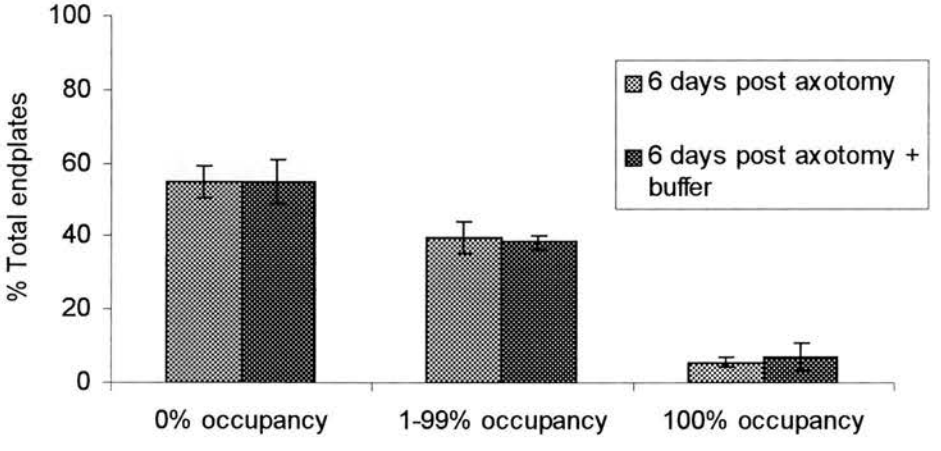


FIGURE 3.2: WITHDRAWAL OF NERVE TERMINAL PROCESSES FROM AXOTOMIZED WLD^s NEUROMUSCULAR JUNCTIONS FOLLOWING BOTULINUM ADMINISTRATION.

The superfusion of botulinum toxin does not prevent nerve terminal withdrawal. Wld^s TA nerve terminal processes remain able to retract from the endplate following nerve lesion.

A and **B** show acetylcholine receptors labelled with TRITC-conjugated α-Bungarotoxin (red), and NF/SV2 staining (green) from the same neuromuscular junction. Nerve terminal ramifications are in the process of withdrawing from the endplate region. Note the preterminal varicosities in **A**, which presumably form prior to retraction, and intact and fully occupied endplates neighbouring withdrawing nerve terminals in **B**.

Scale bar = 20μm

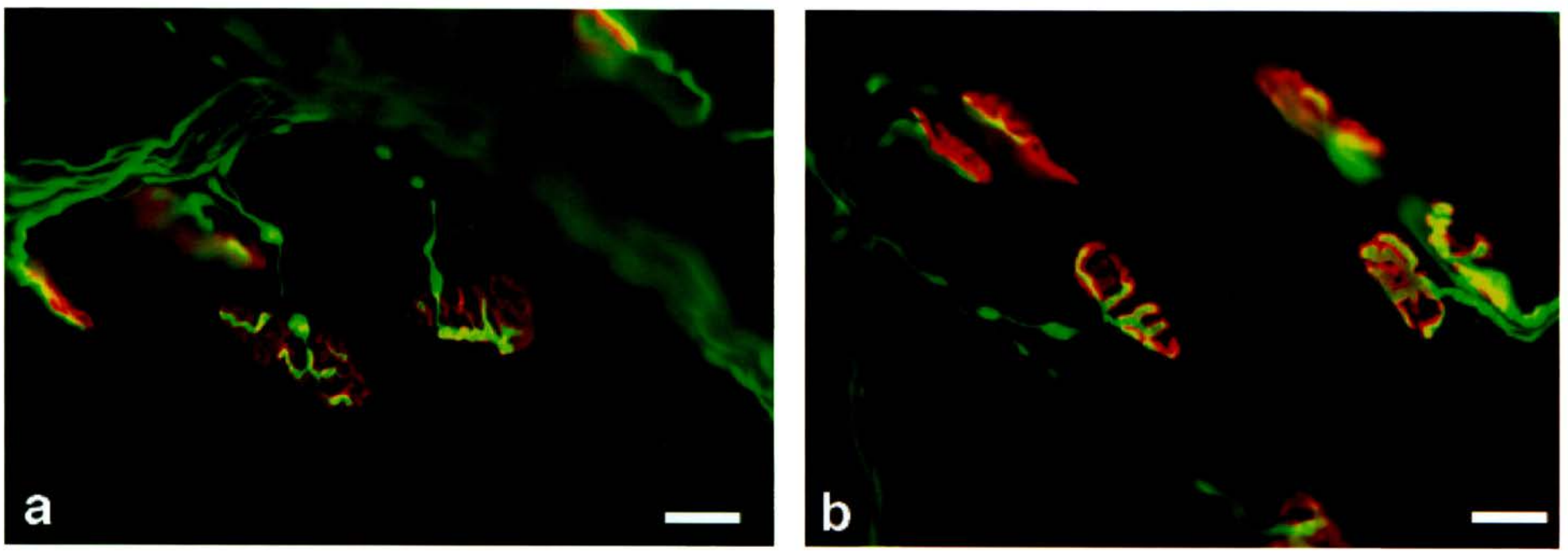

a
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FIGURE 3.3: COMPARISON OF BOTULINUM TREATED AXOTOMIZED AND AXOTOMIZED-ALONE WLD^s NEUROMUSCULAR JUNCTIONS.

Low power images of axotomized-alone and botulinum treated axotomized Wld^s neuromuscular junctions. Botulinum toxin delays Wld^s nerve terminal withdrawal following nerve lesion. All images illustrate an overview of NF/SV2 and α-Bungarotoxin labelled Wld^s TA muscles (x10 magnification).

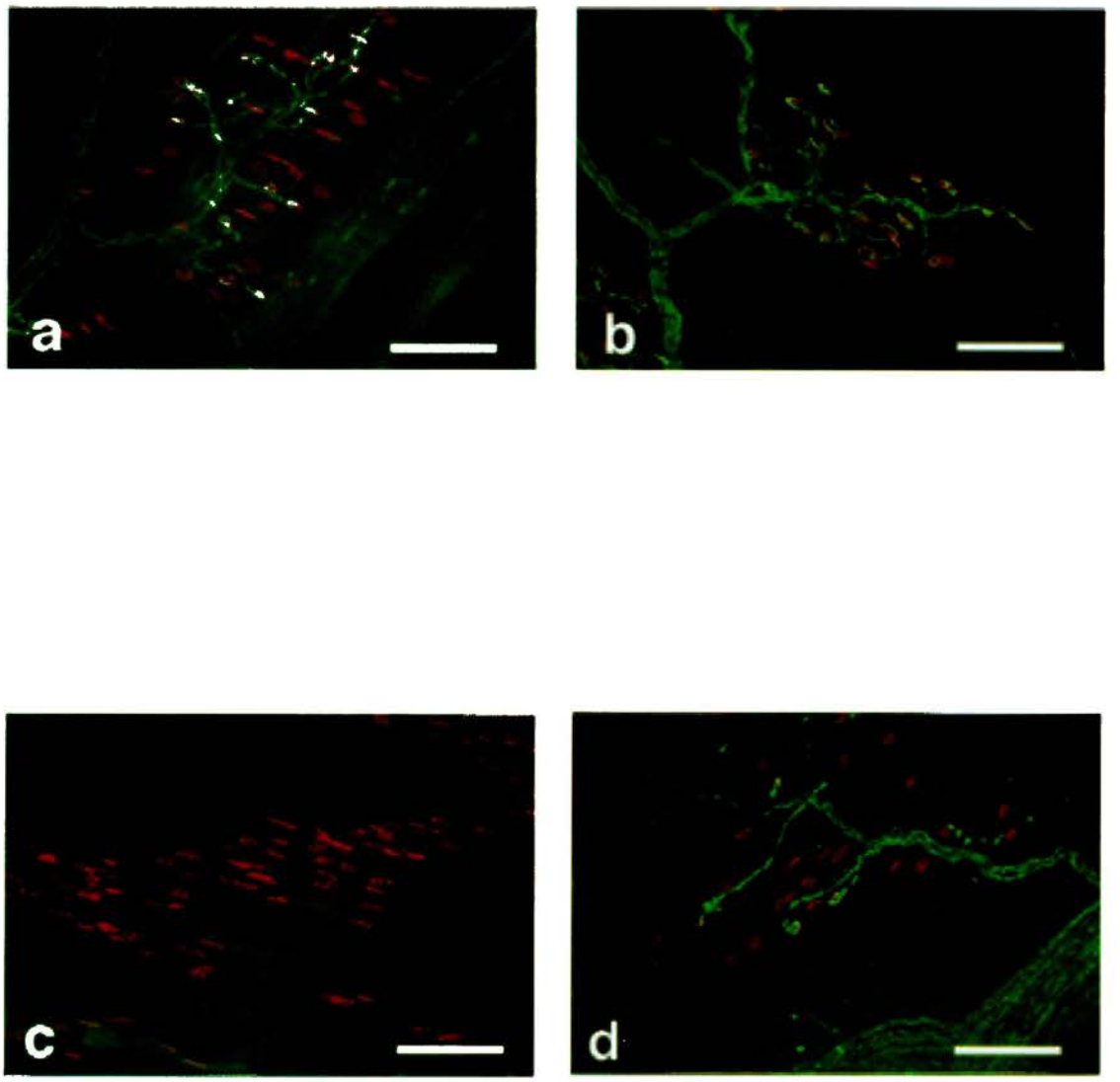
A. 6 day axotomized axotomized-alone muscle. An overview of endplates shows many nerve terminals in advanced stages of withdrawal.

B. Overview of a 6 day axotomized Wld^s TA muscle to which botulinum toxin was added at the time of axotomy. The majority of motor endplates are innervated by axons, and axons in intramuscular nerve bundles are clearly visible. Most endplates show signs of withdrawal.

C. 10 day axotomized-alone muscle. No endplates are contacted by axon processes. The remaining axons are diffusely labelled.

D. 10 day axotomized botulinum treated muscle. Although many endplates are vacant of nerve terminal processes, some endplates remain innervated Note that this image is not representative of the majority of regions in these muscles, but is only used here to illustrate the persistence of nerve terminals in some regions.

Scale bar = 100μm


a
b
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d

FIGURE 3.4: RETRACTION BULBS AT AXOTOMIZED WLD^s NEUROMUSCULAR JUNCTIONS TREATED WITH BOTULINUM TOXIN.

Retraction bulbs are present at various stages after nerve lesion at endplates paralysed by botulinum toxin.

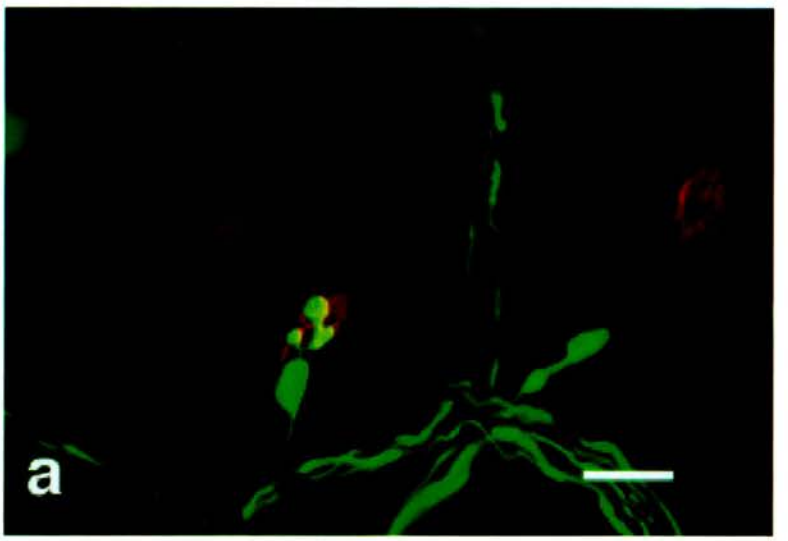
A to **C** depict progressive formation of retractions bulbs in botulinum treated Wld^s TA muscles 10 days after nerve lesion.

A. Centre left of picture: nerve terminal in advanced stages of withdrawal. The nerve terminal ramifications have coalesced in three regions, presumably in preparation for withdrawal from the endplate. A large swelling has formed preterminally. At the left of this image, a retraction bulb appears to be receding from a vacant endplate.

B. Progressive withdrawal of retraction bulbs from the endplate area. Large varicosities with secondary swellings are located next to vacant endplates.

C illustrates two retraction bulbs at different distances from vacant endplates. The retraction bulb located at the top of this image appears to be withdrawing to the intramuscular nerve bundle.

Scale bar = 20μm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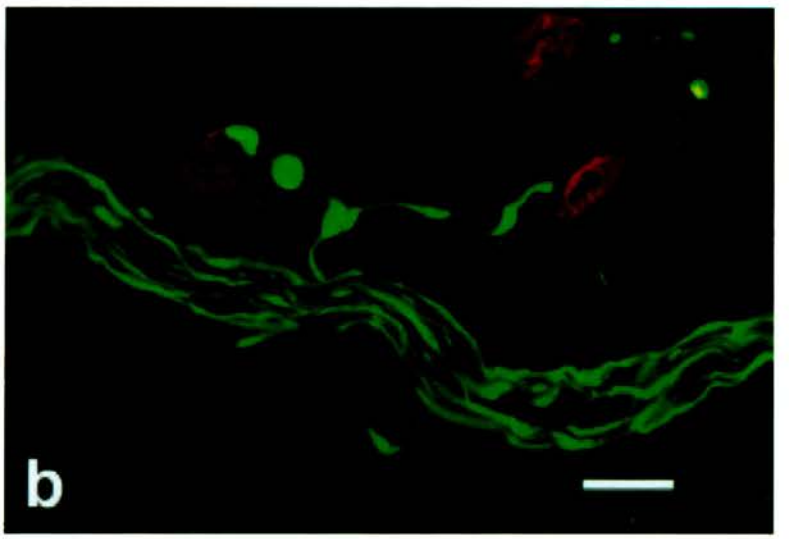
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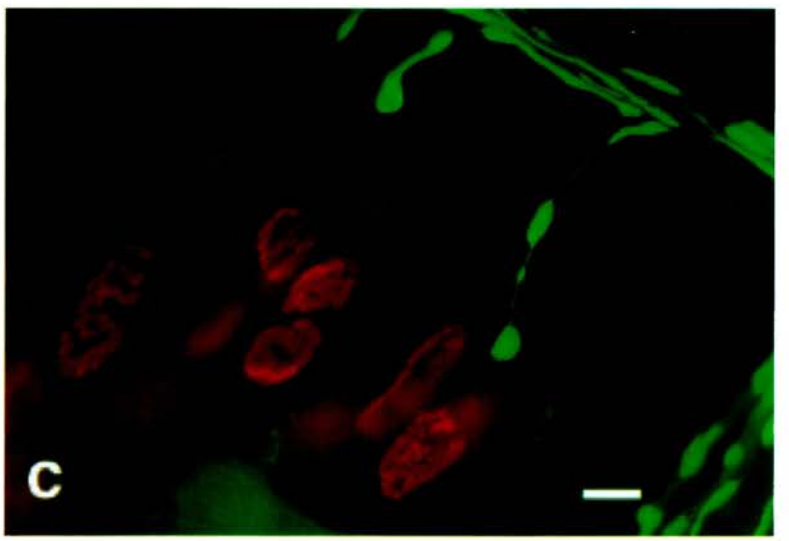
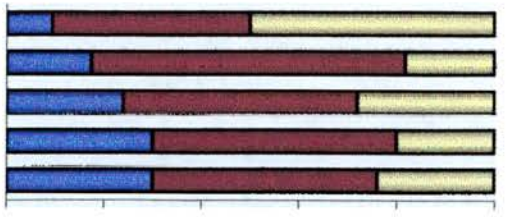
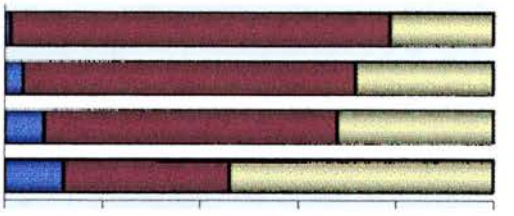

c

FIGURE 3.5: WITHDRAWAL OF AXOTOMIZED WLD^s NERVE TERMINALS IN BOTULINUM TREATED MUSCLES.

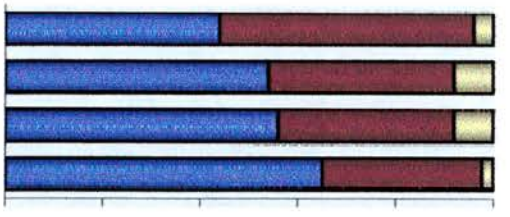
Inhibition of activity delays the withdrawal of Wld^s nerve terminals following nerve lesion. The graphs show axotomized Wld^s TA muscles with and without botulinum toxin treatment. Each bar represents data from one muscle. A total of 1672 endplates from 26 muscles were analysed.



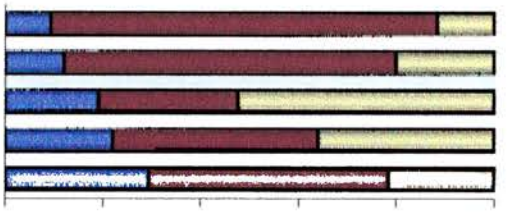
4 days axotomy-alone



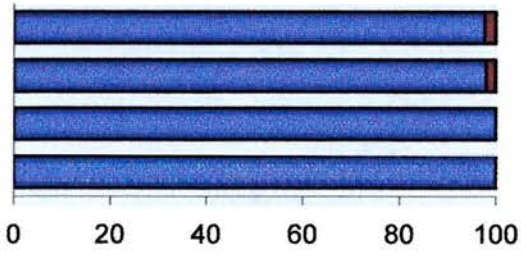
4 days axotomy+BoTX



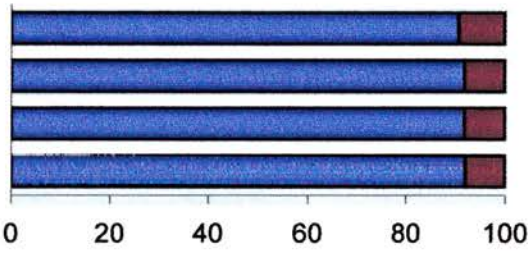
6 days axotomy-alone



6 days axotomy+BoTX



10 days axotomy-alone



10 days axotomy+BoTX

0% Occupancy 1-99% Occupancy 100% Occupancy

FIGURE 3.6: RATE OF WITHDRAWAL FOLLOWING AXOTOMY OF WLD^s NERVE TERMINALS IN BOTULINUM TREATED MUSCLES.

The increase in the number of completely vacated endplates is delayed in botulinum treated Wld^s TA muscles. Data pooled from 841 endplates and 26 muscles. Each point represents one muscle.

An estimation of the delay was made by taking the difference between the regressions at the midpoint (7 days post axotomy). The delay was calculated as 1.14 days for the period investigated.

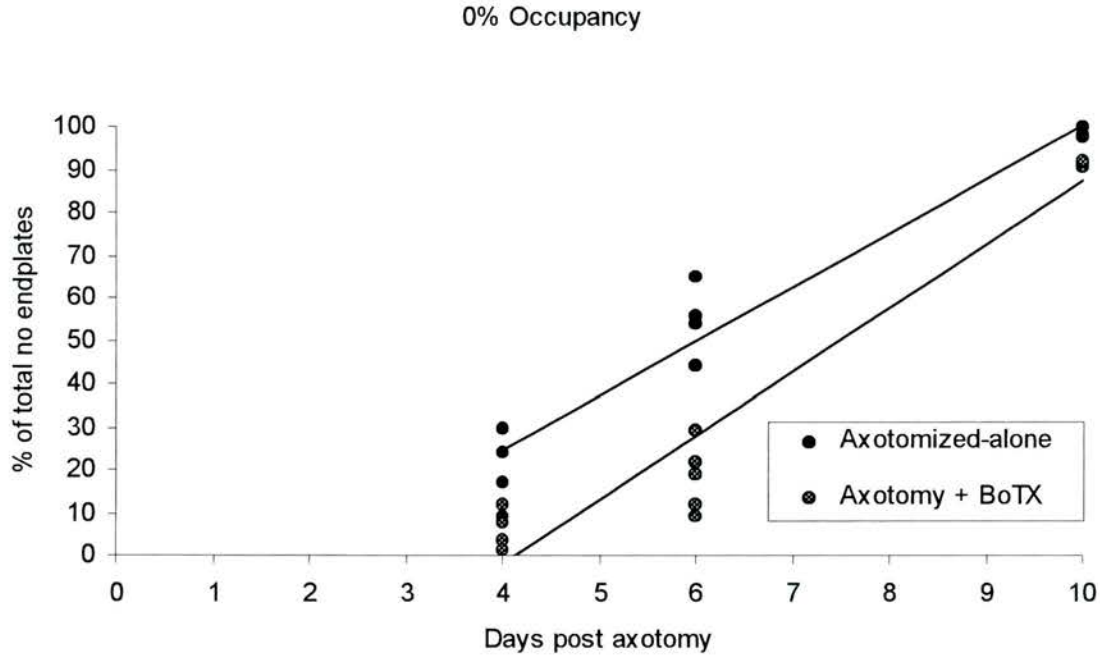


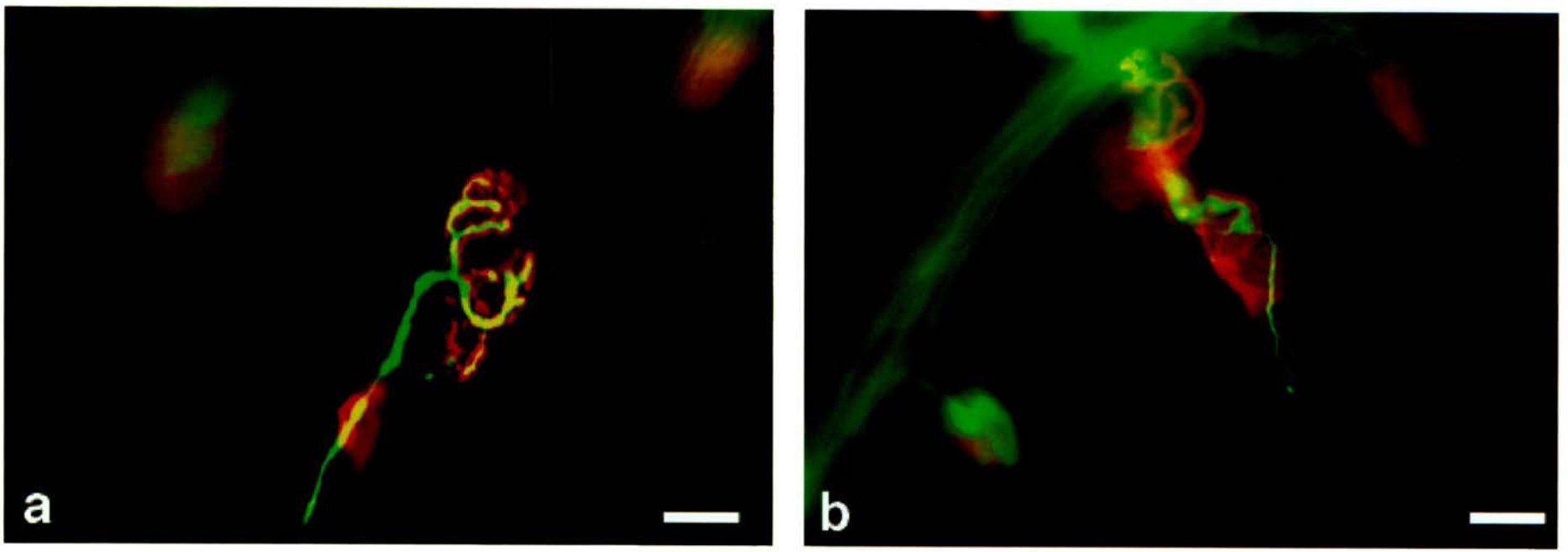
FIGURE 3.7: NERVE TERMINAL SPROUTS AT AXOTOMIZED WLD^s NEUROMUSCULAR JUNCTIONS TREATED WITH BOTULINUM TOXIN.

Wld^s TA nerve terminals extend NF/SV2 labelled processes following axotomy and botulinum administration.

A. Nerve terminal 4 days after axotomy and botulinum treatment. Nerve terminal boutons appear to be withdrawing from this endplate. However, a small process extends from the junctional region, possibly elongating towards a vacant neighbouring endplate.

B. Nerve terminal sprouts extending from 6 day axotomized, botulinum treated neuromuscular junction.

Scale bar = 20μm


a
b

3.4 DISCUSSION

The principal aim of this chapter was to discover the effect of inhibiting activity on the withdrawal of axotomized Wld^s motor nerve terminals. Essentially, this is a study of the prevention of spontaneous MEPP release at Wld^s neuromuscular junctions, and the consequences of this on the retraction of axotomized nerve terminals.

The results show that, in botulinum-treated animals, Wld^s motor nerve terminals withdraw from the neuromuscular junction after nerve lesion. However, there is a significant but small delay in the retraction of nerve terminals following nerve lesion. This overall delay was estimated from the linear regression calculations to be 1.14 days over the period investigated. If the delay was caused by a decline in the rate of retraction of terminal boutons once withdrawal had been initiated, then the linear regression lines depicted in Figure 3. 6 would diverge. In fact, the rate of withdrawal after the initial trigger appeared to be slightly accelerated. The analysis indicates that the decrease in the overall rate of nerve terminal retraction was therefore probably attributable to a delay in the initiation of withdrawal rather than a delay in the progression of the withdrawal process once started.

The delayed response to axotomy in botulinum treated muscles demonstrates that activity influences the withdrawal of Wld^s nerve terminals after nerve injury. This intriguing result indicates that spontaneous activity may play a role in the retraction of terminal boutons. The findings of the chapter 2 indicate that MEPPs persist beyond the ability of the nerve terminal to elicit action potentials or EPPs, suggesting a fairly robust mechanism for spontaneous release of neurotransmitter at axotomized Wld^s neuromuscular junctions (see 2.3.3). Furthermore, MEPP frequencies were similar to those of unoperated controls 4, 5, 6, and 7 days post axotomy, indicating that MEPP rate remained normal for up to 7 days post axotomy. However, MEPP frequencies declined at 8 and 12 days post axotomy, until at 13 days no MEPPs were recorded. Botulinum toxin appears to have a greater effect on the delay in the initiation of nerve terminal withdrawal at 4 and 6 days post axotomy, times at which the MEPP rate remains comparable to unoperated control muscles. The effect of botulinum treatment is lessened at 10 days post axotomy, where the levels of withdrawal were similar to axotomized-alone preparations, and incidentally around the time when MEPPs fail altogether (see chapter 2, 2.4). Therefore, the effect of the

prevention of synaptic vesicle fusion via botulinum toxin is most pronounced during the time when the mechanism for the generation of spontaneous MEPPs remains normal. Botulinum toxin had less effect on nerve terminals which probably did not have normal rates of spontaneous transmitter release, at 10 days post axotomy. This implies that spontaneous transmitter release influences nerve terminal withdrawal at axotomized Wld^s junctions.

The mechanism for this may at first seem obscure. However, consider a structurally persistent nerve terminal that is spontaneously and asynchronously active. MEPPs occur as a result of the spontaneous fusion of synaptic vesicles to the plasma membrane, resulting in the release of transmitter into the synaptic cleft which produces a postsynaptic potential. These spontaneous events take place at different boutons, and therefore different locations of the junction. Spontaneous fusion of a number of synaptic vesicles at one bouton could produce several MEPPs, and may perhaps cause the 'bursting activity' observed at some endplates (see 2.3.3). Furthermore, axotomized Wld^s muscles exhibit a considerable amount of spontaneous contractile activity that resembles fibrillation (see 2.3.3, Ribchester *et al.*, 1995). Intracellular recordings revealed spontaneous action potentials were the cause of this contractile activity, and these were apparently triggered from the rising phase of abnormally large MEPPs (Ribchester *et al.*, 1995). It is not inconceivable that the generation of a number of MEPPs, or a spontaneous action potential, from a particular bouton could establish a form of competition between the synaptic boutons similar to activity-dependent competition between nerve terminals during synapse elimination. In this paradigm, more functionally robust boutons would be more spontaneously active than their neighbours, thus competitively disadvantaging these weaker boutons. These boutons would then retract. However, because overall the axotomized Wld^s synapse is functionally weak (due to the lack of persistent and evoked action potentials) eventually all boutons undergo retraction. Whether synapse withdrawal is a result of a disparity in the levels of activity or not, it is likely that it is the relatively low level of acetylcholine release that predisposes axotomized Wld^s nerve terminals to withdrawal. Axotomized Wld^s nerve terminals are functionally weak in comparison to unoperated controls (see 2.3.3). Inefficaceous synaptic transmission essentially means that the function of the neuromuscular junction, to

induce muscle action potentials, is compromised. Therefore, this is probably the initial signal that induces the retraction of Wld^s nerve terminal processes from the muscle endplate. It is possible that this is due to a decline in trophic support from the muscle, or a decline in the ability to receive trophic support. Various studies have shown that trophic factor release, and trophic factor uptake, are dependent on the ability to release neurotransmitter (see 2.4). A decrease in trophic factor release from muscle fibres due to weak activity, or perhaps a decline in trophic factor receptors on the nerve terminal, could induce nerve terminal retraction. Alternatively, an increase in the release of protease such as thrombin, and a concurrent decline in protease inhibitor (such as protease nexin 1 – see 2.4), may initiate the withdrawal of axotomized Wld^s terminals. This would result in a decline in the adhesivity of the nerve terminal structure to the neuromuscular junction, eventually resulting in retraction of terminal ramifications.

Assuming that the decline in activity, or a disparity in the levels of intra-terminal spontaneous activity, could mediate nerve terminal retraction, how then does botulinum toxin delay synapse withdraw at Wld^s junctions following nerve lesion? Complete abolishment of all activity, which was performed in this experiment using botulinum toxin, results in complete inactivity. This may effect a change in the signalling events that take place at the neuromuscular junction, which removes activity-dependent withdrawal signals, and instead promotes growth signals. This would also explain the tentative formation of sprouts at botulinum treated endplates. It is well established that botulinum toxin produces denervation-like changes in muscle fibres (see Khan, 1995, for review). Note that denervation changes are delayed in axotomized Wld^s muscles (Brown *et al.*, 1991). Denervation of wild type muscle results in changes in the production of growth promoting factors. These include an upregulation of BDNF and downreulation of NT-4 in muscles (Funakoshi *et al.*, 1993), and an upregulation of GAP-43, the p75 NGF receptor, NGF, BDNF and NT-4 and downregultion of CNTF in Schwann cells (Friedman *et al.*, 1992; Sendtner, Stöckli and Thoenen, 1992; Funakoshi *et al.*, 1993; Hassan *et al.*, 1994). Caroni and Schneider (1994) demonstrated that IGF1 and IGF2 increases in denervated muscle, which in turn promotes the proliferation of perisynaptic interstitial cells thought to facilitate growth of axons. The investigators also observed

the same phenomena in botulinum-poisoned muscles (Caroni and Schneider, 1994). Alterations in the expression of any of these molecules may signal a change in the local environment of the endplate, from growth limiting to growth promoting. Thus, complete blockade of neuromuscular activity may inadvertently provide stability for persistent axotomized Wld^s nerve terminals, and cause a delay in the withdrawal of terminal boutons. In sum, protracted absence of muscle activity may initiate cellular and molecular reactions aimed at restoring neuromuscular transmission and preventing withdrawal. Weak and sporadic activity would promote signals that remove the aberrant input from the neuromuscular junction.

However, this hypothesis cannot entirely account for the withdrawal of nerve terminals at axotomized Wld^s junctions, as the withdrawal process was delayed, but not prevented. Therefore, an alternate mechanism must also affect terminal withdrawal. Perhaps a component of the withdrawal of boutons from a neuromuscular junction is activity-dependent, and another component is intrinsic to the nerve terminal. Thus, although nerve terminal retraction is affected by the abolishment of activity, some pre-programmed mechanism executes the final withdrawal of the nerve terminal. This may be similar to a mechanism such as that proposed for intrinsic withdrawal. Intrinsic withdrawal is thought take place during the period of synapse elimination, and involves the withdrawal of an axon from a previously innervated endplate (see 2.4). The mechanism for this phenomenon is unknown. However, it is possible that a similar mechanism, intrinsic to axotomized Wld^s axons, effects the removal of nerve terminal boutons from the endplate.

Alternatively, perhaps activity-mediated mechanisms *are* entirely responsible for the retraction of axotomized Wld^s nerve terminals, but that the superfusion of botulinum toxin at the time of nerve section did not immediately prevent neurotransmitter release. Thus, a period of several days may have ensued during which the underlying activity-dependent nerve terminal withdrawal mechanisms were activated. The subsequent blockade of neurotransmitter may therefore have been less effective than an immediate block after axotomy. This could be tested by the application of botulinum toxin a few days prior to nerve section.

Botulinum toxin mediates the inhibition of transmitter via the cleavage of a protein necessary for the fusion of synaptic vesicles to the presynaptic membrane. Therefore,

it is possible that the delay in terminal bouton retraction is caused by the prevention of exocytosis *per se*. Thus, synaptic vesicles remain in the nerve terminal, and the neurotransmitter packaged within them is retained. If the withdrawal process were mediated intrinsically then it is possible that the retention of transmitter, together with the prevention of vesicle membrane fusion with the presynaptic plasma membrane, may have a protective effect on those terminals in some way.

Overall, it is apparent that the inhibition of vesicle-associated neurotransmitter release results in the delay of the withdrawal of Wld^s nerve terminals following nerve damage. The mechanism for this withdrawal is not known. Three hypotheses proposed at the start of this chapter, namely that botulinum toxin would either prevent, delay, or accelerate nerve terminal withdrawal. Prevention of nerve terminal withdrawal would provide evidence that withdrawal of axotomized Wld^s nerve terminals occurred via a similar mechanism to that of developmental synapse elimination. That this was not the case suggests that these two processes may be dissimilar. However, the initiation of the retraction of nerve terminal boutons was delayed for approximately 1.14 days, implying that spontaneous activity influences the triggering signals that result in nerve terminal retraction. Further manipulation of activity at axotomized Wld^s neuromuscular junctions may yield more clues as to the nature of this process.

Other events occur during synapse elimination that may shed light on the withdrawal of Wld^s nerve terminals after axotomy. For instance, during synapse elimination Schwann cell cytoplasm overlying withdrawing boutons is withdrawn prior to nerve terminal retraction (Culican, Nelson and Lichtman, 1998). In addition, Schwann cell migration away from the neuromuscular junction, induced by topical application of neuregulin during early postnatal life, results in nerve terminal retraction from the areas that were vacated by Schwann cell processes (Trachtenburg and Thompson, 1997). These findings suggest that Schwann cells are intricately associated with withdrawing motor nerve terminals, a subject that will be further explored in the following chapter.

CHAPTER 4

REACTIVE TERMINAL SCHWANN CELLS AT AXOTOMIZED WLD^S NEUROMUSCULAR JUNCTIONS.

CHAPTER 4: REACTIVE TERMINAL SCHWANN CELLS AT AXOTOMIZED WLD^S NEUROMUSCULAR JUNCTIONS.

4.1 INTRODUCTION

Schwann cells are the glial cells of the peripheral nervous system. During development, a common Schwann cell precursor differentiates into two morphological, and functionally distinct types of Schwann cells: myelinating and non-myelinating (for review, see Jessen and Mirsky, 1998). Myelinating Schwann cells, as the name suggests, form the myelin that ensheaths axons, whilst non-myelinating Schwann cells loosely surround axons of a smaller diameter. In the adult, nerve damage causes both types of Schwann cells to de-differentiate and proliferate, and these cells are known as 'reactive' Schwann cells (Cajal 1928; Abercrombie and Johnson, 1946; Pellegrino and Spencer, 1985; Pellegrino *et al.*, 1986; Clemence *et al.*, 1989). These Schwann cells congregate to form the Bands of Bungner (see Chapter 1), which is thought to facilitate the regeneration of axonal processes by providing trophic support (Bunge, 1993; Ide 1996). Regeneration and restoration of function results in the re-differentiation of Schwann cells to myelinating or non-myelinating cells depending on the axons they contact.

Terminal Schwann cells are a type of non-myelinating Schwann cell that caps neuromuscular junctions. However, unlike Schwann cells of the main nerve trunk, which form a permissive route for regenerating axons, the role of terminal Schwann cells in aiding axon re-growth after nerve damage was unclear. Following denervation, it was previously assumed that terminal Schwann cells simply phagocytose degenerating axon terminals, withdraw from the synaptic gutter (Miledi and Slater, 1968, 1970; Manolov, 1974), release small amounts of neurotransmitter (Miledi and Slater, 1968; Bevan *et al.*, 1973; Dennis and Miledi, 1974) and otherwise remain quiescent until the return of axons to the neuromuscular junction (Gorio *et al.*, 1983). However, the recent findings of Thompson and colleagues suggest that terminal Schwann cells actively participate in the guidance of axon sprouts to neuromuscular junctions.

It is well established that regenerating axons reinnervate previously vacated endplates with remarkable specificity, even though the motor endplate represents less than 0.1% of the total muscle fibre surface (discussed in Sanes, 1989). Reinnervating axons usually grow down the old endoneurial Schwann cell tubes leading to each endplate. However, axons can find endplates even when they grow outwith the confines of the endoneurial tube, and the pattern of branching of axon collaterals from the intramuscular nerve to the endplate, established during development, changes after reinnervation (Covault, Cunningham, and Sanes, 1987; Rich and Lichtman, 1989). Also, many axons continue to grow beyond their endplates, forming the so-called 'escaped fibres' described by neuroanatomists earlier this century (Tello, 1907; Ramon y Cajal, 1928; Gutmann and Young, 1944). These axons contact other endplates, resulting in polyneuronal innervation. Therefore, the mechanism for the guidance of axon processes once they had reached the muscle was debatable.

In muscles, various molecules present in basal lamina may provide a suitable substrate for axonal growth, including N-CAM, fibronectin, tenascin, laminin and heparin sulfate proteoglycan (Covault and Sanes, 1986; Reiger *et al.*, 1985; Sanes, Schachner, and Covault, 1986; Bixby, Lillien, and Reichardt, 1987; Fadic, Brandan, and Inestroa, 1990). Denervation induces selective proliferation of interstitial cells in perisynaptic areas, which leads to localisation of adhesive molecules such as tenascin C and fibronectin (Sanes *et al*, 1986; Connor and McMahan, 1987; Caroni and Schneider, 1994). Guidance of axons to the endplate itself may result from the release of diffusible factors, such as insulin-like growth factor from the endplate, establishing a concentration gradient centred at the endplate which preferentially attracts axonal growth cones (Henderson, Huchet, and Changeux 1983; Kuffler, 1989; Caroni and Grandes, 1990). However, in the light of recent findings, it seems probable that terminal Schwann cell processes provide the major guiding substrate for regenerating axon sprouts in muscles.

Reynolds and Woolf (1992), using immunocytochemical techniques to label Schwann cell specific S-100 protein, demonstrated that terminal Schwann cells respond to denervation by not only removing the remains of the degenerating nerve terminal, but also by extending long processes across the surface of the muscle.

These processes, known as terminal Schwann cell sprouts, often reach lengths of several hundred micrometers, and withdraw after reinnervation of the muscle. The initial cue for this sprouting is as yet unknown. However, terminal Schwann cells appear to be extremely sensitive to neuromuscular activity: stimulation of the motor nerve elevates internal Schwann cell calcium concentration (Jahromi, Robitaille and Charlton, 1992; Reist and Smith, 1992), and blockade of synaptic transmission using TTX or ω-conotoxin induces a rapid upregulation of glial fibrillary acidic protein (GFAP) in terminal Schwann cells (Georgiou *et al.*, 1994). Recently, it has been shown that acetylcholine secreted from nerve terminals decreases GFAP in terminal Schwann cells via muscarinic acetylcholine receptors (Georgiou, Robitaille and Charlton, 1999). Therefore, terminal Schwann cells clearly respond to alterations in nerve terminal activity. However, it is not known whether terminal Schwann cell sprouting is as a result of cutoff of transmitter-release or related substances from the nerve terminal, from the degeneration of the nerve terminal itself, or from a signal (or decline in a signal) from the inactive muscle fibre. Interestingly, calcitonin gene-related peptide (CGRP), a molecule released from motor nerve terminals, prevents denervation-induced axonal sprouting in muscle (Matteoli *et al.*, 1988; Tsujimoto and Kuno, 1988).

These reports indicate that terminal Schwann cells respond to nerve terminal degeneration by sprouting. What is the functional significance of this sprouting? Numerous studies have demonstrated that Schwann cells are excellent, even preferred, substrates for axonal growth *in vitro* (Fallon, 1985; Bolin and Shooter, 1994). Son and Thompson (1995a,b) labelled reactive Schwann cells during the course of denervation and reinnervation. They observed that terminal Schwann cells, after nerve terminal degeneration had occurred, dedifferentiate and extend long filamentous sprouts, confirming the original reports of Reynolds and Woolf (1992). Many of these processes fasciculated with others growing from adjacent endplates, thus forming a network of sprouts that interconnect the denervated endplates. Regenerating axons grew back to the muscle and re-occupied endplate sites. However, these axons continued to elongate even after they had contact an endplate, following the previously laid down network of Schwann cell sprouts. This lead the axon sprouts to other endplates, and eventually resulted in polyneuronal innervation

of motor endplates. Similarly, in partially denervated muscles terminal Schwann cells sprout not only from denervated endplates, but also innervated neuromuscular junctions (Son and Thompson, 1995a). However, most Schwann cell sprouts originated from denervated endplates. This led the investigators to propose that in a partially denervated muscle, Schwann cells from unoccupied endplates sprouted to innervated endplates, and led the axonal sprouts back to the denervated endplates. In order to resolve this, a foreign nerve was transected, and placed onto the surface of another muscle three days later (to ensure that no axons would remain in the foreign nerve). Schwann cells from the implanted nerve grew processes towards the endplate zone of the muscle. In some preparations, in which Schwann cell sprouts had not yet contacted endplates, no axonal sprouting was observed. However, in preparations in which Schwann cell processes had fully penetrated the endplate zone rampant axonal sprouting was observed, which in nearly every case followed the exact pathway of Schwann cell processes. Botulinum toxin induces extensive axonal sprouting, in response to inactivity (Brown *et al.*, 1981). Son and Thompson (1995b) reapplied this experimental paradigm to the investigation of Schwann cell sprouting, and observed that not only did Schwann cells extend numerous processes, but that nerve sprouts followed, or were associated with, Schwann cell processes. In fact, in some cases, Schwann cell sprouts were longer than the axonal sprouts associated with them, implying that Schwann cells were directly guiding nerve terminal sprouts. A recent experiment confirmed these findings using a vital dye, Calcein Blue am ester, that labels living mouse terminal Schwann cells without apparently damaging them (O'Malley, Waran, and Balice-Gordon, 1999). Terminal Schwann cells sprouted in response to nerve crush, and revisualisation of the same processes after reinnervation indicated that axon sprouts used existing Schwann cell processes and chains of cell bodies as substrates for outgrowth. Taken together, these studies suggest that terminal Schwann cells are not only permissive for axon growth but also instructive in the guidance of nerve sprouts following denervation.

In addition to their role in the guidance of regenerating axons, terminal Schwann cells are also implicated in the formation and maintenance of stable neuromuscular synapses. Schwann cells express a number of signalling molecules and receptors (Reynolds and Woolf, 1993; Scherer and Salzer, 1996), and respond to synaptic

changes by altering expression levels of neurotrophins (Funakoshi *et al.*, 1993) and CNTF (Friedman *et al.*, 1992; Sendtner *et al.*, 1992). As previously mentioned, terminal Schwann cells respond to alterations in transmitter release. Schwann cells at the neuromuscular junction have been implicated in the regulation of nerve terminal number during synapse elimination (Hirata *et al.*, 1997; Culican, Nelson and Lichtman, 1998). Schwann cells can modify the expression of acetylcholinesterase and butyrylcholinesterase, induce the formation of junctional folds and aggregate acetylcholine receptors at developing cultured rat neuromuscular junctions (Chapron *et al.*, 1997; Koenig, de La Porte, and Chapron, 1998). In addition, exogenous application of neuregulins causes retraction of Schwann cells from the endplate area, which in turn induces nerve terminal withdrawal (Trachtenburg and Thompson, 1997). In sum, terminal Schwann cells actively participate in the formation, maintenance and remodelling of the neuromuscular junction.

4.1.1 Aims and hypotheses

Hypothesis: terminal Schwann cell sprouting is initiated by the absence of motor nerve terminal contact.

This chapter will investigate the response of terminal Schwann cells following axotomy of Wld^s nerves. Specifically, do terminal Schwann cells respond as expected at Wld^s neuromuscular junctions when nerve terminal structure and function persists? To this end, three alternative outcomes are proposed:

1. Terminal Schwann cell sprouting is inhibited at axotomized Wld^s endplates.
2. Terminal Schwann cell sprouting is delayed at axotomized Wld^s endplates.
3. Terminal Schwann cell sprouting proceeds as normal at axotomized Wld^s endplates.

Inhibition of terminal Schwann cell sprouting would suggest that the prolonged presence of intact nerve terminals is sufficient to prevent the initiation of process elongation. A delay in the sprouting response of terminal Schwann cells would suggest that the presence of morphologically and functionally persistent nerve terminals prevents Schwann cell sprouting until that nerve terminal withdraws from the endplate. Alternatively, the withdrawal process that occurs following Wld^s nerve section may not affect terminal Schwann cell sprouting in any way.

4.2 MATERIALS AND METHODS

Animals, surgery, and anaesthesia were carried out as previously described in Chapter 2 (2.2.1, 2.2.2), except where otherwise stated. One month old female C57BL/Wld^s mice and female CBA or C57/6J mice of the same age were used. The nerve supply to the *transversus abdominus* (TA) muscle was resected, and nerve-muscle preparations were dissected 3, 6, 8, and 10 – 12 and 32 days later. The preparations were fixed and stained using an antibody that recognises S-100, a calcium-binding protein present in non-myelinating and terminal Schwann cells (Cocchia and Michetti, 1981; Mata, Alessi, and Fink, 1990), and to a lesser extent in myelinating Schwann cells (Mata, Alesi and Fink, 1990; Mirsky and Jessen, 1996).

Immunocytochemistry

Wholemount preparations were stained as follows (all steps performed on rotating platform, if possible):

1. Fix for 30 minutes in 4% paraformaldehyde in 1x phosphate buffered saline (PBS) solution.
2. Wash for 1 hour in PBS.
3. Incubate at 37 °C with 1mg/ml Collagenase.
4. Wash with PBS x 3.
5. Remove connective tissue from surface of muscle.
6. Inactivate collagenase activity with 0.1% sodium borohydride for 10 minutes (place on rotating platform).
7. Wash for 10 minutes in PBS.
8. Incubate with TRITC conjugated α-Bungarotoxin (5μg/ml) for 20 minutes, followed by 20 minute PBS wash.
9. Incubate with 1% Triton-X for 10 minutes.
10. Preblock (5% Donkey serum in 1% Triton-X solution) for 2 hours, or overnight.
11. Incubate with primary antibody (1:500 rabbit S-100 in preblock solution) for 2 hours.
12. Wash 1 hour with preblock solution.

13. Incubate >1 hour with secondary antibody (donkey anti-rabbit FITC 1:200 in preblock solution). Cover preparation with tinfoil from this point onwards.
14. Wash 1 hour with 1% Triton-X.
15. Mount and coverslip preparation with Vectashield. Store at 4 °C.

4.4 RESULTS

Fixed nerve muscle preparations 3, 6, 8, and 10 to 12 days post axotomy were labelled with antibodies to S-100 protein, a Schwann cell-specific marker (Reynolds and Woolf, 1992; Astrow, Son, and Thompson 1994), and with TRITC conjugated α-Bungarotoxin to visualise acetylcholine receptors. Neuromuscular junctions were examined for evidence of Schwann cell alterations. A morphometric assay for terminal Schwann cell sprouting was performed.

Terminal Schwann cells in unoperated control Wld^s mice were closely juxtapositioned to acetylcholine receptors. In fact, the distribution of terminal Schwann cell staining corresponded directly to that of α-Bungarotoxin labelled acetylcholine receptors (Figure 4.1). This was also the case at unoperated CBA neuromuscular junctions.

Neuromuscular junctions were examined for evidence of terminal Schwann cell sprouts. These were categorised as S-100 positive processes that extended beyond the confines of the neuromuscular junction for distances greater than 20 micrometers. Initial observations of axotomized Wld^s junctions revealed that Schwann cell sprouts were present. Terminal Schwann cell sprouts were very rarely detected at control, unoperated Wld^s junctions (<1%) or CBA/6J junctions (3.8%). Terminal Schwann cell sprouts were observed at CBA and Wld^s junctions as early as 3 days after nerve section. In both wild type and Wld^s muscles sprouts of over 200 micrometers were observed. Thus, axotomy-induced Schwann cell sprouting was not inhibited at Wld^s junctions (Figure 4.2). However, there was a difference between the amount of S-100 sprouts at Wld^s and wild type junctions at 3, 6 and 8 days post axotomy. Schwann cell sprouting at axotomized Wld^s endplates at these time points was significantly lower than sprouting at wild type endplates. These findings are represented in Figure 4.3. This difference was most exaggerated at 6 days: 64% of CBA/6J endplates exhibited terminal Schwann cell sprouts, whilst only 20% of axotomized Wld^s endplates showed signs of Schwann cell sprouting. By 10 to 12 days post axotomy, there was no significant difference between the amount of sprouting in Wld^s and CBA/6J muscles. However, during the period of the experiment, Schwann cell sprouting at Wld^s endplates did not ever reach the maximal level of sprouting

observed at denervated wild type endplates (64% at 6 days post axotomy). This suggests that Schwann cell sprouting at axotomized Wld^s endplates is not only delayed, but also reduced.

Overall, Schwann cells exhibiting sprouts from wild type and Wld^s junctions were generally similar in their morphological characteristics. S-100 labelled Schwann cell processes extended from a central 'body' which may have been one or more Schwann cell nucleus. This body usually occupied less than 100% of the endplate area (Figure 4.2A). However, there were some differences between the axotomy-induced alterations in Schwann cells at wild type and Wld^s endplates. At wild type endplates, Schwann cell cytoplasm withdrew slightly from the boundary of the endplate region. However, Schwann cells at some Wld^s endplates withdrew considerably from the boundary in a pattern reminiscent of axotomized Wld^s nerve terminal withdrawal (Figure 4.4). Unfortunately, triple labelling of nerve terminal (NF/SV2), acetylcholine receptors (α-Bungarotoxin), and Schwann cell (S-100) was unsuccessful.

In sum, the formation of terminal Schwann cell processes following nerve injury was considerably and significantly delayed at Wld^s neuromuscular junctions. The overall quantity of Schwann cell sprouting was less than that observed in wild type muscle, for the duration of the experiment. In addition, terminal Schwann cell withdrawal appears to mirror that of nerve terminal withdrawal at axotomized Wld^s endplates.

FIGURE 4.1: S-100 STAINING AT WLD^s NEUROMUSCULAR JUNCTIONS.

S-100 staining at unoperated control C57BL/6J (**A**) and Wld^s (**B**) TA neuromuscular junctions.

Images **A** and **B** show S-100 labelled Schwann cell processes (green) and TRITC-conjugated α-Bungarotoxin labelled acetylcholine receptors (red) visualised with a triple band pass filter block. A yellow colour appears where α-Bungarotoxin and S-100 staining coincide.

Note the precise overlap between the s-100 stained terminal Schwann cell processes and postsynaptic acetylcholine receptors.

Scale bar = 20μm

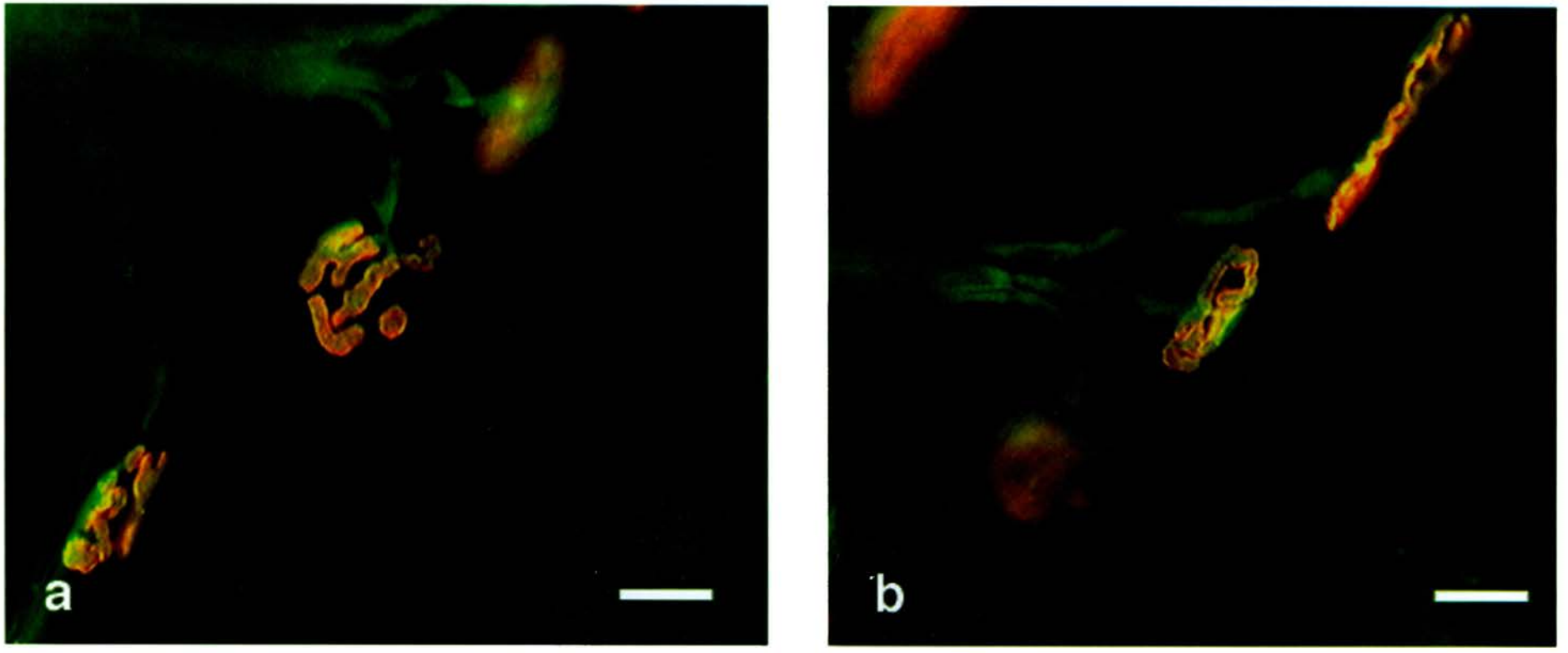

a
b

FIGURE 4.2: TERMINAL SCHWANN CELL SPROUTING AT AXOTOMIZED NEUROMUSCULAR JUNCTIONS.

Terminal Schwann cells sprout at axotomized wild type and Wld^s TA neuromuscular junctions.

A. Images taken from 12 day axotomized CBA muscle. An S-100 positive sprout extends from a neuromuscular junction. This sprout extends for a considerable distance from the endplate. Note that terminal Schwann cell processes have withdrawn from the majority of the endplate region.

B. Two sprouts extend from a 6 day denervated C57BL/6J endplate.

C. and **D**. Terminal Schwann cell sprouts at 8 day axotomized Wld^s neuromuscular junctions.

Scale bar = 20μm

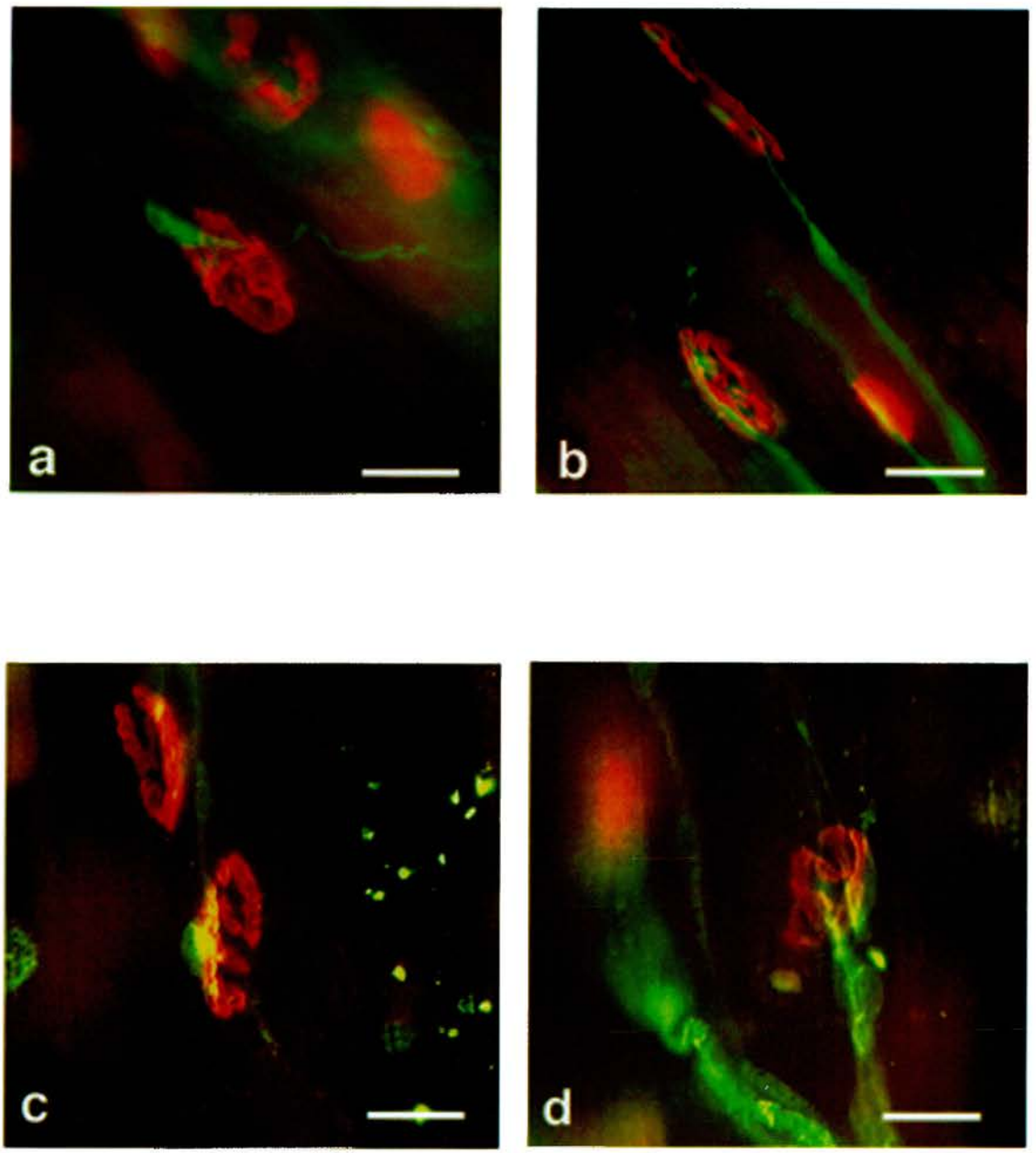

a
b
c
d

FIGURE 4.3: TERMINAL SCHWANN CELL SPROUTING IS DELAYED AT AXOTOMIZED WLD^s NEUROMUSCULAR JUNCTIONS.

Terminal Schwann cell sprouting is delayed at axotomized Wld^s TA neuromuscular junctions in comparison with wild type (C57BL/6J and CBA) TA junctions. Data pooled from analysis of 1372 endplates from 16 Wld^s muscles and 936 endplates from 14 wild type muscles. Statistical analysis was performed using a paired t-test to test significances within groups (i.e. between C57BL/Wld^s and CBA/6J).

* denotes $p < 0.05$

** denotes $p < 0.001$

All other groups were not significantly different.

Error bars are standard error of the mean.

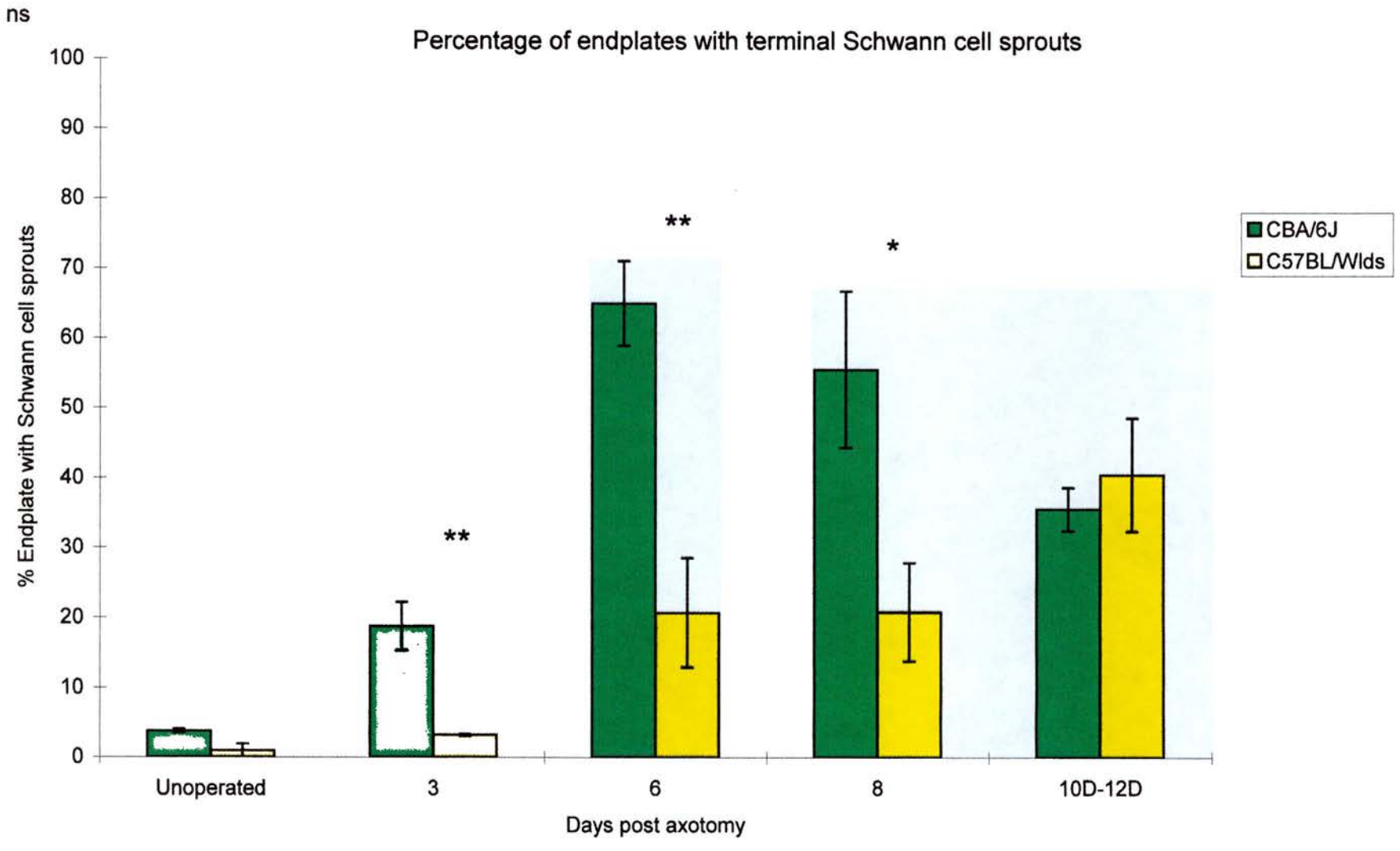
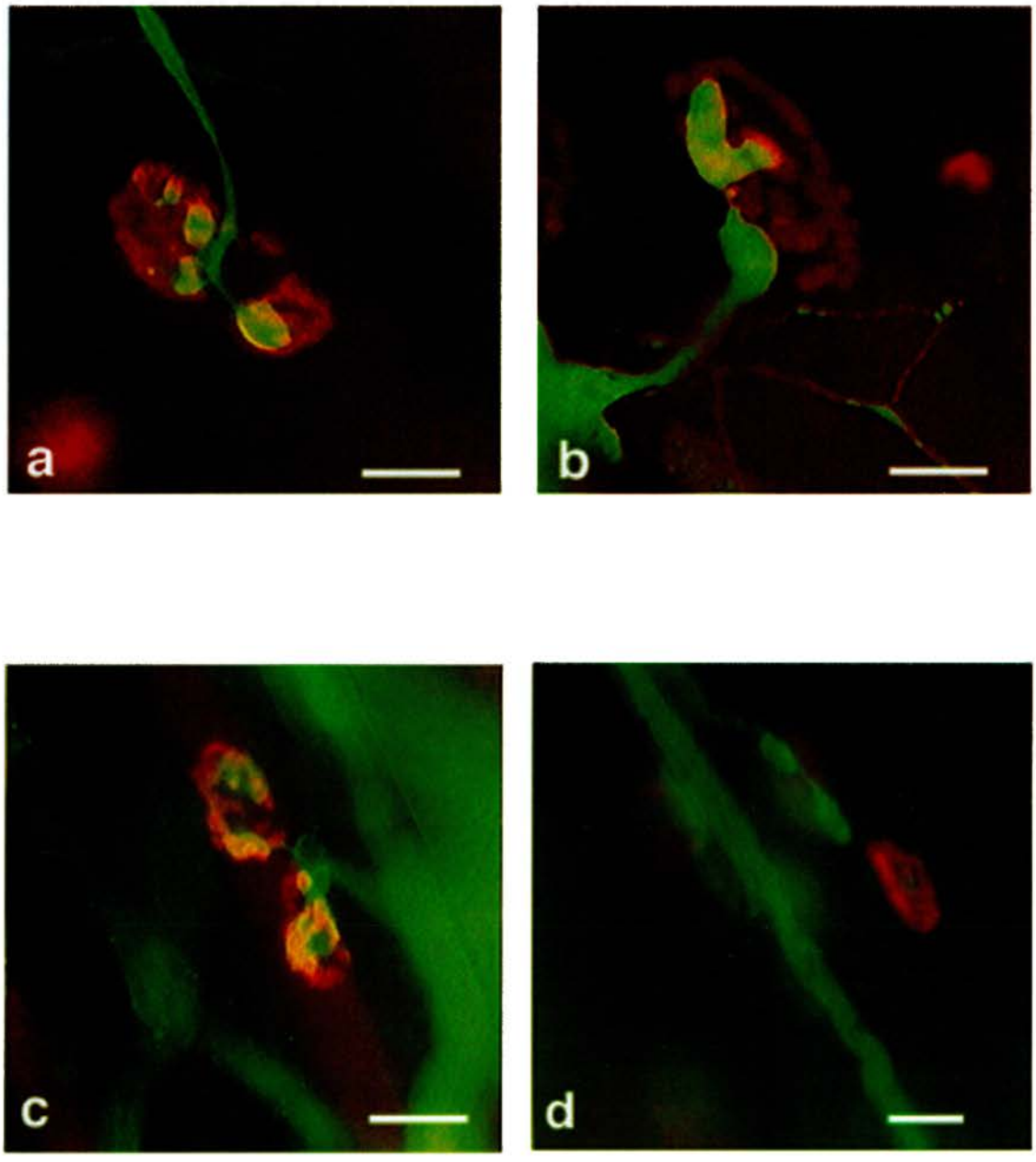

ns
Percentage of endplates with terminal Schwann cell sprouts
100
90
80
70
60
50
40
30
20
10
0
% Endplate with Schwann cell sprouts
**
**
*
CBA/6J
C57BL/Wlds
Unoperated
3
6
8
10D-12D
Days post axotomy

FIGURE 4.4: TERMINAL SCHWANN CELLS PROGRESSIVELY RETRACT AT AXOTOMIZED WLD^s NEUROMUSCULAR JUNCTIONS.

Terminal Schwann cells at axotomized Wld^s TA neuromuscular junctions withdraw in a manner similar to nerve terminal withdrawal.

Images **A** to **D** illustrate S-100 stained terminal Schwann cells withdrawing from the perimeter of the endplate region in a piecemeal fashion. Note the large swellings shown in **B**, **C** and **D**, which are reminiscent of axonal varicosities presented in Figures 2.18 and 2.19. Of particular interest is the Schwann cell process depicted in **D**, which strongly resembles a terminal retraction bulb.

Scale bar = 20μm


a
b
c
d

4.4 DISCUSSION

The results show that terminal Schwann cells at Wld^s endplates remain able to respond to axotomy of distal nerves by extending sprouts from the endplate region. However, the extent of sprouting is delayed, and appears to be reduced in comparison with that observed at denervated wild type junctions.

In unoperated muscles, terminal Schwann cell cytoplasm is very closely aligned with acetylcholine receptor areas (see Figure 4.1), and thus similar to the distribution of motor nerve terminal ramifications (see chapter 2, 2.3; Reynolds and Woolf, 1992; Son and Thompson, 1995a,b; O'Malley, Waran, and Balice-Gordon, 1999). In only a very small number of endplates were Schwann cell processes extending beyond the confines of the endplate area observed. These Schwann cell processes may form as part of the natural and ongoing remodelling of adult neuromuscular junctions (Lichtman, Magrassi and Purves, 1987; Wigston, 1990).

At wild type junctions, Schwann cell processes increased in number from 3 days to 6 days post denervation, thereafter declining (see Figure 4.3). This concurs with a recent study of terminal Schwann cell sprouting following nerve damage by O'Malley *et al.* (1999), which demonstrated that process length and number declined from 10 days onwards. This suggests that the peak of Schwann cell sprouting lies within the period of observation in this study.

At axotomized Wld^s junctions, Schwann cell sprouting was less abundant and did not reach the levels observed in denervated wild type muscles during the period of observation. There are two possible explanations for the delay in terminal Schwann cell sprouting following Wld^s nerve lesion.

First, the presence of an intact nerve terminal may inhibit Schwann cell sprouting at these junctions. Schwann cells are sensitive to variations in the level of activity, and the persistence of spontaneous activity at axotomized Wld^s endplates may be sufficient to prevent sprout formation. Thus, nerve terminal withdrawal and loss of spontaneous activity may dictate the rate of terminal Schwann cell sprouting. Alternatively, perhaps intact but axotomized Wld^s nerve terminals release CGRP, which has been shown to inhibit axonal sprouting (cf. Matteoli *et al.*, 1988; Tsujimoto and Kuno, 1988).

Second, it is possible that terminal Schwann cells may themselves regulate the withdrawal of nerve terminal ramifications, and it is not until after nerve terminal processes withdraw that Schwann cells sprout. Schwann cells have been implicated in the regulation of nerve terminal withdrawal during synapse elimination (Hirata *et al.*, 1997; Culican, Nelson and Lichtman, 1998). Furthermore, the removal of terminal Schwann cell cytoplasm from the endplate region, induced by topical application of neuregulin, causes retraction of nerve terminal boutons underlying the absent Schwann cell process (Trachtenburg and Thompson, 1997). Thus, the withdrawal of Schwann cell cytoplasm observed at Wld^s junctions may induce nerve terminal withdrawal. Both these hypotheses are supported by the fact that withdrawal of Schwann cell cytoplasm from the endplate regions strongly resembles the withdrawal of nerve terminal boutons (see picture). However, it is not possible to verify either of these propositions from this study alone.

The fact that the overall number of sprouts did not eventually compare to that observed in wild type muscles suggests two possibilities. First, that Schwann cell sprouting was not only delayed but also reduced in Wld^s muscles, or second, that Schwann cell sprouting was merely delayed, and if the period of the observation was extended then sprouting would eventually reach that of normal denervated muscle. It is likely that the former suggestion is correct: in a parallel study by Dr. J. Barry, using mouse sternomastoid muscle, Schwann cell sprouting was examined up to 30 days post nerve crush in wild type and Wld^s mice (Barry *et al.*, 1997). In these muscles, sprouting of terminal Schwann cells at axotomized Wld^s endplates, even after 30 days, did not reach the levels of sprouting observed in the wild type muscles. Thus, it appears that not only is sprout formation delayed, but it is also reduced at Wld^s junctions. If this statement is correct, this is further evidence that the Schwann cell reaction to axotomy of Wld^s nerves is not merely delayed, but altered. In the main nerve trunk the wave of mitotic division of Schwann cells, observed after nerve section in wild type mice, occurs in Wld^s mice but the total number of Schwann cells never reaches that observed in wild type mice (Brown *et al.*, 1991b). Thus, that the changes that occur after axotomy in Wld^s mice may be attributable to a separate and distinct phenomenon.

These changes in the reaction of terminal Schwann cells to axotomy in Wld^s mice may be as a result of a change in the function of Schwann cells conferred by the Wld mutation itself. Although the phenotype of terminal Schwann cells has not previously been specifically investigated, Thomson and colleagues (1991) have investigated alterations in the Schwann cells that inhabit the main nerve trunk following axotomy. They observed that downregulation of the P_0 gene, that normally occurs following nerve damage, did not occur in lesion Wld^s nerves. However, dissociated Wld^s Schwann cells cultured without axons reduced expression of the P_0 protein in the normal manner, indicating that in this respect at least Wld^s Schwann cells appear to function normally. Therefore, for this process dissociated Wld^s Schwann cells behave as expected, and given that other experiments have indicated that the Wld^s mutation is intrinsic to axons (see Chapter 1, 1.3.1), it seems unlikely that the Wld^s mutation is conferred specifically and directly to terminal Schwann cells.

The conclusions of this chapter are that Schwann cell withdrawal and process formation induced by nerve damage is affected by the persistence of intact Wld^s motor nerve terminals. Axotomy of Wld^s nerves results in a reduction and delay of Schwann cell sprouting and abnormal retraction of Schwann cell cytoplasm overlying the neuromuscular junction.

CHAPTER 5

GENERAL DISCUSSION.

CHAPTER 5: GENERAL DISCUSSION.

Wallerian degeneration is a fundamental phenomenon involving the disintegration of distal severed axons, and is succeeded by regenerative responses in the peripheral nervous system. The C57BL/Wld^s mutant mouse is unique in that central and peripheral nerve degeneration is slowed. That such a fundamental phenomenon, common to both central and peripheral axons, is profoundly delayed in the C57BL/Wld^s mutant mouse is remarkable, and suggests that Wallerian degeneration may be subject to genetic regulation. The studies of this mouse to date have focused on aspects of the disintegration of axons in peripheral nerves. The main conclusion of these studies is that loss of nerve conduction and disruption of axon structure induced by nerve damage, which occurs relatively rapidly in mammals, is delayed in C57BL/Wld^s mice for considerable lengths of time.

One of the first events of Wallerian degeneration is the disintegration of motor nerve terminals. This occurs rapidly, is complete in rodents after 2-3 days. Ribchester and colleagues (1995) investigated this phenomenon in C57BL/Wld^s mice, and reported that nerve terminal loss was delayed by up to 2 weeks. The initial investigations of this thesis were primarily to further examine the phenomenon of slow Wallerian degeneration at Wld^s neuromuscular junctions. However, the main findings of this thesis demonstrate that, surprisingly, motor nerve terminals did not appear to degenerate at all. Instead, nerve terminals retract progressively from the endplate, and eventually axonal processes withdrew from the endplate region completely. This intriguing observation was verified in several ways - by the use of vital dyes to examine the loss of actively recycling vesicles, by labelling of neurofilament and SV2 proteins, by the periodic examination of living neuromuscular junctions, and finally by the examination of the changes in the functional properties of the nerve terminal. These experiments suggest that severed Wld^s motor axons undergo a progressive retraction of nerve terminal processes resulting in the formation of swollen retraction bulbs, which were eventually destined for reabsorption into the parent axon in the intramuscular nerve. During the withdrawal of nerve terminal boutons there was an concurrent decline in neurotransmitter release. These observations raised the question

that perhaps these changes were similar to those that took place at wild type neuromuscular junctions, but that at wild type junctions this processes was greatly accelerated. A catalogue of previous studies indicate that axotomy of wild type nerves results in *in situ* degeneration of motor nerve terminals rather than progressive withdrawal; there have been no reports of retraction bulbs, and rather than a gradual decline in nerve terminal function, the ability to elicit action potentials/EPPs after nerve stimulation is lost abruptly (Birks, Katz, and Miledi, 1960; Miledi and Slater, 1970; Manolov, 1974; Winlow and Usherwood, 1975; Korneliussen and Jansen, 1976; Riley, 1981; Kawabuchi *et al.*, 1991). However, the techniques employed in this thesis have not previously been used to investigate Wallerian degeneration of wild type motor nerve terminals. It is possible that the use of these modern techniques could reveal similar, but simply more rapid, changes at denervated wild type junctions to those observed at axotomized Wld^s junctions. Thus, degenerating wild type motor nerve terminals were examined using vital staining and immunocytochemical techniques. This study confirmed previous reports of Wallerian degeneration at neuromuscular junctions, and indicated that wild type degeneration appeared to differ in several ways from nerve terminal withdrawal at axotomized Wld^s junctions. Therefore, this thesis proposes that axotomy of Wld^s nerve terminals results in withdrawal of nerve terminal processes which is dissimilar to wild type degeneration. Further experiments are required to conclusively affirm this hypothesis. An electron microscopic study of the ultrastructural changes that occur following axotomy would reveal the alterations in nerve terminal morphology that lead up to the retraction of nerve terminal boutons and formation of retraction bulbs. Photoconversion of FM1-43 such as that employed by Betz and colleaugues (1996), to relocate FM1-43 label ultrastructurally, would prove particularly useful. Further examination of the physiology of nerve terminal withdrawal, and in particular at vitally stained junctions, is needed to fully characterise the changes in synaptic efficacy that take place during the reduction in endplate coverage by the nerve terminal. For instance, how is the vesicle recycling mechanism affected during withdrawal of nerve terminal boutons? This question could be addressed by examining the staining and destaining of synaptic vesicles in axotomized Wld^s nerve terminals with FM1-43.

Assuming that axotomized Wld^s withdraw rather than degenerate, how is nerve terminal withdrawal in axons severed from their cell body regulated, and are there any similarities between withdrawal of axotomized Wld^s nerve terminals and other types of motor axon withdrawal? The possibility that withdrawal of axotomized Wld^s nerve terminals is similar to the retraction of axon arbours from multiply innervated junctions during the developmental phenomenon of synapse elimination is discussed in detail in Chapter 2 (2.4). Motor axons withdraw nerve terminals during synapse elimination, and morphological studies of this process show that, in a parallel to the observations of Wld^s nerve terminals, nerve terminal withdrawal is progressive and piecemeal, and results in the formation of a retraction bulbs, which recedes from the endplate zone (Riley, 1977, 1981; Bixby, 1981; Gorio *et al.*, 1983; Balice-Gordon *et al.*, 1993). Given that morphologically these processes seem so similar, an experiment was devised to test the relationship of synapse elimination to the withdrawal of axotomized Wld^s nerve terminals. During synapse elimination, abolishing activity has the effect of preventing, or in some cases delaying, nerve terminal withdrawal (Benoit and Changeux, 1975; Pestronk, Drachman and Griffin, 1976; Thompson, Kuffler and Jansen, 1979; Brown, Hopkins and Keynes, 1982; Caldwell and Ridge, 1983; Taxt, 1983). Botulinum toxin was used to prevent vesicle-associated transmitter release at Wld^s neuromuscular junctions following nerve lesion. The results show that nerve terminal withdrawal was delayed by 1.14 days, but not prevented. Thus, activity influences the withdrawal of motor nerve terminal boutons in Wld^s mice. These results imply that there may be some underlying homology between activity-dependent synapse elimination and nerve terminal withdrawal in Wld^s mice. Further experiments are required to validate this statement. Manipulation of other factors known to affect nerve terminal withdrawal during synapse elimination should yield further insights into the similarities between these phenomenon. For instance, addition or removal of trophic factors such as GDNF (and others), or proteases such as thrombin and its inhibitor protease nexin 1, at axotomized Wld^s motor nerve terminals may reveal an underlying and homologous fundamental mechanism in synapse withdrawal induced by different conditions.

5.1 A Model of Neuromuscular Plasticity

Figure 5.1 outlines one way in which synapse growth, elimination, maintenance, and withdrawal of axotomized Wld^s nerve terminals could be regulated. This model is based on the supposition that activity can regulate these components of synaptic plasticity at the neuromuscular junction. It is known that muscle fibres that are not innervated, or that are paralysed, can release growth promoting trophic factors such as BDNF and IGF1 and 2 (Funakoshi *et al.*, 1993; Caroni and Schneider, 1994). The release of these factors may result a change of the local environment such that the growth of motor axons, and Schwann cells, is promoted (Figure 5.1A). Thus, absence of neurotransmitter induced activity in muscle fibres may initiate a change in the local environment that promotes restoration of function, preventing withdrawal. This is one mechanism that can account for the delay of nerve terminal retraction in botulinum poisoned Wld^s muscles (see 3.4).

The level of activity strongly influences the process of synapse withdrawal during synapse elimination (see 3.1.2, Figure 5.1B). A number of reports suggest that more active nerve terminals at multiply innervated junctions displace less active inputs (Ridge and Betz, 1984; Ribchester and Taxt, 1983, 1984; Ribchester, 1988; Lo and Poo, 1991; Dan and Poo, 1992; Dunia and Herrera, 1993; Coleman *et al.*, 1997). An attractive explanation for this is that more active inputs successfully compete and are rewarded for a trophic factor, whilst less active inputs receive less of this factor, and withdraw (see Sanes and Lichtman, 1999). A simplistic interpretation of this theory is to suppose that activity of a nerve terminal increases both the release of trophic factor from the muscle fibre (or other cell type), whilst at the same time inducing the upregulation of trophic factor receptor on its own surface membranes. Such a positive feedback mechanism would strongly favour more active inputs, which would swiftly outdistance less active nerve terminals in competitive terms. This mechanism has been implicated in other neuronal systems (Ghosh *et al.*, 1994; Cabelli *et al.*, 1995; Meyer-Franke *et al.*, 1995). Alternatively, less active inputs may be 'punished' by a retrograde factor released from the muscle, again resulting in withdrawal, whilst more active inputs are protected. Candidate molecules for this mechanism include the

protease thrombin, and its inhibitor protease nexin-1 (Nelson *et al.*, 1993; Liu *et al.*, 1994a; Liu *et al.*, 1994b). It is possible that the withdrawal of nerve terminal boutons at axotomized Wld^s neuromuscular junctions are regulated in much the same way (Figure 5.1C). In this paradigm, the nerve terminal is only weakly active (spontaneous activity). This induces a change in the local environment resulting in a decline in trophic factor release, or a decline in the trophic factor receptor expression on the surface of the axotomized nerve terminal. In addition, the nerve terminal is no longer supported by protease inhibitors, and proteases released from the muscle cleave the adhesive bonds that bind the nerve terminal to the neuromuscular junction, resulting in withdrawal.

Finally, strong and synchronous activity at neuromuscular junctions may promote synapse maintenance and stability (Figure 5.1D). The level of trophic factor release is sufficient for the support of the nerve terminal, and the nerve terminal expresses enough trophic factor receptors to receive this support. The action of proteases is negligible as the active nerve terminal induces protease inhibitor release.

It is clear that further experiments are needed to elucidate the relationship between synapse elimination and nerve terminal withdrawal in axotomized Wld^s muscles. Perhaps, although some of the triggers for the withdrawal of nerve terminals may be different, these processes may share the same basic mechanism.

The role of terminal Schwann cell sprouting following nerve lesion was investigated in Wld^s mice. The fact that Wld^s nerve terminals persist for several days following axotomy provides an interesting paradigm for the study of terminal Schwann cell reaction to axotomy. In wild type mice, terminal Schwann cells respond to nerve lesion by extending long sprouts, which are thought to provide a growth substrate for regenerating axons (Reynolds and Woolf, 1992; Son and Thompson, 1995a,b; O'Malley, Waran, and Balice-Gordon, 1999). The trigger for terminal Schwann cell sprouting is as yet unknown. Therefore, it would be interesting to discover how terminal Schwann cells are influenced by the presence of an intact nerve terminal following axotomy. The results indicate that terminal Schwann cell sprouting was delayed in line with the persistence of intact nerve terminals. This implies that, in

response to axotomy at least, terminal Schwann cell sprouting is prevented by intact nerve terminals, and the withdrawal of those nerve terminals allows the subsequent sprouting reaction. Several questions result from this study. For instance, is Schwann cell sprouting prevented by physical contact, or a signal from intact nerve terminal? Observations of terminal Schwann cells at axotomized Wld^s neuromuscular junctions indicate that Schwann cell processes, which usually correspond precisely to the shape of the nerve terminal, retract in a manner identical to that of Wld^s nerve terminals. This suggests that there is a strong relationship between the distribution of Schwann cell processes and retracting nerve terminal cytoplasm. Such a relationship exists between withdrawing nerve terminals and Schwann cells during developmental synapse elimination. Further investigation into the precise overlap between retracting nerve terminals and Schwann cell processes is needed to verify this at axotomized Wld^s neuromuscular junctions. In particular, a multiply staining preparation, enabling visualisation of acetylcholine receptors, nerve terminal, and terminal Schwann cell simultaneously, would elucidate the interactions between these three cell types at the neuromuscular junction. Unfortunately, although this was attempted, it is technically challenging to prepare multiply stained neuromuscular junctions, and was beyond the scope of this thesis. The effect of delaying nerve terminal withdrawal, such as by botulinum toxin administration, would also further our current knowledge of the relationship between withdrawing nerve terminals and Schwann cells.

Further investigation into the precise nature of the Wld mutation should prove beneficial to many fields of study. It is important to understand the precise nature of Wallerian degeneration, and this may become possible with the characterisation of the Wld mutation. Such knowledge may aid in the therapeutic treatment of many neuronal degenerative disease states. Interestingly, the withdrawal of Wld^s nerve terminals resembles that observed in a murine model of progressive motor neuron disease (Haase *et al.*, 1997). Finally, elucidation of the precise mechanism of motor nerve terminal withdrawal in C57BL/Wld^s mice may yield insights into other withdrawal phenomena that take place during synapse development, maintenance and repair.

In sum, this thesis demonstrates that there may be fundamental differences between Wallerian degeneration of motor nerve terminals in wild type mice and axotomy-

induced changes that take place in C57BL/Wld^s mice. Wld^s motor nerve terminals undergo progressive synapse disassembly and withdrawal, which is influenced by neurotransmitter release. In addition, terminal Schwann cell sprouting is delayed.

The fact that this thesis may have raised more questions than it has answered reflects the complexity of the issues surrounding motor nerve terminal withdrawal, and the paucity of our current knowledge of axotomy-induced phenomena in C57BL/Wld^s mice. Many of the results obtained in this study were unexpected, and further investigation of C57BL/Wld^s mice may clarify our understanding of the mechanisms involved in nerve terminal withdrawal.

To conclude, the findings of this thesis suggest a re-examination of the phenomenon of slow Wallerian degeneration of severed distal axons in C57BL/Wld^s mice.

FIGURE 5.1: MECHANISMS OF SYNAPSE FORMATION, WITHDRAWAL AND MAINTENANCE.

A schematic to illustrate mechanisms by which activity may influence the plasticity of neuromuscular synapses.

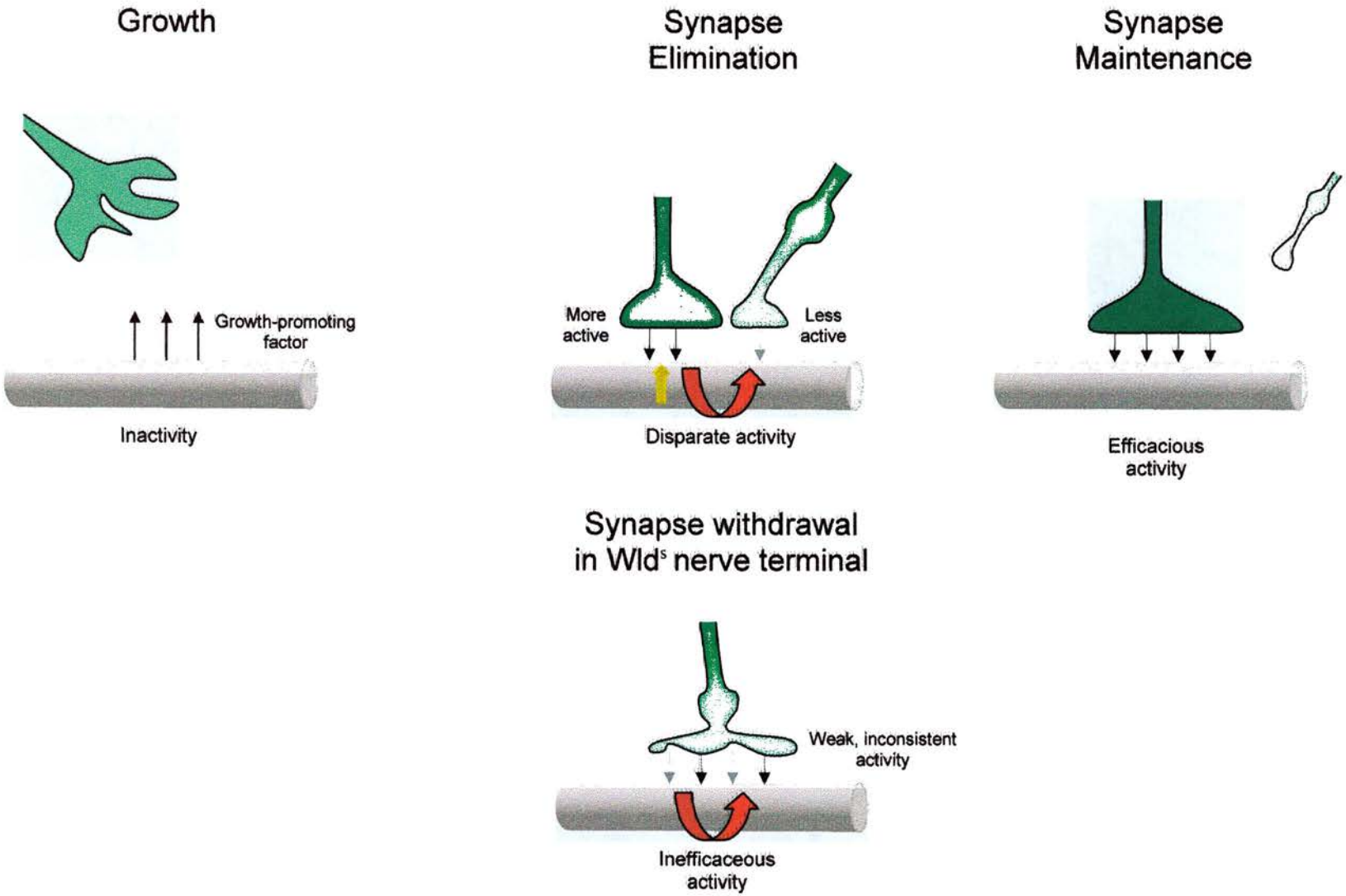
A. Growth-promoting state. Absence of axon-derived activity over the neuromuscular junction results in the upregulation and/or release of factors responsible for the promotion of functional innervation. Such a state may exist during development, or regeneration following nerve lesion, before axons form neuromuscular synapses. Alternatively, inactivity produced by botulinum toxin application, or another type of activity blockade, may result in this state. Competitive interactions leading to synapse withdrawal are suppressed.

B. Disparate activity state - synapse elimination. Multiply innervated junctions are asynchronously active. More active axons successfully compete with less active nerve terminals, resulting in the weakening and retraction of the less active input. Thus, activity rewards the more active input, whilst the less active or efficacious input is removed.

C. Weak activity - synapse withdrawal. As with synapse elimination, inconsistent and weak activity results in the withdrawal of axon arbours from the neuromuscular junction. Inefficacious release of neurotransmitter disrupts the fine balance of factors responsible for synapse maintenance, resulting in a local environment which no longer supports the presence of the nerve terminal. This activates the molecular and cellular machinery responsible for synapse withdrawal.

D. Efficacious activity - synapse maintenance. Synchronous and persistently efficacious activity at the neuromuscular junction results in stable innervation, regulated by a fine equilibrium in the levels of factors responsible necessary for synapse maintenance.

Mechanisms of synapse formation, withdrawal and maintenance



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APPENDICES

APPENDIX A: SOLUTIONS

1. Composition of normal physiological saline (in mM):

NaCl	120
KCl	5
$CaCl_2$	2
$MgCl_2$	1
NaH_2PO_4	0.4
$NaHCO_3$	23.8
Glucose	5.6

2. Composition of elevated potassium saline (in mM):

NaCl	75
KCl	50
$CaCl_2$	2
$MgCl_2$	1
NaH_2PO_4	0.4
$NaHCO_3$	23.8
Glucose	5.6

3. Composition of high magnesium saline (in mM):

NaCl	75
KCl	50
$CaCl_2$	1
$MgCl_2$	3
NaH_2PO_4	0.4
$NaHCO_3$	23.8
Glucose	5.6

APPENDIX B: SUPPLIERS

Animals

C57BL/Wld^s mice	Harlan-Olac, Bicester, UK

All other animals from Department of Medical Microbiology, Edinburgh University, Edinburgh, UK

Software, hardware and materials

Adobe Photoshop	Adobe Systems UK, Uxbridge, Middlesex, UK
CED1410+ interface	Cambridge Electronic Design, Cambridge, UK
Falcon LTC1160 SIT camera	Custom Cameras, Wells, UK
Nikon filter blocks	Nikon Ltd., Kingston-upon-Thames, Surrey, UK
Openlab Software	Improvision, Coventry, UK
WCP software package	Dr. J. Dempster, University of Strathclyde, Glasgow, UK

Solutions and chemicals

FM1-43	Molecular Probes, Eugene, Oregon
S-100 antibody	DAKO Ltd., Ely, Cambridgeshire, UK
TRITC conjugated α-bungarotoxin	Molecular Probes, Eugene, Oregon
Vectashield	Vector Laboratories, Orton Southgate, Peterborough, UK

The monoclonal antibody 2H3 (neurofilament) developed by J.M. Jessel and J. Dodd, and the monoclonal antibody SV2 developed by K.M. Buckley, was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA.

All other chemicals and materials were obtained from Sigma Chemical Company, Poole, Dorset, UK

APPENDIX C: PUBLICATIONS

In preparation:

Ribchester RR, Mattison RJ, Costanzo EM, Thomson D, Parson SH, Barry JA (1999) Progressive withdrawal of synaptic boutons in axotomized Wld^s mouse skeletal muscle. *Neuron*

In submission:

Ribchester RR, Pakiam JG, O'Carroll CM, Thomson D, Mattison RJ, Costanzo EM, Gillingwater TH, Barry JA (1999) Impaired neuromuscular transmission preceding synapse withdrawal in axotomized adult Wld^s mutant mouse skeletal muscle. *J. Physiol. (Lond.)*

Published abstracts:

Ribchester RR, Costanzo EM, Parson SH, Mattison RJ, Barry JA (1998) Regulation of synaptic efficacy and occupancy at mammalian neuromuscular junctions. *J. Physiol.* **511P**:S37

Ribchester RR, Costanzo EM, Mattison RJ, Barry JA, Parson SH (1998) Kinetics of synaptic bouton withdrawal in axotomised Wld(s) mouse muscle. *Eur. J. Neurosci.* **10**:16902

Barry JA, Mattison RJ, Nelson C, Lichtman JW, Ribchester RR (1997) Schwann cell sprouting in a mutant mouse with slowed Wallerian degeneration. *Soc. Neurosci Abstr.* **23**:609

Mattison RJ, Thomson D, Barry JA, Ribchester RR (1996) Sudden death of axotomized motor nerve terminals at neuromuscular junctions in Wld(s) mice. *J. Physiol. (Lond.)* **495P**:152-153

Ribchester RR, Barry JA, Mattison RJ (1996) Activity independence of synaptic competition at reinnervated mammalian neuromuscular junctions. *J. Physiol. (Lond.)* **495P**:S5-S6