Unravelling the molecular and cellular mechanisms contributing to secondary axonal degeneration following traumatic brain injury

by

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Submitted in fulfilment of the
requirement for the Degree of
Doctor of Philosophy

University of Tasmania (September, 2008)

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Part of the work submitted in this thesis has been published or submitted for publication as follows;

Staal JA., Dickson TC., Chung RS., Vickers JC. 2008. Disruption of the ubiquitin proteasome system following axonal stretch injury accelerates progression to secondary axotomy. J Neurotrauma. (In press).

Staal JA., Dickson TC., Chung RS., Vickers JC. 2007. Cyclosporin-A treatment attenuates delayed cytoskeletal alterations and secondary axotomy following mild axonal stretch injury. Dev Neurobiology 67(14) 1831-42

Staal JA, Chung RS, McCormack GH, Dickson TC, Cozens MA, Chuckowree JA, Quilty MC, Vickers JC. 2005. Mild axonal stretch injury in vitro induces a progressive series of neurofilament alterations ultimately leading to delayed axotomy. J Neurotrauma 22(10) 1081-91

Dickson TC, Chung RS, McCormack GH, **Staal JA**, Vickers JC. 2007. Acute reactive and regenerative changes in mature cortical axons following injury. Neuroreport 18(3) 283-8

SUMMARY

Traumatic brain injury (TBI) is currently a leading cause of death and disability in individuals under the age of 45 years in developed nations. Accordingly, the incidence of TBI is high in people during the most productive years of their life and often results in prolonged or life-long impairments in cognition and personality. In this regard, diffuse axonal injury (DAI) is proposed to be a major cause of on-going disability following mild to severe forms of brain injury. As DAI represents an evolving form of axonopathy, taking hours to days to develop, there is a substantial 'therapeutic window' for possible interventions. This thesis introduces a novel *in vitro* method for causing mild-moderate transient axonal shear strain that leads. over 2-3 days, to secondary DAI-like axotomy. This injury method provides important mechanistic insights into the underlying cause of DAI, presenting also an ideal platform for investigating new therapeutic approaches.

Altered membrane permeability, particularly in regard to disrupted calcium homeostasis, and the gradual degradation of the cytoskeleton resulting in axon swellings, are important pathological features of DAI. It has long been proposed that altered membrane permeability, due to mechanical strain, is an initiator for secondary axotomy. This thesis demonstrates, using cell-impermeant tracers, that altered membrane permeability is not an immediate consequence of injury, rather it is associated with the delayed degradation of the axon cytoskeleton. Conversely, this thesis demonstrates, using calcium imaging techniques, immediate increases in intracellular calcium, principally due to release of calcium from intracellular stores. Immunocytochemical analysis further demonstrated the calcium-modulated release of pro-apoptotic proteins, which are also associated with the degradation of the cytoskeleton, at later time-points. The pharmacological inhibition of the calcium-activated phosphatase, calcineurin, attenuated cytoskeletal damage and delayed secondary axotomy. These results suggest the release of calcium from intracellular stores, at the time of injury, initiates the post-injury sequelae, which results in cytoskeletal degradation and secondary axotomy due to calcium activated proteases and phosphatases.

Altered ubiquitin proteasome system (UPS) activity is proposed to play an important role in the secondary degeneration of neurons following injury and in many neurodegenerative disorders. Accordingly, the pharmacological inhibition of the UPS is proposed to attenuate axonal degeneration. This thesis illustrates that UPS activity plays an important role in delaying secondary axotomy, indicated by an accelerated progression to secondary axotomy following inhibition of the UPS. Furthermore, mild to moderate mechanical injury induces neurite sprouting. These highly dynamic neurites contained growth associated proteins, however, lacked growth guidance structures, which may contribute to undirected and abnormal synaptic connections that underlie epileptiform activity following human cases of DAI.

ACKNOWLEDGEMENTS

I primarily wish to thank my supervisors Professor James Vickers, Dr Tracey Dickson and Dr. Roger Chung for their continued support, guidance, patience and enthusiasm. In particular, I would like to extend a special thank you to Professor Vickers and Professor Simon Foote for providing the opportunity to undertake this PhD study at the Menzies Research Institute.

Thank you to all the staff and students of the department for your support, encouragement and humour. A special thank you to my fellow colleagues, Graeme McCormack, Adele Woodhouse, Anna King, Catherine Blizzard, Ruth Musgrove, Yao Liu, Stanislaw Mitew, and Emma Cazaley, for your friendship and making my time in the department enjoyable and memorable. A special thank you to Dr. Rob Gasperini, Dr. Lisa Foa and Dr. Mathew Kirkcaldie. Also to Murray and Marcus at the animal house, thank you for your support.

Finally, I would love to extend an especially huge thank you to Ling San, my Mum, Dad, brother and sister. Each of you has provided me with endless patience and encouragement and for this I am eternally grateful.

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ABBREVIATIONS

AD	Alzheimer's disease
AIF	Apoptosis inducing factor
APP	amyloid precursor protein
BDNF	brain derived neurotrophic factor
CO_2	carbon dioxide
°C	degrees Celsius
CNS	central nervous system
CsA	Cyclosporin A
DAI	diffuse axonal injury
DIV	days *in vitro*
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
F-actin	Filamentous Actin
g	gram
GAP-43	Growth-associated protein 43
GDNF	glial derived neurotrophic factor
GFAP	glial fibrillary acidic protein
IF	intermediate filament
IgG	immunoglobulin
kDa	kilo Dalton
L	Litre
µl	micro litre (10^{-6}l)
µm	micrometre (10^{-6}m)
µM	micro molar (10^{-6}M)
M	Molar
ml	millilitre
mm	millimetre
MAP	microtubule associated protein
MAP-2	microtubule-associated protein 2

MPT	Mitochondrial permeability transition pore
mRNA	messenger ribonucleic acid
MT	microtubule
NFTs	neurofibrillary tangles
NF	neurofilament
NF-H	neurofilament triplet high molecular weight subunit
NF-M	neurofilament triplet medium molecular weight subunit
NF-L	neurofilament triplet light molecular weight subunit
PBS	phosphate buffered saline
PFA	paraformaldehyde
PI	post-injury
PNS	peripheral nervous system
RT-PCR	reverse transcriptase polymerase chain reaction
SEM	Standard error margin
TBI	traumatic brain injury
Ub	Ubiquitin
UPS	Ubiquitin proteasome system
Wlds	Wallerian degeneration slow

INTRODUCTION

1.1 TRAUMATIC BRAIN INJURY

Traumatic brain injury (TBI) is currently one of the most common causes of death and disability in individuals under the age of 45 years in developing nations (McDermott et al., 2004). Accordingly, the incidence of TBI is high in people during the most productive years of their lives and regularly leads to prolonged or life-long impairments in cognition and personality. Mechanical trauma and/or rapid acceleration/deceleration involving the head often results in widespread axonal damage, or diffuse axonal injury (DAI) in which neural circuitry is disrupted or lost throughout the brain. Damage to the brain following injury is further exacerbated due to the poor regenerative capacity of mature neurons. Thus, even the loss of only a few neurons may cause permanent disruption to neural connections and widespread dysfunction of the circuits into which they integrate.

Head trauma commonly results in both immediate (primary) and delayed secondary damage. In the majority of head injury cases, secondary DAI damage is proposed to be a significant contributor to on-going disability (Buki and Povlishock, 2006). As reviewed below, neurons undergo a structured series of alterations following DAI. This response involves a number of intrinsic and extrinsic pathways, which may result in either delayed axotomy, or attempted regeneration of the damaged neuron. Alterations to the axonal cytoskeletal proteins, particularly microtubules and the intermediate filament protein subclass known as the neurofilament triplet proteins, play a pivotal role in the post-injury sequelae. There is still a fundamental lack of information about the underlying cellular and molecular mechanisms that lead to delayed axotomy, mainly due to the inadequacies of previous axonal injury models.

As DAI represents an evolving form of axonopathy that takes hours to days to develop there is a substantial 'therapeutic window' for possible interventions targeted at either impeding the cellular changes that lead to secondary axotomy or promoting possible regeneration. Historically, most interventions following DAI have focused on inhibiting the

detrimental cascades activated after trauma, as regeneration in the mature mammalian brain was believed to be impossible. Current therapeutic interventions have failed to translate into a positive outcome in human cases of brain injury and it is increasingly clear that further interventions need to be assessed in models of axonal injury that induce the full spectrum of pathological features.

It is now clear that the mammalian brain retains remarkable plasticity well into adulthood. As detailed in the literature review below, in mature neurons, regenerative attempts may manifest as the elaboration of new axons or sprouts from pre-existing neurons. This regenerative capacity has been well-documented following axon transection and compared to the growth response during development. The regenerative response of diffusely injured axons remains unknown although, in the majority of head injury cases, axons are not immediately severed. Whether injury-induced axonal sprouting results in the restoration of neural connectivity and function remains contentious, however, it is agreed that if the repair process is purely reliant on endogenous capacity, regeneration is limited.

This thesis will, therefore, seek to investigate the potential of a novel *in vitro* model of mild/moderate axonal injury to replicate the progressive series of pathological features observed in the majority of clinical TBI cases. This will illustrate the long-term neuronal response to injury, particularly in relation to the role of the cytoskeleton and the possible cellular degenerative and regenerative pathways. Additionally, this information will provide insight into possible therapeutic strategies for the treatment of TBI.

1.2 PRIMARY AND SECONDARY BRAIN DAMAGE

Brain trauma may result in tissue deformation at the moment of injury due to mechanical forces (referred to as primary brain damage). This deformation may directly damage blood vessels, axons, neurons and glia, initiating dynamic and evolving processes resulting in complex cellular, inflammatory, neurochemical and metabolic alterations (Blumbergs et al., 1989; Finnie and Blumbergs, 2002). Secondary brain damage arises from complications following primary brain damage. These include ischemic and hypoxic damage concomitant

with cerebral swelling, the consequences of raised intracranial pressure, hydrocephalus and infection (Blumbergs et al., 1989). Inflammatory and cytotoxic mechanisms of injury are often the product of ischemia, which is considered to be one of the largest contributors to secondary brain damage. Post-mortem studies of victims of TBI indicate that cerebral ischemia is a common consequence (Egeler-Peerdeman, 1998). Hypoxemia and hypotension are also common clinical findings in patients with TBI (Chesnut et al., 1993). Cerebral hypotension is one of the principal predictors of a poor outcome after TBI (Chesnut et al., 2000) and it has been documented that a single episode of hypotension can be linked to a 150% increase in mortality rate (Chesnut et al., 1993; Statler et al., 2001). The type and severity of pathological features following TBI is dependant on the biomechanics of head trauma.

1.3 MECHANISMS OF TRAUMATIC BRAIN INJURY

TBI is characterized by a wide spectrum of mechanisms of injury and pathological features. The three major forms of head trauma include penetrating, blunt and inertial injury. These different forms of injury can result in either focal or diffuse brain damage.

Penetrating brain injury involves the breaching of the skull by an object and the consequent piercing of the brain. Penetrating head injuries initiate a combination of contact and inertial forces that result in a sequence of events that vary with the size of the impacting object and the magnitude of the force delivered to the contact point. This combination of forces often results in focal brain damage characterised by laceration of the scalp, extradural haematoma, surface contusions and lacerations, and intracerebral haemorrhage (Graham, 1996). An example of a penetrating head injury is a gunshot wound to the head. Penetrating head injuries, despite their severity, account for the minority of brain injury cases reported in Australia (McDermott et al., 2004).

Blunt and inertial brain injuries account for the majority of head trauma cases and result from the rapid movement of the brain within the cranium (Fig 1.1). In blunt head injury cases this usually occurs following a contact injury, which results from an object striking

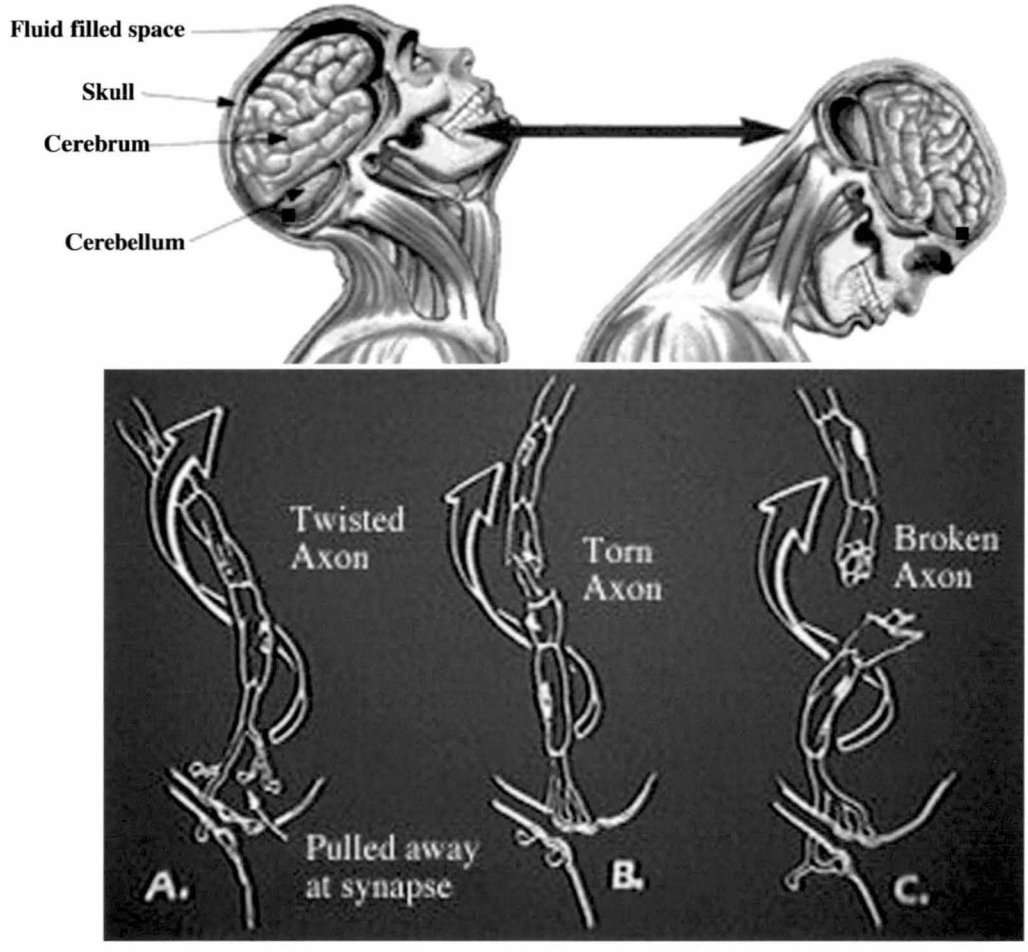


Figure 1.1 TBI occurs following the rapid acceleration/deacceleration of the head. In this regard, the rapid movement of the head causes the the brain to move and strike the skull. Axons are particularly vulnerable to mechanical forces and this can cause them to twist (A), tear (B) and/or break (C). In the majority of DAI cases, there is no immediate severing of the axon.
Adapted from Anatomy and functions of the brain, Mechanisms of closed head injury. www.doctorlawyer.net.

the head (or vice-versa) leading to focal injuries such as scalp laceration, extradural haematoma and skull fractures. Non-contact injuries (inertia brain injury) such as the rapid acceleration/deceleration of the head results in head injuries caused solely by inertia of the brain relative to the movement of the head (translational, rotational, angulation). Under these latter conditions, skull fractures do not occur. Inertial injury forces (can also be referred to as impulsive loading) cause mechanical strains that operate in a "centripetal sequence" (Ommaya and Gennarelli, 1974; Gaetz, 2004). With mild forces, the sequence begins at the surface of the brain and progressively affects the deeper structures as forces become more severe. The physicist Holbourn (1943) examined the effects of acceleration/deceleration force but in a gelatine model of the brain, and described how shear strain occurred in the CNS. He suggested that rotational acceleration forces are the primary cause of injury producing predictable damage to the brain. Subsequent studies illustrated that the direction of rotation also determined the severity of injury and recovery (Gennarelli et al., 1982). Sagittal (front to back) rotational injuries were associated with good recovery, conversely, lateral (side to side) rotational injuries resulted in persistent coma or severe disability.

The two most important and common types of damage caused by inertial forces are acute subdural haematoma resulting from tearing of subdural bridging veins and widespread damage to white matter in the form of diffuse axonal injury (DAI) (Jafari et al., 1997, 1998; Maxwell et al., 1997; Adams et al., 2000; Finnie and Blumbergs, 2002; Longhi et al., 2005). Diffuse brain injuries, particularly DAI, are of particular importance due to their high frequency in most head injury cases and their difficulty to be detected by current scanning techniques (Povlishock et al., 1992, 1996; Pettus et al., 1994; Jafari et al., 1998; Adams et al., 2000; Thompson et al., 2006).

1.3.1 Diffuse axonal injury

Diffuse axonal injury is a common and important pathological feature of TBI. Previously undetectable *in vivo*, DAI affects major axonal bundles within the brain, particularly the

cerebral hemispheres, contributing to a clinical pattern involving slowing, inconsistency, distractibility, and impaired top-down control classically observed in patients with TBI (Levine et al., 2007). Even injuries classified as mild (the majority of reported cases) can result in persistent neuro-behavioural impairment (Kraus et al., 2007). It has been suggested that DAI is primarily initiated by ionic homeostatic disruption and altered permeability of the axolemma rather than instantaneous nerve cell death. In this regard, initial axolemma disruption has been linked to the onset of subtle axonal abnormalities, which become progressively severe over time, ultimately culminating in a delayed neuronal degeneration referred to as secondary axotomy (Povlishock and Christman, 1995; Gennarelli, 1996; Graham, 1996; Maxwell et al., 1997). Secondary axotomy is characterized by focal swelling followed by disconnection of the two axonal segments, the formation of 'retraction balls' and Wallerian degeneration of the distal component. Studies of human (Blumbergs et al., 1989; Grady et al., 1993) and various experimental models (Maxwell et al., 1991; Yaghmai and Povlishock, 1992; Pettus and Povlishock, 1996; Dunn-Meynell and Levin, 1997; Jafari et al., 1997; Povlishock et al., 1997) of delayed axonal pathology following trauma have indicated that abnormalities in the organization of cytoskeletal elements may play an important role in the neuronal response to injury. As with other models of axonal damage, DAI may also be associated with the development of neuronal mitochondrial changes, abnormal lamellar bodies, the disruption and/or loss of microtubules and focal demyelination (Maxwell et al., 1997).

1.4 THE NEURONAL CYTOSKELETON

The cytoskeleton is one of the major components of the neuron. This internal support system not only defines the architecture of the cell (Fig 1.2) but also plays a pivotal role in cell division, cell guidance (particularly in development), cellular transport, anchoring and compartmentalization (Dent and Gertler, 2003). Structural and functional alterations to the neuronal cytoskeleton play an important role in the mechanism of pathological change underlying DAI (Smith and Meaney, 2000).

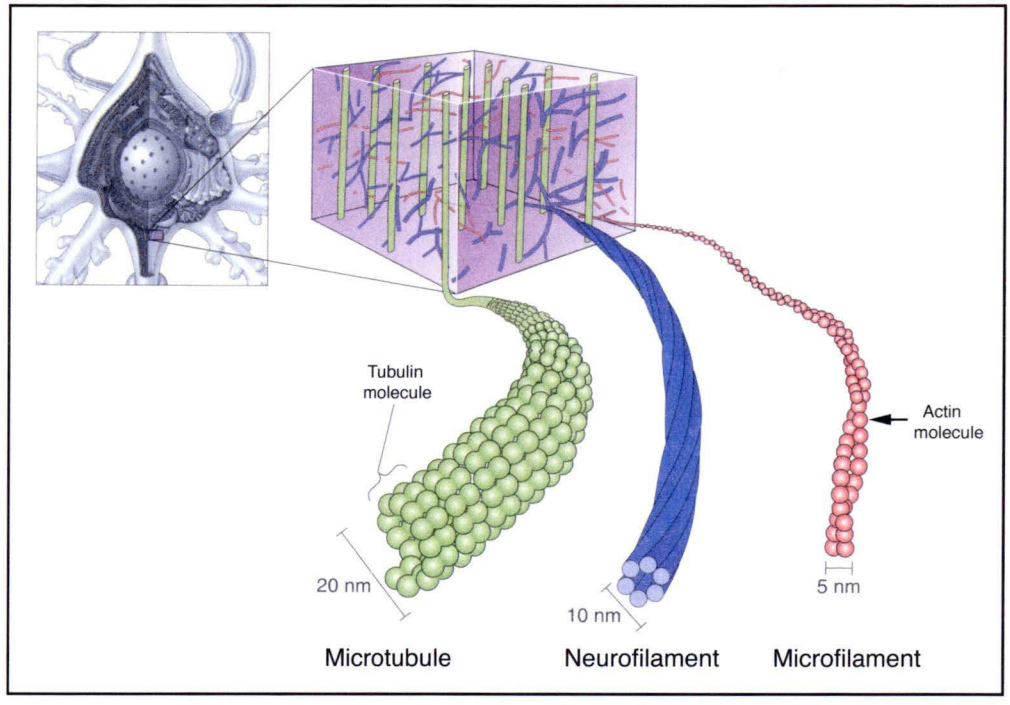


Figure 1.2 Diagrammatic representation of the neuronal cytoskeleton

The neuronal cytoskeleton is composed of three fibrillar systems, microfilaments, neuronal intermediate filaments (neurofilaments) and microtubules. Neurofilaments, unlike microfilaments and microtubules, are composed of subunits with a filamentous structure. The diameter of neurofilaments is intermediate between microfilaments and microtubules.

Adapted from Bear M, Connors B and Paradiso M; Neuroscience: Exploring the brain, Maryland, 2001, Lippincott Williams & Wilkins.

The neuronal cytoskeleton comprises three main fibrillar systems, which are the microtubules (MTs), microfilaments and intermediate filaments (IFs). Each has a characteristic composition, structure and organization that may be further specialized in a particular cell type or subcellular domain. The defining structural elements have long been identifiable in electron micrographs (Dent and Gertler, 2003), and a considerable amount is known about the detailed organization of these components.

1.4.1 Microtubules

Biochemically, MTs are long unbranched polymers of 50-kDa tubulin subunits. Heterodimers of α and β-tubulin are aligned end to end to form protofilaments, which are arranged laterally into a tubular structure, the walls of the MT polymer, with an external diameter of approximately 25nm and inner light core, 15nm in diameter (Hirokawa, 1994).

A variety of post-translational modification sites are available on neuronal MTs and twenty isoforms of tubulin can be identified using isoelectric focusing. For example, the principal modifications include acetylation of α-tubulin by tubulin acetylase, phosphorylation of β-tubulin by casein kinase II and the tyrosination/detyronisation of α-tubulin by tubulin ligase (Mullins et al., 1994; Utreras et al., 2008;). Detyrosinated and acetylated α-tubulins are the most studied modifications and appear to be associated with more stable MT populations (Lanfanchere and Job, 2000). The majority of α-tubulins are expressed with a carboxy-terminal tyrosine residue, which can be cleaved by tubulin carboxypeptidase and then replaced by tubulin tyrosine ligase, in a rapid cycle involving all available tubulin. Since the carboxypeptidase acts only on assembled tubulin and the ligase acts on unassembled tubulin, this tyrosination/detyrosination cycle is linked to MT dynamics (Myers et al., 2006).

MT composition, modification and organization varies according to their localisation to specific cellular compartments, which suggests that MTs exist in specialized forms to perform designated tasks in the unique environments of the neuron. This is particularly

evident for axonal MTs, which contain stable segments that are unusually resistant to treatments that depolymerize MTs in other cells. These stable domains may serve to organize MTs in axons, particularly during regeneration (Brady, 1993).

MTs play an important role in axonal transport in nerve cells. They are responsible for the bi-directional movement of membrane bound organelles along axons and dendrites (Baas et al., 2006). This is facilitated by the molecular motors kinesin (Myers et al., 2006), for anterograde transport and dynein (Hirokawa et al., 1998), which is responsible for retrograde transport. The other proposed functions of MTs include maintenance of neuronal morphology, cell division and motility in development, and importantly neuroplasticity following injury (Bouquet et al., 2004).

MTs are highly dynamic and play an important role in the elongation of axons and the guidance of growth cones (Dent et al., 1999). In this regard, MTs interact with actin filaments within the growth cone (Dent et al., 1999). Initially it was assumed that changes in the organization of MTs at the growth cone occur secondarily, as a consequence of formation and disassembly of actin filaments. However, further studies of cytoskeletal dynamics observed in living growth cones revealed that MTs do not remain passively bundled in the growth cone centre (Dent and Kalil 2001; Schaefer et al. 2002). Other studies have demonstrated that attractive growth cone turning can be initiated by localized MT stabilization in conjunction with actin dynamics (Buck and Zheng, 2002). Recently, it has been shown that MTs themselves can be a direct target of guidance cues in controlling of growth cones and regulating axon elongation (Lee et al., 2004; Zhou et al., 2004). MTs not only play a vital role during development but also modulate the post-injury sprouting response (Chuckowree and Vickers, 2003). However, MTs are not restricted to growth cones and can dynamically reorganize to form axon collateral branches (Dent and kalil, 2001; Schaefer et al., 2002; Wang and Brown, 2002). In this regard, calcium transients are proposed to play an important function in modulating MT dynamics (Dent et al., 2004; Hutchins and Kalil, 2008).

1.4.1.1 Microtubule Associated Proteins

Microtubule-associated proteins (MAPs) are a heterogeneous group of proteins, which co-purify with MTs (Avila et al., 2008). MAPs display a complex expression pattern during development and have a high degree of specificity within different neuronal cell types (Matus, 1998). MAPs associate with MTs rather than free tubulin and remain in constant stoichiometry with the tubulin in MTs through cycles of assembly and disassembly. MAPs are primarily cytoskeleton structural components, involved in MT-dependant organization of the cytoplasm through interactions with other subcellular components (Avila et al., 2008). Besides playing a role in the regulation of MT assembly, MAPs also participate in axonal transport along MTs (Dehmelt et al., 2006).

MAPs are separated into two broad groups based on their molecular weights. Typically, the high weight MAPs include 1A, 1B, 2A and 2B (>270 kDa). The tau proteins are a major component of the low molecular weight MAPs (30-60 KDa) (Maccioni and Cambiazo, 1995; Avila, 2006; Lace et al., 2007), which also include MAP3 and MAP4, and the molecular motors kinesin and dynein, which drive the intracellular transport of membrane-bound organelles along MT tracts. The two groups of MAPs possess similar carboxy-terminal amino acid sequences despite the difference in molecular weights.

1.4.2 Neurofilaments

Neurofilaments are a type of intermediate filament exclusively expressed within neuronal cells, most prominently in large axons. Intermediate filaments are intermediate is size between actin filaments and MTs, only in their average diameters, ranging from 10-15 nm compared to 8 nm for filamentous (F)-actin and 23 nm for MTs. NFs interact with each other both *in vitro* and *in vivo* without the aid of other proteins, to form bundles and networks mediated by long flexible polypeptide chains, called sidearms that emanate from the surface of the core polymer (Fig 1.3). Six distinct classes of proteins are now regarded as potential mammalian NF subunits, and these are classified on the basis of similarities in

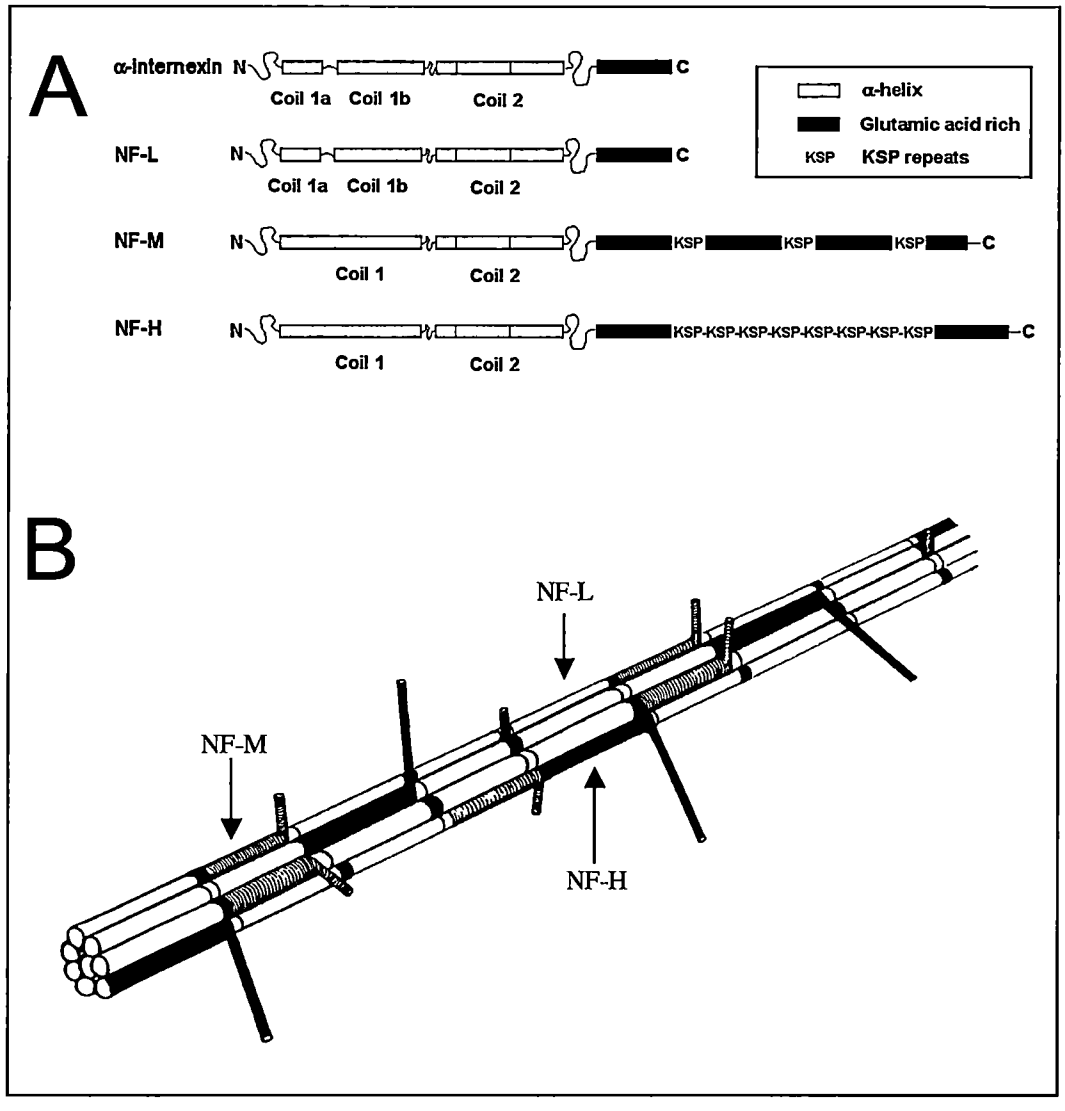


Figure 1.2 Diagrammatic representation of type IV neuronal intermediate filament proteins. *A,* Type IV neuronal intermediate filament proteins include the neurofilament triplet proteins, NFL, NFM and NFH, commonly referred to as neurofilaments, as well as α-internexin. These proteins comprise the main intermediate filament component of the cytoskeleton of most mature CNS neurons. Neuronal intermediate filaments have a distinct filamentous structure. *B,* Neurofilament triplet proteins are polymers of NFL, NFM and NFH subunits. NFM and NFH possess carboxy terminal tail domains (or ' side-arms') that protrude from the core filament, vary in length between sub-units and undergo phosphorlylation to increase inter filament spacing.

Adapted from Lariviere R and Julien J-P: Functions of intermediate filaments in neuronal development and disease: J.Neurobiol, 2004, 58 131-148.

sequence and gene structure. These classes are: vimentin (class III), peripherin (class III), α-internexin (class IV), nestin and a group of three related NF subunits, known as the NF triplet proteins. The NF triplet proteins are designated as NFL, NFM and NFH for, respectively, low- (68kDa), medium-(160kDa) and high-molecular-weight (205kDa) subunits (Perrot et al., 2008). The NF triplet subunits share sequence and structural similarity in a coiled core domain, but differ in the length and sequence of their N-termini and more dramatically of their C-termini, which in the case of the medium (NFM) and heavy chains (NFH) of NFs form the flexible extensions that link NFs to each other and to other elements in the cytoplasm.

NFs role, together with microtubules and microfilaments, is to enhance structural integrity, cell shape, and cell and organelle motility. In addition, a major function of NFs, particularly in phosphorylated forms, is related to control of axonal calibre (Nixon and Shea, 1992). A central role for NFs in maintaining and regulating axon calibre has been highlighted by the failure of radial axon growth following targeted deletion of the NFL protein (Zhu et al., 1997). This is not only of structural significance but also important because the speeds of conductivity of an impulse down the axon is proportional to its calibre (Hirokawa and Takeda, 1998; Julien, 1999). Studies have analysed the importance of the number and stoichiometric proportion of each NF subunit in the axonal calibre determination (Perrot et al., 2008). The over-expression of NFL in transgenic mice, which leads to a two to threefold increase in the number of NFs, did not significantly alter the diameter of the axons (Monteiro et al., 1990; Xu et al., 1996). This suggests that NF number alone is unlikely to be the principal determinant of axon calibre. However, the co-overexpression of either NFL–NFM or NFL–NFH increases the axonal calibre (Xu et al., 1996; Meier et al., 1999). Altogether these results indicate that both the number of NFs and a precise stoichiometry of their subunits are essential for radial growth of the axon.

As part of complex and dynamic network, NFs also interact with a number of substrates, and these are principally mediated through NF-associated proteins that can modulate structure (linker proteins) and function (enzymes) (Perrot et al., 2008). Linker proteins are responsible for the interaction between the different filaments or organelles, whereas

enzymes (which include mainly kinases and phosphatases) modulate NF architecture, assembly and spacing (Perrot et al., 2008). There is a dynamic relationship between NFs and MTs, particularly within the axoskeleton, which is modulated by phosphorylation (Leterrier et al., 1984; Minami et al., 1984; Miyasaka et al., 1993). Other studies have illustrated a direct interaction between motor protein dynein and kinesin and NFs (Yabe et al., 1999, 2000; Shah et al., 2000; Wagner et al., 2004; Theiss et al., 2005; Motil et al., 2006). Additionally, it has been reported that NFs were able to bind DNA, RNA and histone H1 nuclear protein, suggesting a possible role of NF in the regulation of transcription processes (Traub et al., 1987; Wang et al., 2001; Perrot et al., 2008). In regard to organelles, NFs have been shown to form cross-bridges with mitochondria (Hirokawa, 1982; Leterrier et al., 1994) and dynamic complexes with smooth endoplasmic reticulum (Metuzals et al., 1995). It is important to note that the majority of these interactions are regulated by NF phosphorylation, which will be further discussed later.

NFs may also have a neuroprotective effect and promote cell survival. This was demonstrated by crossing transgenic mice over-expressing human NFH and mice expressing a mutant superoxide dismutase ($SOD1G^{32}R$) linked to human amyotrophic lateral sclerosis (ALS), which resulted in an increase in the lifespan of the progeny by 65% (Couillard-Despres et al., 1998). Additionally, the double transgenic mutant mice over-expressing peripherin and human NFH transgenes were almost completely protected from peripherin-mediated degeneration of neurons *in vivo* (Beaulieu and Julien, 2003). The over expression of human NFH resulted in the movement of the intracellular peripherin localization from the axonal compartment to the perkaryal compartment of spinal cord motor neurons. These results suggest that NFH plays a role in the sequestering of peripherin, preventing the detrimental development of intermediate filament inclusions, which may result in disruption to axonal transport. There is also significant evidence to also suggest that NF proteins play complex roles in the control of ionic balance. For example, modifications to NFH and NFM is shown to be particularly capable of assimilating stress-related adducts, such as the release of toxic aldehydes generated from oxidative damage, to balance cellular conditions, until they are degraded at the synapse or modifications are reversed in the cytoplasm (Wataya et al., 2002; Chou et al., 1998; Leski et al., 2001).

Both NFM and NFH tails consist of four regions of random coils. The second acidic amino-acid-rich region produces an extended structure, which may imply a spacing function possibly ensuring that the carboxy-terminus portion of the subunits are positioned remote from the rod domain (Fig. 1.3B). These projecting tail regions may also correspond to the unique side arm structures observed in ultrastructural studies. A series of multiple lysine-serine-proline (KSP) repeats are located beyond this region, which may be reversibly phosphorylated. The phosphorylation of NFs induces conformational changes and is involved in modifying NF size, morphology and dynamics, especially following damage and in diseased states (Julien, 1999; Brownlees et al., 2002; Shea et al., 2003).

1.4.2.1 NF Phosphorylation

NF phosphorylation plays a critical role in the regulation of filament translocation, formation and function and may also be involved in the pathogenesis of certain neurodegenerative diseases. The phosphorylation of NFs is topographically regulated, with a proximo-distal gradient consisting of an intense phosphorylation in axons and little or no phosphorylation in cell bodies and dendrites (Nixon and shea, 1992; Nixon et al., 1994). The phosphorylation sites on the three NF subunits are located on the amino-terminal and carboxy-terminal domains, and these are targets of second messenger- dependant and independent kinases respectively (Sihag and Nixon, 1989, 1990; Perrot et al., 2008).

NF phosphorylation has multiple important roles. The major function is the regulation of axonal radial growth. There is extensive phosphorylation of the KSP repeats in the tail domain of NFM and NFH in axons. This results in the formation of NF sidearms, increased interneuronal spacing, radial growth of axons and, consequently, increased conduction of velocity (de Waegh et al., 1992; Nakagawa et al., 1995; Yin et al., 1998). As mentioned above NF phosphorylation also regulates MT-NF and NF-dynein/kinesin interactions (Motil et al., 2006; Perrot et al., 2008). The loss of NFM and NFH phosphorylation in demyelinated axons, suggests a link between myelination and NF phosphorylation (de

Waegh et al., 1992). The role of NF phosphorylation in axonal transport supported by studies, some analysing the movement of GFP-tagged phosphorylation mutants of NFH in neurons, illustrating NFH side-arm phosphorylation as a regulator of NF transport (Ackerley et al., 2000; Shea et al., 2003; Yabe et al., 2003).

Alterations to NFs have been associated with many neurodegenerative diseases and also may have a significant role in the secondary degeneration of injured axons (Chuckowree et al., 2004; Povlishock and Katz, 2005; Kesavapany et al., 2007). Indeed, the pathological accumulations of NFs are an important pathological feature of DAI and (Dickson et al., 2000; Smith et al., 2003; Chen et al., 2004) neurodegenerative disorders such as Alzheimer's disease (Vickers et al., 1996; Dickson et al., 1999), Parkinson's disease (Spillantini et al., 1998 Trimmer et al., 2004), and amyotrophic lateral sclerosis (Murayama et al., 1992).

1.5 NEURONAL RESPONSE TO INJURY

The neuronal response to injury involves a number of cellular and molecular mechanisms and is directly dependant on the severity of the injury. Immediate severing of axons at the time of injury, termed primary axotomy, results in rapid neuronal degeneration (Smith and Meaney, 2000). This only occurs at the most severe levels of injury, and in most circumstances, mechanical trauma induces a delayed cell death (Maxwell et al., 1997). This delayed cell death has been demonstrated in a number of human and animal studies, yet it is evident that this process involving a number of cellular and molecular pathways is more complex that previously believed (Smith and Meaney, 2003; Povlishock and Katz, 2005). It has been proposed that the neuronal response to injury is characterized by altered axolemma permeability, cytoskeletal disruption, and the induction of a number of cell death pathways ultimately resulting in cell death (Buki et al., 1999; Smith and Meaney, 2003).

1.5.1 Altered axolemma permeability

Many *in vitro* and *in vivo* DAI studies, utilising either extracellular tracer infusion techniques or tracers typically excluded from the neuronal cytoplasm, have illustrated microscopic mechanoporation of the axolemma (Geddes et al., 2003; Prado et al., 2005). This mechanoporation was believed to play a significant role in DAI and result in the detrimental influx of ions, particularly calcium, through the compromised cell membrane (Singleton and Povlishock, 2004). Altered axolemma permeability to calcium has long been associated with TBI (Povlishock and Pettus, 1996; Maxwell et al., 1997; Okonkwo et al., 1999). The implications of increased intra-axonal calcium will be discussed in detail in the following section, but it is important to note that current thought holds that injury induced mechanoporation of the axolemma results in abnormal influx of calcium which in turn triggers various proteases and phosphatases resulting in cytoskeletal degradation and/or neuronal death (Buki et al., 2000; Buki and Povlishock, 2005).

Evidence for the possible involvement of calcium in TBI is based on the finding that calcium in the cerebrospinal fluid decreases from 1 to 0.01mM following head trauma (Young, 1992; Nilsson et al., 1993). Additionally, total brain tissue calcium uptake is increased following *in vivo* injury (Hovda et al., 1992; Fineman et al., 1993). These results support the notion that total intracellular calcium is elevated in the cells of the CNS after TBI. However, there are several intracellular calcium stores within the neuron including calcium bound to the plasma membrane, cytoskeletal components, and calcium-binding proteins; calcium stores in the endoplasmic recticulum, mitochondria, and nucleus; and intracellular free calcium (Kostyuk and Verkhratsky, 1994; Weber et al., 2001). It is unclear what the distribution of calcium in these stores are following TBI (Weber et al., 2001).

The role of altered axolemma permeabilty in the eventual death of the neuron following DAI is controversial (Farkas et al., 2006). Following the early studies of injury induced disruption to axolemma permeability (Povlishock and Christman, 1995; Gennarelli, 1996; Graham, 1996; Maxwell et al., 1997), recent studies have revealed that neurons respond heterogeneously to injury induced axolemma disruptions (Farkas et al., 2006). Specifically, some neurons with membrane disruption had ultrastructural evidence of neuronal necrosis,

as determined using Fluoro Jade. In contrast, other neurons sustaining membrane disruption did not show comparable signs of overt cell damage (Farkas et al., 2006; Farkas and Povlishock, 2007). Additionally, different populations of injured neurons, at the same time and in the same brain foci, did not illustrate axolemma disruption yet they demonstrated either the induction of heat-shock protein expression and/or calpain mediated spectrin proteolysis (Farkas and Povlishock, 2007). These divergent responses to axolemma damage suggest that mechanoporation may not ultimately lead to neuronal death or that there is a potential for membrane resealing and recovery following injury.

Despite divergent results on the significance of axolemma alterations following injury, other studies have illustrated increased intracellular calcium levels within injured axons and suggest an extracellular source for the calcium influx (Iwata et al., 2004). Disruption to axolemma permeability is not seen following axonal stretch injury (Smith et al., 1999) and further studies by Wolf and colleagues (2001) also indicated no evidence of calcium entry through mechanically produced pores. However, calcium-mediated proteolytic cleavage of tetrodotoxin-sensitive sodium channels resulting in an influx of calcium has been illustrated following stretch injury but the initial source of increased cytosolic calcium remains to be elucidated (Iwata et al., 2004). Traumatic stretch injury to cultured cortical neurons resulted in alterations in intracellular calcium stores and capacitative calcium influx (Weber et al., 1999, 2001, 2002). In this regard, intracellular calcium stores located on the endoplasmic reticulum are reported to play an important role in the post-injury intracellular calcium homeostasis (Weber et al., 2001, 2002). Although intracellular calcium levels return to basal levels following release from stores (induced either by stretch injury or thapsigargin treatment), these calcium disruptions alter calcium signalling population dynamics, which could affect normal neurotransmission in the brain and contribute to some of the pathology of TBI (Weber et al., 2001, 2002).

1.5.1.1 Calcium dysregulation

The influx of calcium ions through voltage-dependant and ligand-gated channels in the plasma membrane is a critical signal for the release of neurotransmitters from presynaptic

terminals and for responses of the postsynaptic neuron (Majewska et al., 2000; Burnashev and Rozvov, 2005; Hartmann and Konnerth, 2005). The intracellular concentration of calcium increases only transiently during normal physiological functioning. The regulation of these calcium fluxes is disrupted following injury, in diseased states and more insidiously in normal aging (Weber et al., 2001). In these circumstances the ability of neurons to control calcium fluxes, recovering from an increased calcium load, results in oxidative stress. Additionally, calcium can affect the integrity of the neuronal cytoskeleton. Calcium alone is capable of precipitous disassembly of cold-labile microtubules (Pettus and Povlishock, 1996). Although not all microtubules are disrupted directly by calcium, microtubules are depolymerised when calcium interacts with calmodulin under normal physiological conditions (Job et al., 1981). Calcium both prevents microtubule assembly and induces a rapid disassembly of pre-existing polymers (Maxwell et al., 1997).

Increased intracellular calcium levels may also result in the activation of cysteine proteases (calpains) and phosphatases (calcineurin and calmodium). Calpains degrade a number of substrates including cytoskeletal proteins, membrane receptors and metabolic enzymes (Bi et al., 1996; Caba et al., 2002; Guttmann et al., 2002) and may also activate of a variety of caspases (Leist et al., 1997; Volbracht et al., 2001), which is a pivotal component in the initiation of apoptosis. Calpain activation and decreased calpain inhibition by calpatatin have been linked to NF degradation, although this may only be a posttranslational modification (Povlishock, 2000). Activated calpains seem to play a role in the early degradation of neuronal spectrin (Okonkwo et al., 1998; Povlishock, 2000). In this regard, calpain mediated spectrin proteolysis has also been shown to directly contribute to the proteolytic sidearm modification of NFs resulting in NF compaction (Buki and Povlishock, 2005). Relatedly, axotomy of Mauthner axons in the lamprey induces a large influx of extracellular calcium that correlates well with the temporal pattern of NF sidearm proteolysis (Strautman et al., 1990; Hall and Lee, 1995).

Alternatively, calcium-induced activation of calcineurin can occur, which may modify the phosphorylation state of NF sidearms, thereby altering the repelling forces of the sidearms, resulting in NF compaction (Okonkwo et al., 1998; Buki and Povlishock, 2005).

Calcineurin is a heterodimer, which consists of a catalytic subunit (calcineurin-A) with a molecular mass of about 57-59 kDa and a regulatory calcium-binding subunit (calcineurin-B) with a molecular mass of 19 kDa (Klee et al., 1988). Ubiquitously distributed in eukaryotes and widely distributed in the brain, calcineurin, is highly expressed in the hippocampus, except for interneurons (Sik et al., 1998), and the caudate putamen (Klee et al., 1988; Steiner et al., 1992; Halpain et al., 1998). Calcineurin-A is found in cell bodies, postsynaptic densities, dendrites, axons, and spines commonly located in the cytosol or associated with the plasma membrane within the cell (Klee et al., 1988; Wu et al., 2007). Calcineurin has also been shown to be associated with the cytoskeleton. This finding is of interest given that several substrates of calcineurin are co-localised to the cytoskeleton including tau (Garver et al., 1999; Goto et al., 1985; Kayyali et al., 1997), microtubule-associated protein 2, tubulin (Goto et al., 1985), dystrophin (Michalak et al. 1996), and dynamin (Liu et al., 1994). As a serine/threonine protein phosphatase, calcineurin acts as an effector of calcium signalling by regulating the phosphorylation state of these cytoskeletal proteins. As a result calcineurin activity is linked to calcium-induced cytoskeletal damage following injury. The general implications of cytoskeletal alterations, including NF compaction will be discussed in detail in the following sections, but one of the more significant calcium-mediated cytoskeletal alterations is damage to the mitochondria. Spectrin can cross-link mitochondria with other organelles (Peters et al., 1991) and following injury calcium mediated spectrin proteolysis appears on the surface of swollen mitochondria (Buki et al., 1999). This may suggest that the proteolysis of spectrin is related to mitochondrial damage.

1.5.1.2 Mitochondrial damage and release of cytochrome-C

Axonal injury is associated with alterations to the mitochondria including swelling and the rupture of the mitochondrial cristae (Buki et al., 1999). These mitochondrial alterations are reminiscent of those induced by calcium mediated opening of the mitochondrial permeability transition (MPT) pore and is proposed to be mediated by the excessive sequestration of calcium following injury (Buki and Povlishock, 2005). Mitochondrial

injury provides a further energetic and homeostatic challenge for the injured neuron. The release of calcium from mitochondrial stores may also further exacerbate the neuronal response to injury. Mitochondrial injury can also result in the activation of the caspase death cascade due to MPT-mediated release of pro-apoptotic substances such as cytochrome-C and apoptosis inducing factor (AIF) (Jiang and Stys, 2000).

Cytochrome-C release from mitochondrial stores following injury is a critical event in the mechanism of neuronal death. When released into the cytosol cytochrome-C forms the apoptosome with Apaf-1 and pro-caspase-9 resulting in caspase-3 activation. Despite its importance in neuronal cell death very little about the mechanism(s) of cytochrome-C release is known. Most studies have focused on the release of cytochrome-C via mitochondrial permeabilization and its regulation by members of the Bcl-2 family (Buki et al., 1999; Buki and Povlishock, 2006). Recent insights have emerged on the underlying mechanism for cytochrome-C mobilization from the mitochondrial inner membrane, which is a prerequisite for its release. Under physiological conditions cytochrome-C can function as a mitochondrial electron carrier. However, following a pro-apoptotic insult cytochrome-C may play a role as a specific perioxidase (Kagan et al., 2006). The majority of cytochrome-C is loosely attached to the inner mitochondrial membrane and can be easily detached using an ionic salt. This fraction of cytochrome-C participates in electron transport and superoxide scavenging (Okouchi et al., 2007). The remaining amount of cytochrome-C is tightly bound to a phospholipid unique to the mitochondrial inner membrane known as cardiolipin (Okouchi et al., 2007). The cytochrome-C bound cardiolipin exhibits peroxidase activity that eliminates mitochondrial derived H_2O_2 and is important in apoptosis (Chen et al., 2002). The disruption of mitochondrial electron transport following a calcium associated traumatic insult induces the generation of O_2^- and H_2O_2, which promotes the peroxidase activity of the cytochrome-C bound to the cardiolipin. This initiation of peroxidase activity results in the dissociation of cytochrome-C. This release of cytochrome-C is referred to as "two step" (Gogvadze et al., 2006) with the first step involving cytochrome-C detachment from cardiolipin at the inner membrane upon oxidation of the phospholipids. The second step involves cytochrome-C release into the cytosol through Bax/Bak outer membrane permeabilization (Ott et al., 2002).

The use of potent mitochondrial pore blockers, such as cyclosporin-A, to prevent the release of cytochrome-C provided not only profound mitochondrial protection but also a significant blunting of the above described calpain-mediated spectrin proteolysis and related cytoskeletal changes (Büki et al., 1999b; Okonkwo and Povlishock, 1999; Okonkwo et al., 1999). However, not all cytoskeletal alterations, for example NF compaction (Okonkwo and Povlishock, 1999), were attenuated following injury highlighting the complexity of DAI and the possible involvement of other calcium-mediated pathways, which need to be further examined.

1.5.2 Cytoskeletal response to injury

As an integral support component of the neuron, NFs are particularly at risk to damage. NFs stabilize the neuronal structure and provide tensile strength. An increased population of NFs within a neuron will provide neurons the mechanical strength necessary to resist the compressive forces of the local environment (Cleveland et al., 1991). The NF alterations following injury have been extensively studied in both *in vitro* (Dickson et al., 2000; Chuckowree and Vickers, 2003) and *in vivo* (King et al., 2000; 2001) models involving axon transection (Meller et al., 1993). These studies have demonstrated accumulations of NFs into ring- and bulb-like structures in severed axonal stumps. Both structures lacked microtubules and appeared to contain a dense core of organelles, surrounded by a whorl (forming a ring-like structure) or dense ball (forming a bulb-like structure) of NFs. Morphologically similar structures were also observed in a subtype of Aß plaque-associated dystrophic neurites in Alzheimer's disease (King et al., 2000). The accumulations of NF proteins at the transected axonal tips often persist for long periods of time, with the NFs remaining bundled (Hall and Lee, 1995; Nixon, 1998).

There is a spectrum of pathologic changes that are observed in non-disruptively (unsevered) injured axons, which is based on the severity of the injury as well as the size of the axons. However, it is well understood that in mild injuries, in which there is no disruption to the

axolemma, there is a misalignment of the NFs and microfilaments without any loss of microtubules (Pettus et al., 1994; Pettus and Povlishock, 1996; Jafari et al., 1997; Maxwell et al., 1997). This may support the finding that at milder levels of compression injury, axons can completely recover (Maxwell et al., 1997). At moderate to severe injuries there is a compaction of the NFs (Pettus et al., 1994; Pettus and Povlishock, 1996; Jafari et al., 1997), reducing the spacing between adjacent NFs, and loss of microtubules. NF compaction, leading to an increased density of NFs within the axoplasm, may occur within 5 minutes of injury persisting for months, and it is established that there is little change in the compaction between this time period (Pettus et al., 1994; Pettus and Povlishock, 1996). There is quantitative and morphological evidence of microtubule loss in both moderate and severe levels of axonal injury (Hoffman et al., 1984, 1985; Jafari et al., 1997, 1998; Maxwell and Graham, 1997; Adlard et al., 2000). The post-injury reduction in microtubules has been demonstrated in large pontomedullary nerve fibres in cats (Povlishock et al., 1995), smaller optic nerve fibres of the guinea pig (Maxwell et al., 1994; Maxwell, 1995, 1996) and in myelinated axons of the human cortex (Castejon and Acurero, 2004). The loss of microtubules is most notable at the nodes of Ranvier (Maxwell. 1996) where, in non-injured controls there is significantly higher levels compared to NFs (Maxwell and Graham, 1997). Fast axonal transport continues in areas where microtubules remain intact, distant to the focal area of their loss. Microtubules and their associated proteins are fundamental to the process of axonal transport, continuously moving membrane bound vesicles to distal axonal regions. It is still unclear whether there is morphological and functional recovery from microtubule misalignment, but recent studies demonstrated pharmacological microtubule destabilization of sensory neurons in cell culture induces retraction bulb formation (Erturk et al., 2007).

1.5.2.1.1 Axonal transport disruption

Impairment of axonal transport and the subsequent development of axonal swellings appear to be associated with a sequence of axonal changes underlying DAI. Two mechanisms of axonal damage have been suggested to lead to the impairment of axonal transport. The first

mechanism involves axon swelling due to a change in ionic influx. The pathological influx of sodium through sodium channels has been well characterized in both models of white matter anoxia and dynamic stretch of axons (Stys et al., 1992; Waxman et al., 1992; Waxman et al., 1994; Wolf et al., 1999; Li et al., 1999; Agrawal et al., 1996). This disruption to ionic homeostasis may result in osmotic swelling of axons due to sodium influx; the resultant increase in intracellular calcium may also induce the deleterious activation of proteases leading to additional cytoskeletal damage as previously discussed (Saatman et al., 1996; Pike et al., 1996; Buki et al., 1999; McCracken et al., 1999). Although the disruption in ionic homeostasis may play an important role in the development of axonal swellings and contribute to the eventual demise of the injured axon, it is not considered to be the principal pathological feature of traumatically injured axons. Rather, the accumulation of axonal transport proteins within axon swellings is reported to be a major contributor to cell death following injury. Studies utilizing a variety of markers have illustrated the accumulation of fast transport β-amyloid precursor protein (βAPP) and the slow transport NF proteins (Yaghmai et al., 1992; Gentleman et al., 1993;Grady et al., 1993; Blumbergs et al., 1995; Pierce et al., 1996).

The second proposed mechanism of axonal damage relates to perturbation in the organization of NF subunits and microtubules as a direct result of the mechanical forces underlying TBI. Single-labelling immunocytochemical studies using antibodies that may indicate NF compaction, RM014, and antibodies to APP, a marker of impaired axonal transport, have shown that axonal injury involves both NF compaction and disruption of axonal transport (Buki et al., 1999; Okonkwo et al., 1999). Initially these events were proposed to occur within the same injured axon. Further studies, however, demonstrated that not all traumatically injured axons progressed to form axonal swellings and in some axons NF compaction occurred independently of impaired axonal transport and swelling (Stone et al., 2001). In this regard, Marmarou and colleagues (2005) provided quantitative analysis of the relationship between NF compaction and impaired axonal transport. This study illustrated that in the majority of damaged axons, NF compaction was not associated with impaired axon transport and further demonstrated the importance of multiple markers when evaluating the extent of axonal injury (Marmarou et al., 2005). Recently, the fate of

traumatically injured axons with NF compaction was analysed using electron microscopy (Gallyas et al., 2006). Quantitative analysis of the injured axons revealed that around 50% of the compacted axons recovered in 1 day, and a further 10% did so in 1 week. Electron microscopy revealed that the non-recovering compacted axons underwent a sequence of degenerative morphological changes including homogenisation, fragmentation and resorption of the fragments (Gallyas et al., 2006). Together with alterations to NF structure the disassembly of microtubules in axons may also contribute to disruption to axonal transport.

It is currently accepted that these two mechanisms of axonal damage that result in axonal transport deficits are not mutually exclusive, and that a number of cellular processes may be operating at the same time. The exact physiological changes following TBI are still unknown. However, it is clear that disruption to axonal transport resulting in the development of axon swellings is a significant contributor to the eventual death of the injured neuron. Wallerian degeneration and apoptosis mediated by the ubiquitin proteasome system (UPS) are two cell death mechanisms proposed to be involved in the secondary degeneration of injured neurons following DAI.

1.5.3 Wallerian degeneration

Wallerian degeneration is stereotyped more as sequence of degenerative events following injury, than as a cell death mechanism (Zhai et al., 2003; Vargas and Barres, 2007). The initial illustration of Wallerian degeneration was observed following transection of a nerve (Waller, 1850). In this study, there was progressive degeneration of the distal nerve within days following the injury. In the PNS, rapid Wallerian degeneration results in an extracellular environment that is conducive to axon regeneration (Griffin et al., 1992; Vargas and Barres, 2007). In contrast, Wallerian degeneration within the CNS results in the presence of myelin-associated inhibitors that are likely to provide an inhibitory environment to axon regeneration (Gillingwater et al., 2006; Buss et al., 2005). Although the underlying mechanisms of Wallerian degeneration are still unclear there is evidence of

a disrupted intracellular calcium homeostasis, which may result in the activation of the calcium-dependent protease calpain (George et al., 1995; Kerschensteiner et al. 2005 Buki and Povlishock, 2006; Vargas and Barres, 2007). The pivotal event in the degradation of the axon is considered to be the breakdown of the axonal cytoskeleton described as granular disintegration of the cytoskeleton, in which fine particulate and amorphous debris replaces the axoplasm (Maxwell et al., 2003; Beirowski et al., 2005; Buki and Povlishock, 2006; Vargas and Barres, 2007). Smith et al (1999) found that there was still the possibility that axons could recover from axonal swellings, proving that axonal bulb formation is the end stage event. Initially, it was believed that Wallerian degeneration was a passive process, possibly as the result of "starvation" of the cell due to the disruption in axonal transport (Zhai et al., 2003). The discovery of a spontaneous mutation in mice called Wallerian degeneration slow (Wlds), however, suggests that the degeneration may be an active process (Lunn et al., 1989; Tsao et al. 1999, Raff et al., 2002; Vargas and Barres, 2007). In these mice, Wallerian degeneration in both the PNS and CNS is greatly delayed. Remarkably, the distal portion of a transected Wlds axon can remain viable, and can conduct action potentials for up to 3 weeks, as the result of an intrinsic property of the mutant axon (Perry et al., 1990; Glass et al., 1993). Later studies have illustrated the involvement of the UPS, at least in the early stages of Wallerian degeneration (Zhai et al., 2003). The pharmacological inhibition of the UPS results in a slowing of Wallerian degeneration (Zhai et al., 2003). The mechanisms underlying the UPS involvement in Wallerian degeneration remains to be defined, however, it is proposed that the activation of the UPS may mediate the degradation of key protein(s) that can then trigger the secondary phase of the Wallerian degenerative process (Zhai et al., 2003).

1.5.4 Ubiquitin proteasome system

Evidence of ubiquitinated material within axon swellings following DAI and axotomy may be indicative of UPS involvement (Schweitzer et al., 1993). In the majority of cells, large amounts of newly synthesized proteins are defective "off-pathway" products. This may be the result of protein misfolding due to mutations or inefficient assembly (Schubert et al.,

2000). To avoid these misfolded proteins forming aggregates of high molecular weight oligomers, the proteins can be degraded via the UPS system (Berke et al., 2003). Ubiquitin (Ub) is a 76 amino acid polypeptide, which mediates a number of cellular processes, including degradation, through covalent modification of protein. Addition of a single Ub (mono-ubiquitination) regulates protein activity and trafficking within a cell. By contrast, the addition of Ub in growing chains (poly-ubiquitination) targets the protein for destruction by the multi-subunit 26S proteasome (Ehlers, 2004).

Ubiquitin is conjugated to proteins through an isopeptide linkage involving the C-terminal carboxylate of Ub and the ε-NH_2 group of a lysine side chain of the target protein (Glickman and Ciechanover, 2002). At least three distinct sets of enzymatic activities are involved in the ubiquitination of substrates. These include ubiquitin-activating enzyme (E1), which forms a thiol ester with the carboxyl group of Gly76, activating the C-terminus of ubiquitin. The activated ubiquitin molecule is carried by ubiquitin-conjugating enzyme (E2) and transferred to the substrate lysine residue by ubiquitin-ligases (E3). Additional ubiquitin molecules can be added to form poly-ubiquitin chains via the terminal carboxyl of each ubiquitin linked to the ε-amino group of a lysine residue of an adjacent ubiquitin in the chain. Ubiquitin can form chains *in vivo* at all seven lysine residues (K6, K11, K27, K33, K29, K48, K63) (Peng et al., 2003). Poly-ubiquitination of chains via the lysine residue K48 is the primary signal for protein degradation (Pickart, 2003). K63-linked chains, however, are involved in DNA repair, ribosome function, mitochondrial DNA inheritance, the stress response and targeting of proteins for endocytosis (Pickart, 2003). For substrate recognition and subsequent degradation by the 26S proteasome, a chain of at least four ubiquitin moieties need to be attached to the target protein (Glickman and Ciechanover, 2002). Additionally, a number of enzymes mediate ubiquitination events by removal of Ub. These de-ubiquitinating proteases remove Ub from proteins about to be degraded by the proteasome, recycle monomeric Ub from polymeric Ub chains, and edit inappropriately ubiquitinated proteins (Wilkinson, 1997; Chung and Baek, 1999; Ehlers, 2004).

1.5.4.1 The UPS and axonal injury

Axonal degeneration in both the CNS and PNS occurs in many pathological and developmental settings (Raff et al., 2002; Coleman and Perry, 2002). Multiple lines of genetic evidence demonstrate an intrinsic role of the UPS in axon pruning (Hoopfer et al., 2008). For example, loss-of-function mutations of the E1 or proteasome subunits in the Drosophila mushroom body neurons during metamorphosis blocked axon pruning (Watts et al., 2003). Additional roles of the UPS in axon guidance have also been demonstrated (Campbell and Holt, 2001). However, recent studies have also identified the UPS as a mediator of Wallerian degeneration. Initial evidence for this came from explanted superior cervical ganglion cells with physically transected axons (De Stefano et al., 1998). Under normal (untreated) conditions the transected axons would undergo complete degeneration within 8-10 hrs. However, when axons were treated with pharmacological inhibitors of the UPS or when the de-ubiquitinating enzyme UBP2 was expressed in the transected axons (to prevent poly-ubiquitination), these axons survived for 16 hrs or longer. The survival of the treated cells was at least partially selective for proteasome-mediated proteolysis, as inhibitors of caspases and serine proteases had no neuroprotective effect. A similar neuroprotective effect was observed following *in vivo* ischemic retinal injury in gracile axonal dystrophy (*gad*) mice with an exon deletion for the neuron specific de-ubiquitinating enzyme ubiquitin carboxy terminal hydrolase-L1 (UCH-L1) (Zhai et al., 2003). The *gad* mice showed reduced ubiquitin induction after light stimuli and ischemia and increased expression levels of anti-apoptotic proteins Bcl-2, XIAP and pro-survival brain derived neurotrophic factor proteins (Harada et al., 2004). UPS involvement in axon degeneration following injury was indicated by the accumulation of ubiquitin-like material in rat spinal cord following compression injury of varying degrees. Accumulation of immunoreactive ubiquitin material was localized to regions of axon swellings. This accumulation was proposed to reflect a disruption to axonal transport possibly arrested anterograde transport (Li and Farooque, 1996). This is supported by evidence of disassembly of cytoskeletal proteins, resulting in disrupted axon transport, within axon swellings (Price et al., 1998; Erturk et al., 2007; Serbest et al., 2007). Additionally, sequestered aggregates of proteins (referred to as aggresomes) are known to be actively

transported along microtubules, with redistribution of the IF protein to form a cage surrounding the core of aggregated, ubiquitinated protein (Johnston et al., 1998).

It is therefore likely that the axonal cytoskeleton may be a substrate for ubiquitination and UPS degradation. A combination of immunocytochemical and biochemical analysis revealed major cytoskeletal fragmentation following *in vitro* transection (Zhai et al., 2003). Specifically, both microtubule and NFs are dissembled as the axons swell, bead up and fragment, as seen in early experiments (Finn et al., 2000; Schlaepfer et al., 1984; Wang et al., 2001). Application of proteasome inhibitors dramatically slowed the degradation of both NFs and microtubules following transection, supporting UPS-mediated events. This is further supported by evidence of aberrant accumulations of cytoskeletal-associated proteins caused by a defect in the UPS (Yang et al., 2007). Microtubule disruption is the earliest (~4 hrs) feature of cytoskeletal damage following injury, followed later by NF disassembly (~8-12 hrs). Microtubule destabilization is also one of the earliest features of Wallerian degeneration, and is sufficient to induce axon degeneration in some circumstances (Wang et al., 2001), which may indicate that activation of the UPS leads in a linear causative fashion to microtubule depolymerisation and subsequent NF degradation before degeneration. This may be due to the Ub-dependant degradation of microtubule associated proteins or regulatory molecules (Ehlers, 2004).

1.5.5 Axonal sprouting

Neuronal injuries due to cortical trauma and stroke results in axonal sprouting in the hippocampus and the neocortex (Sutula et al., 1998; Esclapez et al., 1999; Golarai et al., 2001; Santhakumar et al., 2001; Buckmaster et al., 2002; Marchenko et al., 2004). This may provide evidence of an adaptive sequence of alterations aimed at healing and remodelling (Stroemer et al., 1998; Carmichael et al., 2001; Carmichael, 2003; Lee et al., 2004). Although these processes may be involved in regeneration there is also the possibility that they may also contribute to pathophysiological processes such as

epileptogenesis (Dudek and Spitz, 1997; McKinney et al., 1997; Prince et al., 1997; Prince, 1999).

Collateral sprouting during development may provide important insights into the formation of regenerative sprouts following trauma. The refinement of neuronal circuitry is regulated by the formation of collateral axon branches, sprouting from regions behind the distal growth cone (Gallo and Letourneau, 1998; Dent et al., 2004; Tang and Kalil, 2005). The sprouting of new motile structures, filopodial and lamellae, from previously quiescent regions of a neuron is fundamental to the generation of these collateral axon branches. Dynamic reorganization of the cytoskeleton underlies the formation of these motile structures (Gallo and Letourneau, 1998; Tang and Kalil, 2005) particularly in regard to actin filaments, which are also vital for maintenance of these motile structures. Actin dynamics have not only been implicated in the growth of collateral branches but also in guidance (Gallo and Letourneau, 1998). The location of collateral sprout formation, at least during development, has been attributed to calcium transients (Hutchins and Kalil, 2008).

Many studies have demonstrated collateral sprout formation following axon transection (King et al., 2001; Raineteau and Schwab, 2001; Edgerton and Roy, 2002; Edgerton et al., 2004; Hagg, 2006; Sananpanich et al., 2007). Indeed, these studies illustrated that plasticity in the cortex, sub-cortical nuclei and the spinal cord itself can contribute to functional recovery in animal models as well as in humans with spinal cord injury (Raineteau and Schwab, 2001; Edgerton and Roy, 2002; Edgerton et al., 2004; Hagg, 2006). However, collateral sprouting in the severe axon transection models are not directly translatable to DAI, which does not involve immediate axotomy. Accordingly, in non-transectional models of axonal injury, collateral sprouting has been reported following stroke and ischemic cortical injury (Carmichael et al., 2001; 2002; Carmichael, 2003). Additionally, axonal sprouting also is present in partially isolated neocortex, a chronic model of posttraumatic epileptogenesis (Salin et al., 1995; Graber and Prince, 2006; Jin et al., 2006). On one hand, post-stroke axonal sprouting and axon-growth gene expression are seen in rodents, experimental primates and humans and correlate in location and magnitude with functional recovery (Carmichael, 2003; Carmichael et al., 2004, 2005; Dancause et al.,

2005; Li et al., 2005; Li and Carmichael; 2006). Conversely, the recurrent excitatory synapses formed by sprouting axons of chronically injured pyramidal neurons in rats, is proposed to contribute to epileptogenesis (Wuarin and Dudek, 1996, 2001; Jin et al., 2006). Altogether, these studies illustrate that non-transected injured axons are capable of regenerative sprouting. It remains to be elucidated whether mechanical injury, without immediate axotomy, is capable of inducing axon collateral sprouting. This response may reduce functional deficits by enhancing innervation, via collateral sprouting around the injury site by spared fibres, following injury (Hagg et al., 2005). However, sprouting may not necessarily be beneficial and possibly involved in the genesis of post-traumatic epilepsy as discussed above.

1.6 EXPERIMENTAL MODELS OF TBI

A diverse range of models has been used in an attempt to replicate human TBI, particularly DAI. It is often difficult to reliably reconstruct the events leading to primary and secondary lesions of varying severity and regional distribution that constitutes TBI (Blumbergs et al., 1989a). Animal models have been particularly beneficial in the study of TBI as they allow for mechanical input that is quantifiable, subject to manipulation, with the head injuries produced under a controlled set of experimental conditions (Finnie and Blumbergs, 2002). However, there is no single animal model that can reliably replicate the full spectrum of human TBI (Maxwell et al., 1997), and this is due to the differing spatial distribution of damaged axons in most models. This concept is also important in the comparison of the evolving axonal response to trauma in human and animal models. There is increasing evidence to show that the rate of axonal change in small animals is faster than that seen in large animals (Maxwell et al., 1997). The most broadly used animal models of TBI have been classified by Finnie and Blumbergs (2002) as:

- Direct brain deformation
- Impact acceleration models
- Inertial (non impact) acceleration models

1.6.1 *In vivo* models of TBI

In vivo models have been widely used to try and understand the specific responses of the brain to TBI. The advantage of using *in vivo* models is the ability to asses the damage to the brain in a three dimensional manner, as well as observing the interactions between a variety of cells present in the brain. *In vivo* models of TBI provide a more accurate representation of the processes that occur in humans, as they incorporate the physiological conditions found in the brain of a patient with brain injury. Additionally, manipulating cerebral perfusion and oxygenation to generate diffuse ischemia may be useful to differentiate the neuropathological features of global ischemia from DAI (Povlishock et al., 2005) aiding in the design of targeted therapeutic interventions (Saatman et al., 2008).

Most *in vivo* models involve the application of a mechanical force to the head of an animal, followed by a recovery period, and the removal of the brain in order to observe any damage. The most common *in vivo* model used in the early studies of TBI is the impact acceleration model. This has been used in primates (Gennarelli et al., 1982; Maxwell et al., 1993), sheep (Finnie et al., 2000; Finnie et al., 2001; Finnie et al., 2002), rats (Bakay et al., 1977; Marmarou and Shima, 1990) and cats (Tornheim et al., 1984; Tornheim et al., 1990). Impact acceleration models typically involve direct impact to the brain using a piston, a humane stunner or captive bolt, a calibrated pendulum, or a weight drop onto the skull (Finnie and Blumbergs, 2002). These models replicate closed head injury commonly associated with falls, assaults and motor vehicle accidents, which involve the rapid movement of the brain within the skull following impact. The main disadvantage with this model is that it sometimes fails to produce a highly repeatable injury (Finnie and Blumbergs, 2002).

Inertial acceleration models involve rapid acceleration and deceleration of the head without impact. These models induce a repeatable pathologic response, especially when the direction and distance of the head is constrained. Many features of human TBI has been reproduced in this model, including coma and DAI in the white matter (Ommaya and Gennerarelli, 1974; Gennerarelli and Thibault, 1982; Gennerarelli et al., 1982,1990),

however, unlike most human motor accidents it does not involve impact. In this model of head trauma the acceleration required to produce injury experimentally is found only in human TBI when impact occurs (Lighthall et al., 1989).

In recent years, direct brain deformation models, particularly involving fluid percussion, have become the most common model of TBI. These models usually involve a fluid pulse to rapidly compress the exposed dura or cortex through a craniotomy site (Chytrova et al., 2008; Marmarou et al., 2005, 2006). Indeed, fluid percussion models have been implemented in the study of injury pathology (Erb and Povlishock, 1988; Conti et al., 1998), physiology (McIntosh et al., 1994;Albensi et al., 2000), and pharmacology (Faden et al., 1989; Faden et al., 2003) in a wide range of species including, rats (Dixon et al., 1988; Faden et al., 2003), mice (Carbonell et al., 1999), cats (Zauner et al., 2002), pigs (Gibson et al., 2002), rabbits (Hartl et al., 1997) and sheep (Millen et al., 1985). These types of injury generally provide reproducible levels of localized damage to cortex and the brain stem (Cernak, 2005). Additionally, alterations in cerebral blood flow and increased permeability of the blood-brain barrier have been illustrated in these models (Muir et al., 1992; Tanno et al., 1992; Schmidt and Grady, 1993; Qian et al., 1996). The lateral fluid percussion model provides an injury that replicates clinical contusion without skull fracture, and shows a direct relationship between the majority of pathological alterations and injury severity (Cernak, 2005). As a result, direct brain deformation models are widely used in mechanistic studies and for drug screening (Cernak, 2005). The limitations of *in vivo* direct brain injury models is the increased levels of injury severity, which often results in mortality due to large amounts of damage to the brainstem.

The difficulty in using *in vivo* models is that the total amount of axonal injury in the brain may be the result of a combination of mechanical and ischemic injury, it is therefore mandatory for continuous physiological monitoring to ensure that there is no complicating hypoxic episodes that supervene, as there are no current histological methods for distinguishing between the different types of injuries. Injured axons have also been shown to be more vulnerable to ischemia than uninjured axons (Fehlings et al., 1989). Additionally, although all the in vivo models integrate different methods of applying

mechanical force to the brain, each one of them basically induces localized morphological alterations. As a result of limited diffuse injuries, the majority of these models do not cause significant long-term deficits that would be of importance to clinical TBI (Cernak, 2005). Accordingly, these models are more helpful in studies investigating the pathophysiology of localized contusions and/or for the control of treatments aimed at reducing the size of focal lesions (Lighthall et al., 1989).

1.6.2 *In vitro* models of TBI

Although the diverse range of *in vivo* models of DAI have yielded encouraging results in the laboratory, these have largely failed to translate effectively into humans. As a result, another approach has been to further develop newer supplementary methodologies to explore the mechanisms underlying DAI and subsequently develop hypothesis-based strategies on *in vivo* models (Kumaria and Tolias, 2008). In this regard, although *in vitro* models are simplified platforms in the investigation of DAI, they are able to complement *in vivo* models them and allow the exploration of different aspects. Accordingly, *in vitro* models are advantageous as there is better control over experimental variables, they are cheaper and easier to use, and there are fewer ethical issues (Kumaria and Tolias, 2008). Additionally, in the study of the milder forms of DAI, where there is no immediate cellular damage, it is easier to monitor the consequences of mechanical injury to a particular cell type using *in vitro* models, and also increase the screening of therapeutic agents (Morrison et al., 1998a).

One of the earliest and still widely used *in vitro* models of DAI is the axon transection model (for *in vivo* models see pioneering work of Cajal et al., 1928). Typically, in more recent studies, these models involve the culturing of neurons (with or without glia) on to substrate coated substrates, and transection of the cultured axons using a scalpel (King et al., 1997; Dickson et al., 1999, 2000, 2005; Vickers et al., 2000; Chuckowree and Vickers, 2003; Haas et al., 2004; Blizzard et al., 2007). These models of axonal injury are reflective of penetrating brain injuries and are useful also for investigating the regenerative sprouting

response (Chuckcowree and Vickers, 2003; Chuckcowree et al., 2003, 2004; Haas et al., 2004; Blizzard et al., 2007), and the pathology underlying Alzheimer's disease (King et al., 1997; Vickers et al., 2000). The advantages of these types of models are that they are less expensive, and require less skill, thereby making them more amenable to the study of multiple novel therapeutic agents. A disadvantage of axon transection models is that generally they do not account for mechanical stimuli that might relate more closely to DAI such as force, strain and strain rate (Morrison et al., 1998b). However, probably the largest drawback of these models is that primary axotomy only occurs at the most severe levels of injury and may not be yielding to intervention as delayed secondary axotomy *in vivo* (Povlishock and Christman, 1995), resulting in a less clinically translatable concept (Kumaria and Tolias, 2008). Extending on these studies, other *in vitro* models have utilised compression injuries to cell cultures (Murphy and Horrocks, 1993) or organotypic brain slices (van de Pol et al., 1990; Sieg et al., 1999), similar to that seen in *in vivo* spinal cord injury models (Ballentine et al., 1988). However, it is often difficult to reliably measure strain field at the site of impact (Morrison et al., 1998b).

As discussed previously, shear forces created by inertial loading of the brain are manifest during DAI or contusion (Sahuquillo and Poca, 2002). In an attempt to model these forces, cultured neurons and glia were subjected to three tangential accelerations over 3-5 second time intervals (Lucas and Wolf, 1991). However, in this model, deflection of the axon was not measured directly (Kumaria and Tolias, 2008). Building on this study, Nakayama and colleagues (2001) designed an *in vitro* model that involved the oscillation of cells in a single plain over a varied amount of time. Although the injured cells could be directly observed by light microscopy, the hydrostatic pressures involved in this type of model would be difficult to translate to an *in vivo* or clinical setting.

A major disadvantage of all the models of DAI detailed above is that they do not portray the current understanding of the pathophysiology of TBI. In this regard, axonal injury is proposed to be an initiator of cellular cascades that ultimately result in a delayed secondary neuronal death. Accordingly, sublethal neuronal injury is increasingly appreciated as an important target in the investigation of interventions to prevent the ensuing secondary cell

death. Currently, models of axonal stretch injury are regarded as the gold standard in the study of the post-injury sequelae following DAI (Kumaria and Tolias, 2008). The first axonal stretch injury model was developed by Cargill and Thibault and involved the culturing of NG108-15 neuroblastoma cross-glioma cells on custom-built wells, which were deformed with a vacuum pulse generating strains as high as 0.45 with strain rates between <1 and 10/sec (Cargill and Thibault, 1996). Further developments to this model resulted in strains up to 0.65 at strain rates between 0.04 and 15/sec (Morrison et al., 1998b). However, probably the best-characterized and most widely used model of axonal stretch injury is that designed by Smith and colleagues (1999). In this model, neurons derived from a N-Tera2 cell line were cultured on a deformable substrate, which was then stretched using an air-pulse generating system (Smith et al., 1999). The strains achieved by this model were in the range of 0.58 – 0.77 and applied to the axon at times between 26 and 35/sec. Importantly, this model was not only the first to report the thresholds for primary axotomy, but also induce a sublethal mechanical strain that allowed investigation of the acute temporal evolution of axonal alterations in response to injury (Smith et al., 1999).

The axonal stretch injury model produced by Smith and colleagues has been widely used to demonstrate a number of novel findings. These include the high tolerance of axons to stretch injury, and no overt alterations to axolemma permeability unless there is axotomy (Smith et al., 1999). Additionally, this model was utilised to illustrate that traumatic deformation of axons induces abnormal sodium influx through mechanically sensitive sodium channels, which subsequently triggers an increase in intra-axonal calcium via the opening voltage-gated calcium channels and the proteolytic cleavage of voltage-gated sodium channels (Wolf et al., 2005; Iwata et al., 2004). These studies have highlighted the possible therapeutic potential of agents like tetrodotoxin and protease inhibitors (Iwata et al., 2004). Another interesting caveat derived from results of this model is the new approach to nervous system repair, which involves the development of transplantable nervous tissue constructs comprised of mechanically stretch-grown axons (Pfister et al., 2006). Over recent years, axonal stretch injury models have characterized stretch in three dimensions, a variable that adds further credence to the clinical scenario (LaPlaca et al.,

2005). Interestingly, these models have reported that biaxial stretch injury induced more damage compared to uniaxial stretch injury (Geddes-Klein et al., 2006).

In summary, the majority of *in vitro* stretch injury models, yet not inducing primary axotomy, still simulate more severe levels of injury (Kumaria and Tolias, 2008). Regarding the findings mentioned above, it appears that milder forms of injury are more realistic targets for intervention at the present time. Indeed, the delayed nature of secondary axotomy provides an ideal opportunity for therapeutic intervention.

1.7 PROJECT AIMS

One of the greatest clinical challenges in the field of neurotrauma is the minimization of secondary neuronal damage following TBI. Currently, there are no successful treatment strategies for DAI, despite positive therapeutic outcomes in animal models of head injury. In this regard, *in vivo* DAI models, to date, have revealed encouraging results that have failed to be translated into a clinical setting. Over recent years, a new approach has been to utilise *in vitro* models of DAI to characterise specific pathophysiological mechanisms, providing better-defined hypotheses to be tested *in vivo*, which are more likely to subsequently translate into future neuroprotective therapies. Furthermore, previous studies have illustrated that milder forms of TBI, which is the predominate form of head trauma (Povlishock and Christman, 1995), are more realistic targets for intervention due to the increased vulnerability of diffusely (unsevered) injured neurons and that the death of these neurons constitutes most or all of secondary injury (Smith et al., 2003; Buki and Povlishock, 2006). Indeed, DAI is an evolving form of axonopathy, developing over an extend period of time, which provides an opportunity for therapeutic interventions aimed at impeding the cellular alterations that lead to secondary degeneration (Buki and Povlishock, 2006).

Current *in vitro* axonal stretch injury models have provided important mechanistic insights into the axonal response to severe shear/strain injury, however, little is known about the response of axons to milder forms of stretch injury. Additionally, it is often difficult to locate and image mildly injured axons in vitro, as there is no immediate significant damage to the cell. The development of a primary cortical cell culture technique that results in the formation of neuronal cell clusters interconnected by long axonal bundles has provided an ideal platform to image and characterise the response of particular axons to injury (Dickson et al., 1999; Chuckowree et al., 2003; Blizzard et al., 2007). Therefore, this thesis will implement this primary cortical cell culture technique to investigate the important mechanistic insights underlying DAI, utilising a novel animal model capable of replicating the mechanical forces involved in mild to moderate DAI.

AIM 1

To develop a novel* in vitro *model of mild to moderate axonal injury capable of delayed secondary axotomy.

The majority of animal models of axonal injury involve higher levels of mechanical trauma often resulting in either immediate substantial axotomy or the rapid severing of the axon within one hour. As the majority of human TBI cases do not involve immediate axotomy at the time of injury, rather the activation of cellular and pathological cascades resulting in delayed axotomy, less severe models of axonal injury need to be investigated. To achieve this aim a novel *in vitro* axonal injury was developed. This model of axonal injury utilises neocortical neurons from rat embryos that are grown in culture to relative maturity. The growth conditions of this system promote neuronal aggregation and axonal bundle formation, which will be the site of acute stretch injury. To characterise the axonal response to injury, the injured axon bundles will be fixed at predetermined time-points and analysed using immunocytochemical techniques to determine if there is secondary axotomy.

AIM 2

To investigate the neuronal response to mild-moderate axonal stretch injury, specifically, characterising the progressive cellular alterations prior to secondary axotomy.

There has been no comprehensive study that has characterised the sequence of events that precede secondary axotomy following mild to moderate axonal injury. In this regard, the mechanical trauma does not induce any immediately detectable axonal change and may rather result in subtle changes to the axon cytoskeleton. Therefore, immunocytochemistry will be utilised to determine the cytoskeletal alterations from the moment of injury, up to secondary axotomy. To further investigate the underlying cause of cytoskeletal damage following injury, the integrity of the axolemma will be assessed, using cell impermeant tracers, to determine whether alterations to the axolemma permeability is linked to the axonal damage following trauma. Calcium imaging techniques will also be utilised to establish if intra-cellular calcium alterations play a role in post-injury sequelae.

AIM 3

To investigate the mechanisms of axonal degeneration associated with secondary axotomy and possible therapeutic interventions.

Recently, the activity of the UPS and calcium-dependant pro-apoptotic pathways, which include release of cytochrome-C and the activation of calcineurin, has been implicated in axonal degeneration following primary axotomy. Calcium induced release of cytochrome-C from mitochondrial stores has been linked with the activation of caspases and calpains, which may be responsible for the degradation of the cytoskeleton prior to secondary axotomy. Additionally, inhibition of the UPS has been shown to delay Wallerian degeneration following the transection of axons. These two degenerative pathways may provide insight into the mechanisms involved in the delayed secondary axotomy. Therefore, immunocytochemical techniques will be utilised to investigate whether there is activation of the UPS or release of cytochrome-C from mitochondrial stores. Additionally, pharmacological inhibitors of UPS function and the MPT pore, which is involved in the release of cytochrome-C, will be examined to determine the role of these two pathways in the induction of secondary axotomy. Pharmacological inhibition of calcineurin will also be utilised to demonstrate whether this calcium-activated phosphatase plays a role in the degradation of the cytoskeleton following injury.

AIM 4

To investigate the regenerative capacity of axons following non-severing axonal stretch injury.

It is proposed that loss of synaptic connectivity (deafferentation) following DAI, sets the stage for subsequent neuroplastic alterations that can lead to either adaptive or maladaptive changes (Buki and Povlishock, 2006). This has been difficult to study not only because neuroplasticity is complex to follow in the human brain, but also because this is difficult to replicate in an experimental setting. There is limited data that suggests that in the case of severe in vivo TBI, where there is axon severance, diffuse deafferentation initiates sprouting of adjacent intact nerve fibres that in some cases leads to the recovery of significant synaptic input to the previously deafferentated neuronal domains (Erb and Povlishock, 1991). Indeed, the generation of post-traumatic epilepsy, which is seen

following moderate to severe TBI, has been linked to aberrant axonal sprouting (Benardo, 2002). It is unclear whether mechanically injured neurons can initiate an axonal sprouting response, without primary or secondary axotomy. Therefore, the axonal sprouting response of mildly stretch injured axons will be determined using live-imaging. To accomplish this aim, injured axons will be loaded with a lipophillic cell tracer dye (DiI), at set time-points prior to secondary axotomy and imaged using a fluorescent microscope. Immunocytochemical labelling will also be used to illustrate the cytoskeletal composition of these axonal sprouts and determine whether they contain growth-associated proteins (GAP43). Additionally, scanning electron microscopy will be utilized to determine if axonal sprouts have growth cone-like guidance structures, which are important for controlled axon growth (Blizzard et al., 2007). Using inhibitors of actin dynamics and calcineurin, latrunculin-A and FK506 respectively, the role of actin reorganization and calcium signalling pathways in the post-injury sprouting response will be determined.

2 MATERIALS AND METHODS

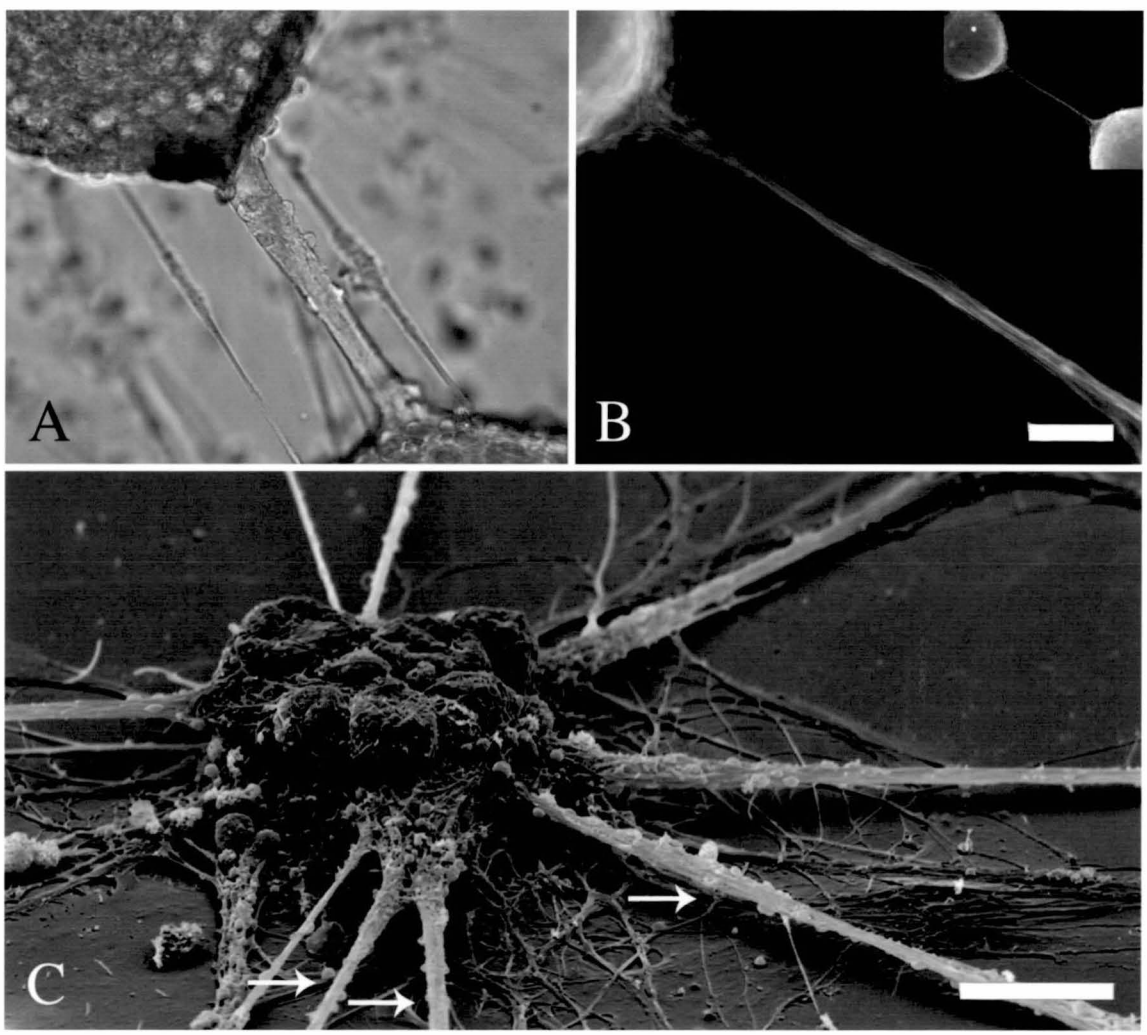
2.1 CELL CULTURE

Time-mated Hooded-Wistar rats carrying pups of 18 days gestation (E18 with sperm-positive day = E1) were sacrificed by carbon dioxide exposure, in accordance with the guidelines stipulated by the University of Tasmania Animal Ethics Committee. Rat pups were immediately removed, placed on ice and decapitated. The cortex was exposed using fine scissors, the meninges pulled away and tissue dissected from the neocortical region using fine forceps and collected in 5 ml of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (Sigma), at 37°C. Enzymatic digestion was carried out with 0.025% trypsin for 15 minutes at 37°C. Tissue was washed three times with HEPES and mechanically dissociated using a pipette. Staining with trypan blue (Sigma) was used to assess cell viability and concentration. Cells were then plated onto glass coverslips (132 mm^2) pre-coated overnight with 0.01% poly-L-lysine, at a cell density of 5 x 10^4 cells/well. Cultures were maintained at 37°C in humidified air containing 5% CO2. Neurons were initially plated into a culture medium consisting of Neurobasal™ medium, 2% B27 supplement (Gibco), 10% foetal calf serum (Gibco), 0.5 mM glutamine, 25 μM glutamate and 1% antibiotic/antimycotic (Gibco). At 24 hrs post-plating, the media was replaced with similar media, but with no fetal bovine serum. Culture media was replaced every 3 days. The fresh medium contained factors that inhibited substantial glial cell proliferation (Chuckowree and Vickers, 2003). As initially reported (Dickson et al., 2000; Chuckowree and Vickers, 2003), this long-term culture technique results in the formation of large neuronal clusters across the coverslip interconnected by thick fasciculated axonal bundles (Fig. 2.1). These axonal bundles form synapses, and contain a cytoskeletal architecture characteristic of mature axons (Chuckowree and Vickers, 2003).

2.2 AXONAL STRETCH INJURY

At 20 days *in vitro* (DIV), a single coverslip was placed into an individual 35-mm tissue culture (Petri) dish with 2 ml of fresh culture media. The next day, the culture dish was placed upon a Leica DMIL inverted microscope. The tip of a glass micropipette (bore thickness 5-10 μm; obtained from P-97 flaming micropippete puller with programmable

Figure 2.1 Neuronal cultures at 21 days *in vitro* had formed large clusters, interconnected by fasciculated bundles of axons (indicated by arrows), shown by bright-field microscopy (*A*), immunocytochemical neurofilament labelling (*B*), and scanning electron microscopy (*C*). Note the highly organized arrangement of parallel neurofilament-immunoreactive processes within the axonal bundle (B). Scale bar, 10 μm (*A*,*B*), 20 μm (*C*).


A
B
C

microprocessor controller, Sutter Instruments) was submerged into the media and moved until just touching the surface of the axon bundle, retracted 100 μm, and a single pulse of media was applied at 20 psi over a period of 13–20 msec using the Picospritzer III (Parker Instrumentation). This resulted in a deflection of a localized segment of the axonal bundle, with the axon bundle responding like a rubberband, such that after stretching it returned back to its original position. A digital movie covering the axonal deflection was captured using an Ikegami (ICD46E) video camera (for an example see Supplementary Movie 1). Only deflections resulting in a relatively minor stretch of 1–6% increase in original axon length are reported. It is notable that deflections of this magnitude did not result in primary axotomy, while at deflections greater than 10% the entire axon bundle was immediately broken.

2.3 IMMUNOCYTOCHEMISTRY

2.3.1 Fixation

Neuronal cultures were fixed using a solution of 4% paraformaldehyde (PFA, Sigma)/4% sucrose (Sigma) in 0.1 M phosphate buffer for 30 minutes at room temperature. Fixation was followed by three ten minute 0.01M PBS washes, prior to immunocytochemistry. Table 2.1 shows commonly used primary antibodies.

2.3.2 Indirect fluorescence immunocytochemistry

Following fixation, primary antibodies, which recognise and bind to specific epitopes of cellular proteins, were applied to specimens at appropriate concentrations (Table 2.1), diluted in a 0.3% Triton-X (Fluka) solution, which was used to permeabilise cell membranes. Optimum antibody concentrations were individually determined for each antibody and control experiments, omitting primary antibodies, eliminated all immunoreactivity. Primary antibodies were incubated on an orbital shaker for two hours at room temperature, then overnight at 4°C, followed by three 0.01M PBS washes. Species and isotype specific AlexaFluor fluorescent secondary antibodies (Table 2.2) were applied

Table 2.1 Primary antibodies

Antigen	Fixation method	Cell culture dilution	Type and Source	Immunising agent
Alpha-internexin	4% PFA	1:1,000	M (Clone 2E3) Chemicon	Full length recombinant rat alpha-internexin
Alpha-internexin	4% PFA	1:5,000	R Novus Biologicals	Full length recombinant rat alpha-internexin
Beta-III Tubulin (neuron specific)	4% PFA	1:10,000	M (Clone 5G8) Promega	C terminus β-III tubulin (EAQGPK)
CD11b/c	4% PFA	1:100	M (Clone CE-1) CalTag	Rat glial membranes and whole brain white matter
Cytochrome c	4% PFA	1:1,000	M (Clone 6H2.B4) BD Biosciences	Rat cytochrome c
Ferritin (activated microglia)	4% PFA	1:1,000	R Dako	Ferritin from human liver
Growth-associatted protein-43 (GAP43)	4% PFA	1:1,000	M (Clone GAP-7B10) Sigma	GAP43 from neonatal rat forebrain membranes
Glial fibrillary acidic protein (GFAP)	4% PFA	1:1,000	M (Clone 6F2) Chemicon	Purified GFAP from porcine spinal cord
Glial fibrillary acidic protein (GFAP)	4% PFA	1:5,000	R Dako	GFAP from cow spinal cord
NFH	4% PFA	1:1,000	R Serotec	Purified bovine neurofilament proteins
NFL	4% PFA	1:1,000	R Novus Biologicals	Native NFL purified from pig spinal cord
NFM	4% PFA	1:1,000	R Serotec	Purified primate neurofilament proteins
NFM/NFH dephosphorylated (SMI32)	4% PFA	1:2,000	M (Clone SMI32) Sternberger	Hypothalamus
NFM/NFH phosphorylated (SMI312)	4% PFA	1:10,000	M (Clone SMI312) Sternberger	Hypothalamus

Antigen	Fixation method	Cell culture dilution	Type and Source	Immunising agent
Spectrin (neuron-specific)	4% PFA	1:1,000	R Chemicon	synaptic/axonal membranes from perfused mouse brain.
Synaptophysin	4% PFA	1:100	R Dako	Human synaptophysin peptide-ovalbumin fusion protein
Tau	4% PFA	1:20,000	R Dako	C-terminal human tau
Ubiquitin	4% PFA	1:1,000	R Dako	Ubiquitin from cow erythrocytes

M, Mouse monoclonal; R, Rabbit polyclonal

Table 2.2 Secondary antibodies

Emission	Reactivity	Species	Supplier
488	Mouse IgG	Goat	Molecular Probes
594	Mouse IgG	Goat	Molecular Probes
488	Rabbit IgM	Goat	Molecular Probes
594	Rabbit IgM	Goat	Molecular Probes
488	Rabbit	Goat	Molecular Probes
594	Rabbit	Goat	Molecular Probes

at a dilution of 1:1000 and incubated in the dark, on an orbital shaker for 90 mins at room temperature. Secondary antibodies were removed and cells were stained with 0.0001% nuclear yellow (Molecular Probes) in PBS for 20 mins at room temperature in the dark. Cells were washed three times with PBS, followed by rinsing in MilliQ® and mounting onto microscope slides using Permafluor mounting medium (Beckman Coulter).

2.4 MICROSCOPY AND ANALYSIS

Fluorescent immunolabelled slides were examined using a Leica DMLB2 immunofluorescence microscope, equipped with a cooled CCD Magnafire (Optronics) digital camera. Images were acquired with Magnafire (version 1.0) software, and figures prepared using Adobe Photoshop® (version 7.0) where necessary. For analyses images were acquired at identical exposure settings and adjusted in an identical manner. Subsequent analysis was performed with the aid of NIH ImageJ (version 1.33u). Graphs and statistical analyses were performed with Graphpad Prism4® software or Microsoft Excel (Mac Os X). For statistical analyses data was obtained from at least three separate cultures and means are reported ± standard error of the mean (SEM). P values were determined using either one-way or two-way ANOVAs (Graphpad Prism4® software) with a value less than 0.05 were considered statistically significant.

3 MILD AXONAL STRETCH INJURY IN VITRO INDUCES A PROGRESSIVE SERIES OF NEUROFILAMENT ALTERATIONS ULTIMATELY LEADING TO DELAYED AXOTOMY

3.1 INTRODUCTION

Diffuse axonal injury typically results in a series of subtle axonal abnormalities, which progressively develop over a period of time, ultimately leading to delayed axonal disconnection and degeneration (Maxwell et al., 2003). DAI is considered an important pathological neuronal alteration linked to clinical outcomes following TBI (Povlishock, 1992; Maxwell and Graham, 1997; Smith and Meaney, 2001). In an attempt to identify the sequelae of axonal alterations in response to DAI, researchers have developed animal models to experimentally induce mechanical axonal stretch or compression injuries (Balentine et al., 1988; Smith et al., 1999; LaPlacca et al., 2005). These studies have yielded considerable insight regarding axonal alterations in response to DAI, and illustrate the advantages of *in vitro* models in the overall elucidation of the entire sequence of pathological axonal changes prior to secondary degeneration.

Although bearing the limitation of being limited to a largely two-dimensional environment, *in vitro* models of TBI have also provided some valuable insight into axonal alterations in response to injury. Such models include direct physical transection of the axon (Dickson et al., 2000; Chuckowree et al., 2003), mechanical stretching of axons of a neuronal cell line NTera2 (Smith et al., 1999) or of primary cortical neurons (Iwata et al., 2004), and physical oscillation of neurons in a single plane over a short period of time (Nakayama et al., 2001). A significant advantage of *in vitro* models is the ability to observe the progression of axonal alterations of individual axons over a period of time, and to test the effect of therapeutic agents directly upon injured neurons.

The studies described above utilise *in vitro* models of injury that result in relatively severe axonal injury and, as a consequence, usually result in axotomy within 24 hrs, which in a comparative sense is likely to be indicative of only the most severe cases of head trauma. In

the current study a novel model of transient axonal stretch injury involving pressurized fluid deflection of axons was developed in order to improve understanding of the sequelae of events in response to mild axonal trauma. Similar models of transient axonal stretch injury have been reported previously, which have determined that the tensile threshold for axotomy within 24 hrs lies at a 15–65% increase in original axon length (Smith et al., 1999). It is important to note that these models involve stretching of a long segment of the axon (typically greater than 100 μm). The current model involves subjecting axons of neurons matured in long-term culture to significantly milder tensile strain across a small axonal length (less than 100 μm), ranging from a 1% to a 6% increase in original axon length. To assess the progression of axonal changes following this relatively mild form of DAI, cytoskeletal changes were examined using immunocytochemical techniques over a period of 72 hrs.

3.2 MATERIALS AND METHODS

3.2.1 Cortical neuron cultures and experimental axonal deflection

Neuron cultures were prepared according to the procedures outlined in chapter 2.1.1 (Materials and Methods). Axonal bundles ranging from 10 to 16 μm in diameter were selected. *In vitro* stretch injuries were performed as described in chapter 2.2 (Materials and Methods; for an example, see Fig. 3.1; see also Supplementary Movie 1). For this study, a total of 175 injuries were performed over five different neuronal cultures.

3.2.2 Measuring the Degree of Axonal Deflection

By capturing digital movies of the deflection event, it was possible to measure the degree of axonal stretching. This was performed for all of the axonal bundles that were injured during the course of this study. To do this, the digital movie was expanded to all of the individual frames (using NIH Image), and the frame displaying the maximal axonal deflection selected. Using this frame, the maximal length of the stretched axon bundle was measured (using NIH image), and the tensile strain applied was calculated as the percentage increase in axonal bundle length compared to the original length of the axon bundle. In all cases, axonal bundles were measured along their entire length, from one cellular cluster to the next. Stretch injuries of 1–20% were attainable.

3.2.3 Fluorescent immunocytochemical analysis of cytoskeletal changes in response to axonal deflection

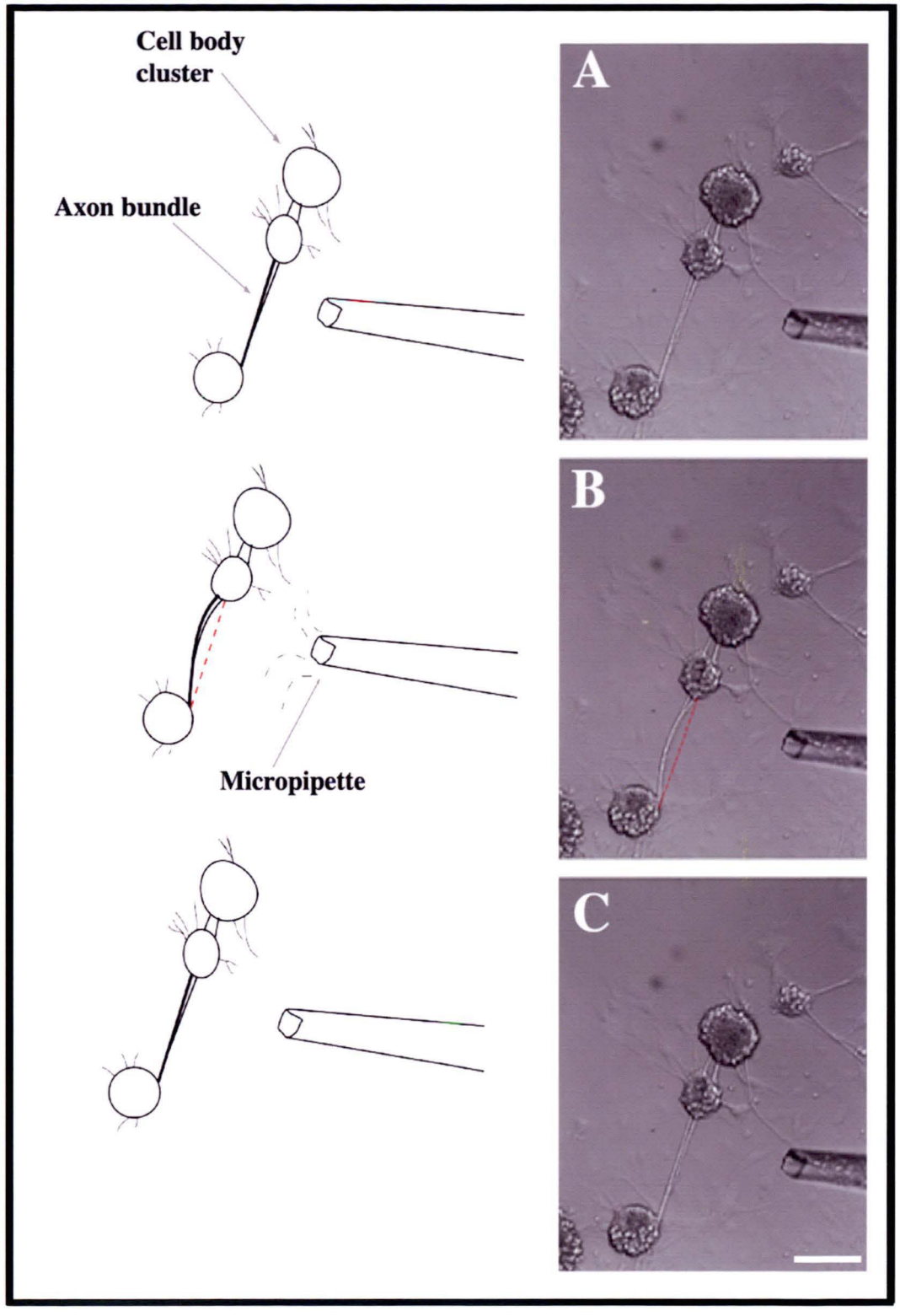
Fixation and immunocytochemistry was performed using the standard procedures in chapter 2.3 (Materials and Methods). Stretch injured axon bundles were fixed at 24 hrs, 48 hrs and 72 hrs PI and immunolabelled with antibodies targeting phosphorylated neurofilaments (SMI-312) It has previously been shown that SMI312 immunoreactivity

does not co-localize with MAP-2 labelled dendrites in this model (Chuckowree and Vickers, 2003), indicating that the SMI312 antibody cocktail identifies axons primarily. Indeed, in this culture system, we have reported previously that there are almost no MAP2–positive dendrites within the fasciculated axonal bundles that were injured in this study (Chuckowree and Vickers, 2003).

3.2.4 Three-dimensional reconstruction of injured axonal bundles

In some circumstances (approximately 20% of all injured cultures, chosen at random) following immunofluorescent labelling, rather than coverslips being mounted onto glass slides, coverslips were placed cell-side up into a glass Petri dish immersed with PBS and observed on an inverted fluorescent stereomicroscope (Leica DMIRB). For three-dimensional (3-D) reconstruction, a series of digital images were captured at 2-μm z-axis intervals (Optronics Magnafire digital camera) through the entire thickness of the injured axonal bundle (ranging from 10 to 50 μm), and the entire stack of images underwent 3-D restoration under a nearest-neighbour algorithm using OpenLab™ software (Improvision). To clearly visualize immunocytochemically labelled cytoskeletal features within the injured axonal bundle, the 3-D reconstructed stack of images was compiled and then rotated in the x- and y-axis using the 3-D rendering tool within OpenLab™, and the resultant data presented in QuickTime™ movie format.

Figure 3.1 An individual axon bundle was targeted with a single pulse, and the resultant deflection was captured by video camera. In the selected series of frames (*A–C*), the axon bundle is rapidly stretched, before recovering to its original position (indicated by dashed red line). For reference, a diagrammatic representation of the injury is also included. Scale bar, 50μm.

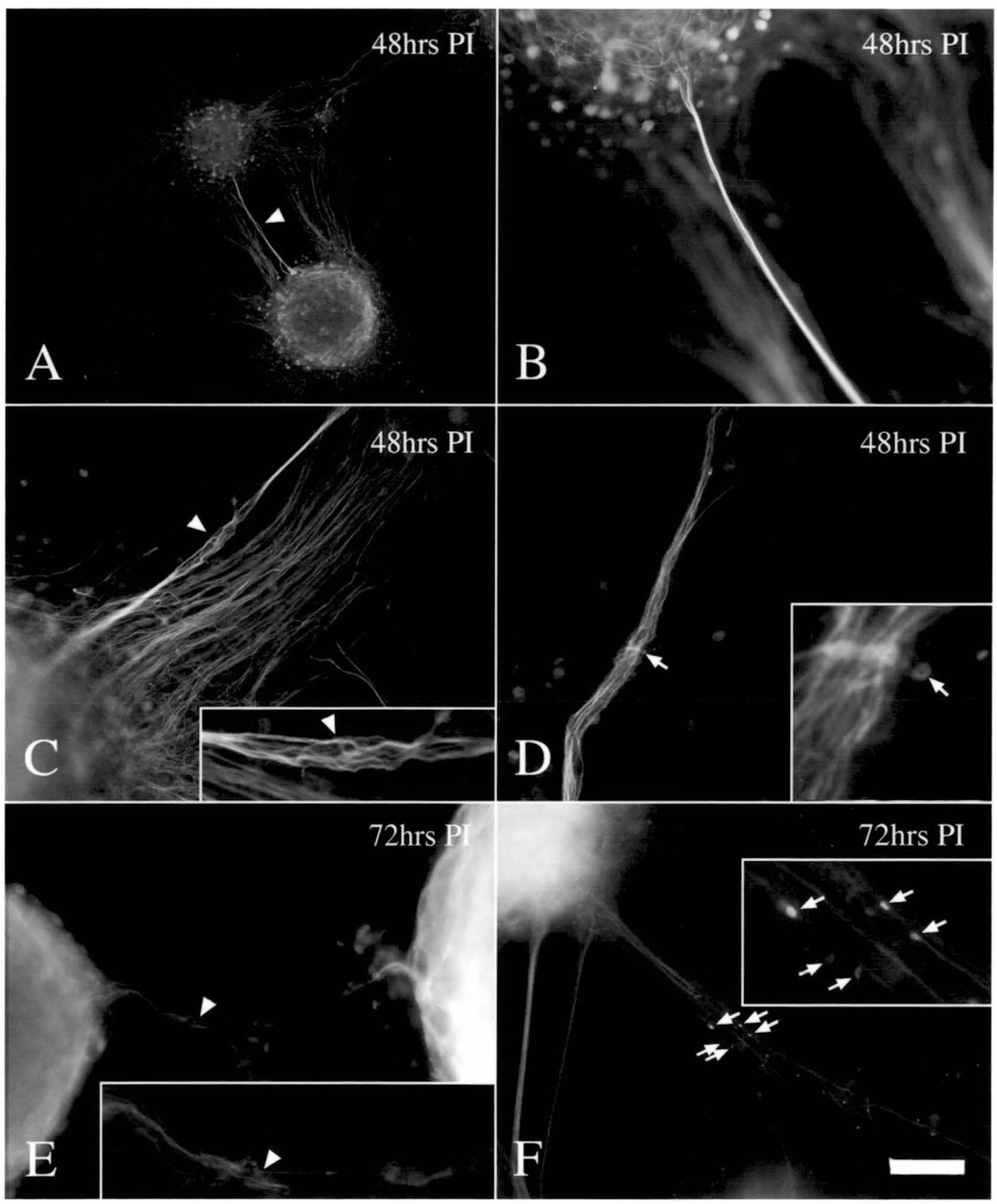

Cell body
cluster
Axon bundle
Micropipette
A
B
C

3.3 RESULTS

3.3.1 Characterizing the temporal sequence of cytoskeletal alterations in response to mild axonal stretch injury

Post-fixation immunocytochemical analysis demonstrated that, by 24 hrs PI, there was no discernable difference between stretched and unstretched axonal bundles; stretched axons remained smooth in profile and axons maintained an ordered arrangement within a relatively straight bundle formation (Table 3.1). However, by 48 hrs PI, NF immunoreactivity was consistently higher in injured axon bundles compared to surrounding uninjured axon bundles (51% of all stretched axonal bundles displayed increased NF immunoreactivity; Table 3.1, Fig. 3.2A,B). Furthermore, at the site of stretch injury, some axonal bundles (25%; Table 3.1) lost their ordered arrangement, with individual axons segregating from each other, often resulting in a frayed appearance of the bundle (Fig. 3.2C). However, it is important to note that individual axons did not appear to be axotomised, rather they became distorted, appearing undulating and wavy within a highly localized region that correlated with the stretched region of the axon (Fig. 3.2C). Small ring-like NF structures were observed within the frayed region of a minority (7%; Table 3.1, Fig. 3.2D) of axon bundles by 48 hrs PI. By 72 h PI, increased NF staining was less evident (in only 27% of cases; Table 3.1), but segregation of individual axons within the bundle was more pronounced, and at higher magnification many axons within the bundle were found to be completely disconnected (axotomy was observed in 70% of cases; Table 3.1, Fig. 3.2E). At this time, ring-like NF structures were present within the frayed region of the bundle (30% of axonal bundles; Table 3.1), often appearing to be located at the terminating tip of injured axons (Fig. 3.2F). These results clearly demonstrate the temporal progression of axonal changes in response to stretch injury, from increased NF immunoreactivity to severe alterations such as delayed axotomy. Note that the SMI312 antibody used to identify NF proteins non-specifically binds to nuclei, as reported previously (Chuckowree and Vickers, 2003). Given that the same fluid pressure (20 psi) was applied for all injuries, it is likely that the individual injury level will vary depending

Figure 3.2 Neurofilament immunoreactivity in stretched axon bundles was significantly increased in comparison to uninjured axons at 48 hrs PI (*A*, enlarged in panel *B*; indicated by arrowhead). At this time, axon bundles regularly became disorganized at the site of injury, with individual processes separating from each other and becoming wavy along a segment of their length (*C*; indicated by arrowhead). Occasionally, ring-like neurofilament structures were observed within the site of stretch injury (*D*; indicated by arrow). By 72 hrs PI, a number of those axons that had been stretched became completely disconnected (*E*; indicated by arrowhead), and a greater number of ring-like neurofilament structures were present (*F*; indicated by arrows). Note that the SMI312 antibody, used for neurofilament labeling, non-specifically binds to nuclei, which is apparent in these images. Scale bar 15μm.


48hrs PI
A
48hrs PI
B
48hrs PI
C
48hrs PI
D
72hrs PI
E
72hrs PI
F

Cytoskeletal alteration	*24 h PI*	*48 h PI*	*72 h PI*
Increased NF immunoreactivity	15% (8/53)	51% (44/85)	27% (10/37)
Axonal distortion (frayed appearance)	5% (2/53)	25% (21/85)	62% (23/37)
NF ring-like structures	2% (1/53)	7% (6/85)	30% (11/37)
Axotomy	0% (0/53)	2% (2/85)	70% (26/37)

Table 3.1 A number of different axonal bundles ranging in thickness from 10 to 16 μm in thickness were deflected, resulting in an increase in original axon length of 1–6%, and assessed at either 24, 48, or 72 hrs post-injury (PI) for the presence or absence of cytoskeletal alterations in neurofilament (NF) protein distribution. These results are presented as percentages, with the actual number of injured axon bundles displaying a cytoskeletal abnormality recorded in brackets. The time-point at which each characteristic cytoskeletal alteration was observed the most is shaded.

Table 3.2 Increased neurofilament immunoreactivity

Strain level	*24 h PI*	*48 h PI*	*72 h PI*
100–103%	0.5%	8%	78%
103–106%	0.5%	16%	12%
106%+	2%	2%	1%

Table 3.3 Axonal distortion

Strain level	*24 h PI*	*48 h PI*	*72 h PI*
100–103%	0.5%	25%	6%
103–106%	0.5%	27%	43%
106%+	5%	3%	22%

Table 3.4 Ring-like neurofilament immunoreactive structures

Strain level	*24 h PI*	*48 h PI*	*72 h PI*
100–103%	0.5%	25%	6%
103–106%	0.5%	27%	43%
106%+	5%	3%	22%

A number of different axonal bundles ranging in thickness from 10 to 16 μm in thickness were exposed to the same fluid pressure (20 psi). The increase in original axon length of each axonal bundle was measured and assessed at either 24, 48, or 72 hrs post-injury (PI) for the presence or absence of cytoskeletal alterations in neurofilament protein distribution, namely increased neurofilament immunoreactivity (Table 3.2), axonal distortion (Table 3.3), and the presence of ring-like neurofilament immuno-reactive structures (Table 3.4). These results are presented as percentages, with the time-point at which each characteristic cytoskeletal alteration was observed the most highlighted (lighter highlighting represents less than 50% occurrence; darker highlighting greater than 50% occurrence). Note that this table is derived from the same experimental data as Table 3.1, however extra data from higher intensity stimulus (106%+) is also included.

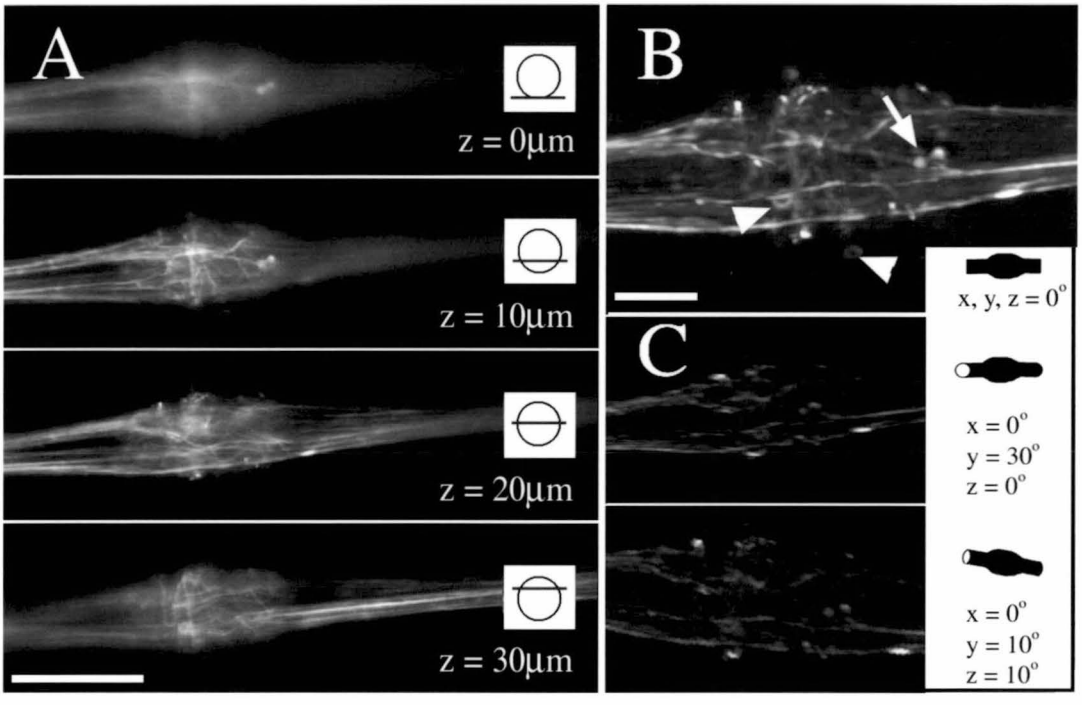
on the length of the injured axons. To account for this, the actual degree of axonal deflection was calculated for each injured axonal bundle (from the same experimental samples discussed above), and the same morphological parameters of axonal injury applied above were used for analysis. In the case of increased NF immunoreactivity, this was most apparent at 48 hrs PI for all degrees of injury strain (Table 3.2), and particularly at the 100–103% and 103–106% levels of strain. For injuries of 106% and higher, there was a much lower number of axons displaying this morphology, since a number of the axon bundles had become axotomised at this time. Similarly, the number of axons displaying increased NF immunoreactivity was dramatically lower by 72 hrs PI, since the majority of axons had undergone secondary axotomy by this time (Table 3.1). In the case of axonal distortion, this was most apparent at the higher strain levels (>103%) and at longer times PI (Table 3.3). Interestingly, the presence of ring-like NF immunoreactive structures was most apparent at the mildest strain level, and became more evident at 72 hrs PI (Table 3.4). Indeed at greater strain levels there was very little development of these ring-like structures, even at time-points up to 72 hrs PI.

3.3.2 Three-dimensional reconstruction of injured axonal bundles

3-D reconstruction was performed by compiling a series of digital images captured at 2μm z-axis intervals through the entire thickness of an injured axonal bundle at 48 hrs PI (some of these images captured at different z-axis depths are presented in Fig. 3.3A). The entire stack of images was compiled into a single, "flattened" image representing a 3-D "looking down" view through the entire axonal bundle (Fig. 3.3B). Following 3-D reconstruction of the individual images, the entire stack was sequentially rotated in a series of intervals in either the x-, y- , z-planes or a combination of axial changes simultaneously, and combined into a QuickTime™ movie to allow accurate 3-D spatial representation of immunohistochemically labelled cytoskeletal features within the injured axon bundle (360-degree x-axis rotation in Supplementary Movie 2). This methodology demonstrated a complex 3-D environment of axonal and cytoskeletal disorganization within the site of injury (selected frames from the Supplementary Movies are shown in Fig. 3.3C). This was

highlighted by an obvious swelling of the axon bundle at the point of injury, caused by the disorganization of the normally parallel-running and closely packed axons within the bundle. In this regard, a number of axons were distorted, and axotomised neurites were present (Fig. 3.3B,C). Furthermore, individual axons with several bulb-like NF swellings were present within the injury site (Fig. 3.3B). Occasionally, several NF ring-like structures, unattached to axons, were also localized within the site of injury (Fig. 3.3B).

Figure 3.3 To perform three-dimensional reconstructions through an entire injured axon bundle that had been immunolabeled for neurofilament proteins (SMI312), individual digital frames were captured in 2μm z-axis intervals through the entire bundle at 48 hrs PI (*A*; cartoons indicate the z-axis depth of the individual image). These images were reconstructed using Open-Lab^{TM} software to form an accurate three-dimensional spatial representation of immunocytochemically labeled cytoskeletal features within the injured axon bundle (*B*). Within the injured axon bundle, an individual axon with several bulb-like neurofilament swellings was visualized within the injury site (arrow). Several neurofilament ring-like structures, unattached to axons, were also found within the site of injury (arrowheads). Using OpenLab^{TM} software, it was possible to rotate the compiled image in either the x-, y-, z-axis or axial combinations to fully observe the spatial relationships of immunolabeled cytoskeletal structures within the injured axon bundle. Several examples of these are presented (*C*; cartoons detail the axial rotations for each image). Scale bar, 50μm.


A
z = 0µm
z = 10µm
z = 20µm
z = 30µm
B
C
x, y, z = 0°
x = 0°
y = 30°
z = 0°
x = 0°
y = 10°
z = 10°

3.4 DISCUSSION

This thesis has presented a simple, reproducible *in vitro* model of transient axonal stretch injury of cultured primary neurons. This model has a number of methodological advantages, including a modifiable and highly reproducible method of injury, and the ability to selectively injure and visualize a highly localized region of the axon. To validate this model, we have investigated the response of axons and NF proteins to mild axonal stretch injury and observed a stereotypy of changes reminiscent of those observed during TBI that leads to a high rate of secondary axotomy after 3 days post-injury.

Previous *in vitro* studies have performed relatively severe (ranging from 15% to 65% stretch of an axon over its initial length) stretch injuries to axons of neuron-like cell lines and noted dramatic axonal responses within 24 hrs including axotomy. However, we have utilized a methodological approach specifically aimed at performing mild transient axonal injury (1–6% stretch) to assess the slow progression of axonal alterations that are characteristic of diffuse axonal injury (DAI) evolving over several days. In this regard, almost no discernable difference in cytoskeletal arrangement was noted between unstretched and stretched axonal bundles by 24 hrs PI. It is important to note that the SMI312 cocktail of monoclonal IgGs that we have used labels phosphorylated NFs of all subclasses (-H, and -M,). Hence, we were unable to ascertain subtle alterations in the phosphorylation state of individual NF subunits or phosphorylation sites following trauma. However, by 48 hrs PI, there was a stereotypical response involving a number of characteristic cytoskeletal alterations that bear similarities to *in vivo* neuronal responses associated with DAI that have been reported previously. For instance, NF immunoreactivity was significantly greater in stretched axons compared to surrounding unstretched axons. It is possible that this increased NF immunoreactivity reflects their compaction within axons, which has been noted previously following DAI (Jafari et al., 1997; Povlishock et al., 1997; Okonkwo et al., 1998). It is also possible that this increased SMI312 immunoreactivity reflects an alteration in phosphorylation state of some of the NFs present within the injured axons. In many cases, at the focal site of the deflection, the well-defined organization of NF-positive processes within axon bundles was lost at this time, with individual fibres

segregating from each other and becoming undulating and wavy. This is most likely a direct, if delayed, response to stretch injury since this "fraying" effect only occurred in regions that were directly subjected to stretching. By 72 hrs PI, the majority of injured axon bundles appeared to have suffered secondary axotomy, becoming completely severed at the site of initial stretch injury. Hence, these results clearly demonstrate a temporal progression of axonal changes in response to mild axonal stretch injury, from increased NF immunoreactivity to severe axonal pathology and delayed axotomy.

While the basic analyses discussed above would suggest that all axons undergo a similar stereotypical response to mild mechanical insult, this fails to take into account that the actual injury levels are most likely different for each axonal bundle injured. Since the same fluid pressure (20 psi) was applied for all injuries, it is likely that the injury level for each independently injured axonal bundle will be related to the length of the axons. To account for this, the actual degree of axonal deflection was calculated for each injured axonal bundle, and the same morphological parameters of axonal injury applied above were used for analysis. Based upon this analysis it was possible to differentiate between mildly and severely injured axons based upon morphological characteristics. For instance, mild strain resulted in increased NF immunoreactivity (which may reflect a partial post-traumatic alteration in phosphorylation state of some NFs) and the marked development of ring-like NF immunoreactive structures within axonal bundles that were rarely axotomised. Conversely, at greater strain levels increased NF immunoreactivity was less apparent (which may reflect total dephosphorylation of NFs), while axons often became distorted and disorganized within axonal bundles and eventually became completely disconnected. This suggests that axons do not respond in a stereotypical manner to a mechanical strain insult, and indeed that variable degrees of mechanical injury activate different responses within axons, with dramatically different outcomes. This is a possible explanation for the recent report that impaired axonal transport and altered axolemmal permeability occur in distinct populations of damaged axons following TBI (Stone et al., 2004), since it may reflect the fact that axons undergoing different degrees of strain in the TBI model respond differently. Furthermore, our study supports the notion presented by Stone and colleagues (2004) that multiple approaches are required to assess axonal response to injury, and indeed

the cytoskeletal characteristics that we have used in this study may be useful parameters for discriminating between mildly and severely injured axons following TBI.

Using 3-D imaging technology, the injured axon bundle was reconstructed from a series of individual images to provide a clear spatial representation of immunolabelled cytoskeletal features following injury. This new and novel methodology provided a significantly improved resolution of imaging, and highlighted the substantial disorganisation of axons following stretch injury. Intriguingly, NF ring-like structures were often completely separate from any nearby axons. It is unclear how these structures develop, or indeed how they become disconnected from axons. These abnormal features appear to be associated with injury and require further investigation, since they have observed previously in a range of conditions resulting in axonal injury including localised axonal transection *in vitro* (Dickson et al., 2000; Chuckowree and Vickers, 2003), physically induced axonal injury *in vivo* (King et al., 1997, 2001) and in the Alzheimer's disease brain (Dickson et al., 1999). Furthermore, the reported presence of abnormal bulb-like NF-immunoreactive swellings along injured axons has been illustrated previously, and is considered a hallmark of axonal injury (Povlishock and Christman, 1995; Maxwell et al., 1997).

There are several excellent studies that have been reported previously investigating different experimental models for stretching axons, which have determined the threshold for axotomy, which ranges from 33% in peripheral nerves (Gray and Ritchie, 1954), to 15–24% in a model of inertial injury to guinea pig optic nerves (Meaney et al., 1995), and to 65% in a cultured human neuron cell-line (Smith et al., 1999). For comparison, the strain rate (calculated as 1/initial axon length x Δaxon length/ time) applied by Smith et al. (1999) was in the range of 26–35 sec^{-1}, while we have applied strain rates of considerably lower magnitude, from 0.8 sec^{-1} (100–103% increase in original axon length) to 4.2 sec^{-1} (106%). It is rather surprising then, given the high tensile strains reported previously, that in the current study even very small levels of mechanical strain (1–3% increase in original axon length) cause dramatic changes in axonal structure. A possible explanation for this is that the studies in the literature have involved stretching of a relatively long length of the axon (greater than 100 μm), in either optic nerve stretch models (Maxwell, 1996; Maxwell and

Graham, 1997; Jafari et al., 1997) or *in vitro* uniaxial stretch of axons (Smith et al., 1999), while in this study the injuries were performed upon axons spanning the relatively short distance between neuronal clusters (typically less than 100 μm). Another possible explanation is that as a consequence of the relatively short length of axonal bundles injured, these injuries are performed in close proximity to the axon hillock, which may exacerbate axonal responses to trauma.

In summary, this thesis presents a simple, reproducible model of axonal stretch injury that results in several axonal alterations characteristic of DAI and, in particular, delayed axotomy. Furthermore, using this model this thesis has demonstrated that differing degrees of injury result in different axonal responses, supporting the idea that axons respond heterogeneously to trauma under certain situations. Hence, this model has a number of possible applications for further research into understanding neuronal responses to physical trauma, and in developing and testing therapeutic agents that specifically target some of the aberrant alterations underlying the pathological cascades contributing to DAI. In this regard, axolemma permeability alterations are proposed to play an important role in the post-injury alteration of the axon cytoskeleton, through the activation of cytoskeletal-degradative cascades. Thus, it is important to investigate whether the NF alterations observed in this chapter are linked to disruptions to axolemma permeability.

4 ACUTE CALCIUM INFLUX INITIATES DELAYED CYTOSKELETAL AND AXOLEMMA PERMEABILITY ALTERATIONS VIA ACTIVATION OF CALCINEURIN.

4.1 INTRODUCTION

As illustrated in the previous chapter, axonal stretch injury results in the progressive degradation of the axon cytoskeleton, ultimately leading to delayed secondary axotomy (Maxwell et al., 1997; Smith et al., 2003). However, the mechanisms underlying this evolving form of axonopathy, particularly in regard to cytoskeletal damage, remains undefined. One proposed mechanism is that axon shear stress may cause focal axolemmal permeability alterations, leading to increased intracellular calcium and the harmful activation of calcium-dependant cascades, involving calpain, caspase, and calcineurin, which might contribute to cytoskeletal damage (Povlishock, 1993; Povlishock and Pettus, 1996; Wolf et al., 2001). Indeed, evidence of calcium-induced mitochondrial damage, resulting in the release of cytochrome-C, and activation of calcium-modulated spectrin degradation following DAI (Buki et al., 1999, 2000) supported the pivotal role of calcium dysregulation in the post-injury sequelae. However, cytoskeletal damage in the absence of axolemma permeability disruption following *in vitro* axonal stretch injury suggested that another, more subtle, mechanism may underlie the progressive deterioration of the axon cytoskeleton (Smith et al., 1999). In this regard, the source of intracellular calcium dysregulation following axonal stretch injury remains to be investigated.

The perturbation of the cytoskeleton in relevant experimental models has been linked to impaired axonal transport and, consequently, the formation of axonal swellings (Povlishock and Pettus, 1996; Okonkwo et al., 1998; Saatman et al., 1998, 2000, 2003; Postmantur et al, 2000). The role of specific cytoskeletal changes in axon transport deficits, such as compaction, is unclear (Stone et al, 2001). However, there is increasing evidence of structural and functional damage to mitochondria following DAI (Lifshitz et al., 2003, 2004, 2006), which may contribute to the pathophysiology of TBI via either metabolic

dysfunction and/or the release of pro-apoptotic factors such as cytochrome-C (Sullivan et al., 1999, 2002). In this respect, inhibition of the formation of the mitochondrial permeability transition (MPT) pore with cyclosporin-A has been reported to prevent cytoskeletal alterations and axonal degeneration following *in vivo* impact acceleration brain injury (Büki et al, 1999; Okonkwo et al., 1999a&b; Suehiro and Povlishock, 2001). However, cyclosporin-A's therapeutic mode of neuroprotection remains to be clearly defined as it is also an inhibitor of calcineurin. Importantly, inhibition of calcineurin with FK506 has also been shown to attenuate cytoskeletal damage following axonal injury (Marmarou and Povlishock, 2006).

The present study utilizes the *in vitro* model of transient stretch injury characterised in the previous chapter to investigate the temporal relationship between delayed cytoskeletal damage, axolemma disruption, alterations to intracellular calcium concentration, mitochondrial disruption (cytochrome-C release) and secondary axotomy. Furthermore, this study will determine whether cyclosporin-A, a MPT pore and calcineurin inhibitor, attenuates cytoskeletal damage and secondary axotomy.

4.2 MATERIALS AND METHODS

4.2.1 Cortical cell culture

Neuron cultures were prepared as outlined in section 2.1 (Materials and Methods). Immunolabelling of cortical cultures with antibodies to glial fibrillary acidic protein, GFAP (DAKO), Ferritin (ICN Biomedicals Inc.), CD11b/c (a marker of microglia, White et al. 1998) and staining with Nuclear Yellow (Molecular Probes) were utilised to detect the presence of non-neuronal cells (Table 2.1).

4.2.2 Experimental axon stretch injury and immunocytochemical analysis

Axonal stretch injury was conducted according to the procedures presented in chapter 2.2 (Materials and Methods). As detailed in the previous chapter, stretch injured axon bundles were fixed at 24 hrs, 48 hrs, and 72 hrs PI and immunolabelled with antibodies targeting α-internexin, NFM, neuronal spectrin, ubiquitin, and cytochrome-C, according to the procedures in chapter 2.3 (Materials and Methods). Immunolabelled preparations were visualized under an upright fluorescing microscope (Leica DMLB2) with images taken using an Optronics Magnafire digital camera as outlined in chapter 2.4 (Materials and Methods). Additionally, a series of digital images were captured at 1.2μm z-axis intervals through the entire thickness of the injured axonal bundle using an Optiscan F900e krypton/argon confocal scanning system attached to an Olympus BX50 epifluorescence microscope. These images were then constructed into a single stack image using NIH ImageJ (version 1.37v) software. Thirty-six axon bundles were injured per time point, and to ensure reliability, three sets of injuries were conducted at each time point in different cultures (total of 324 injured axon bundles).

4.2.3 Axolemmal permeability

Axolemmal permeability following stretch injury was assessed using Alexa 488 hydrazide (Molecular Probes), a membrane impermeant fluorescent dye with a molecular weight of 570 Da. Intracellular accumulation of the dye demonstrates abnormal axolemma permeability to small molecules (Smith et al., 1999). Briefly, prior to injury, 200μl of 580μM Alexa 488 hydrazide dissolved in control saline solution (CSS; 120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 15 mM glucose, and 25mM HEPES, pH 7.4) was added to culture medium. The axons were stretched in the presence of the dye solution. Prior to analysis on a fluorescence microscope (Leica DMLB2), the dye solution was washed off using fresh CSS. High (numerical aperture 0.50) and low (numerical aperture 0.30) magnification images were collected using an Optronics Magnafire digital camera to determine the percentage of stretch injured axons with dye accumulation at each time point. As a positive control, uninjured neuron cultures were permeabilised with a single rinse of 0.005% Saponin (Sigma) in control saline solution and incubated for 5 minutes with the dye solution. As a negative control, the cultures were incubated in the dye solution without injury or Saponin, according to the post-injury observation time intervals. The observations quantative analysis was not blinded, as the evaluator knew which axons were injured. In this regard, the same fluorescent threshold for the control (uninjured) axon bundle was used to determine a positive out outcome inn injured axon bundles (on the same coverslip).

4.2.4 Calcium imaging

Calcium imaging was utilised to investigate whether there is disruption to intracellular calcium following axonal stretch injury. At 1 day prior to imaging, cultured monolayers, growing on glass coverslips were transferred to individual 35mm glass petri dishes. The Neurobasal™ media, in which the neurons were growing, was gradually replaced with artificial cerebrospinal fluid (ACSF, 137 mM NaCl, 5 mM KCl, 5.6 mM glucose, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 20 mM HEPES, 0.6 mM KH_2PO_4, 0.5 mM Na_2HPO_4, 1.4 mM $CaCl_2$, 0.9 mM $MgCl_2$ over a time course of 1 hr prior to imaging, to promote neuronal survival when imaging. Cells were then loaded with Fluo-4AM calcium indicator (1μM, Molecular Probes) for 7 min at 37 °C, washed with fresh ACSF and incubated at 37°C for a further 15-20 min prior to imaging.

Images (5-25 msec exposure) were captured every 3 secs, 5 min before (including at the moment of injury) and after axonal stretch injury, using a cooled CCD camera (ORCA, Hamamatsu) and fluorescence intensities were analysed using ImageJ (NIH) and custom software applications (Matlab, Mathworks). Calcium activity was measured as the average pixel intensity (F) in a specified region-of-interest (ROI) of the axon bundle divided by the baseline fluorescence intensity in the same ROI at the commencement of the observation period (Fo). Background fluorescence was subtracted from all frames using the average pixel value of an identically shaped ROI close to the axon bundle being observed. Graphs representing calcium changes were scaled according to the following formula: (F-Fo)/Fo. Spontaneous event frequencies were obtained using a modified Daubechies 4 discrete wavelet transformation and analysis algorithm (MatLab, Mathworks). Thapsigargin (1μM) was added to positive control cultures and results in the release of calcium from intracellular stores (Weber et al., 2001). Axonal stretch injury and calcium imaging was also conducted in calcium-free ACSF (ACSF without $CaCl_2$ and with 1mM EGTA added).

4.2.5 Determination of cytochrome-C release from axonal mitochondria following injury

MitoTracker™ green dye (Invitrogen) was added to cultures at predetermined time-points to label axonal mitochondria and determine whether cytochrome-C was localized to mitochondria or the cytosol following injury. Prior to injury and at 24 hrs, 48 hrs, 72 hrs PI, the medium from the cultures was removed and replaced with fresh pre-warmed Neurobasal™ medium containing 100nM MitoTracker™ green. The cells were incubated in this medium for 20 mins, the medium removed, and the cells were washed in pre-warmed medium without MitoTracker™ green. The cells were then fixed and immunolabelled for cytochrome-C. Cells retained the MitoTracker™ green following fixation and permeabilization.

4.2.6 Cyclosporin-A and FK506 treatment

Cyclosporin-A, a known inhibitor of calcineurin and the MPT pore (Büki et al., 1999), was added to cultures prior to injury to investigate the potential role of post-injury mitochondrial disruption relative to the release of cytochrome-C and cytoskeletal alterations. One hour prior to injury, neuronal cultures were treated with 20nM cyclosporin-A (Sigma) (Hansson et al., 2004; Mironov et al., 2005). This concentration of cyclosporin-A is within the range of the lowest dose concentration capable of producing a therapeutic effect (Brustovetsky and Dubinsky, 2000; Hansson et al., 2004; Mironov et al., 2005). Additionally, as cyclosporin-A was to remain in the culture for a period of 96 hrs, the lowest possible concentration was investigated.

Tacrolimus (FK506, 1μM), a potent inhibitor of calcineurin (Bavetta et al., 1999) was added to cultures prior to injury to determine whether calcineurin inhibition alone would result in an improved survival following axonal stretch injury. One hour prior to injury, neuronal cultures were treated with 0.1 μM cyclosporin-A (Sigma) (Lyons et al., 1994). FK506 and cyclosporin-A remained in the medium until fixation with 4% paraformaldehyde. Following fixation, the treated cells were incubated with antibodies to cytoskeletal proteins as described in chapter 2.3 (Materials and Methods). Kaplan-Meier survival analyses were conducted using the Graphpad Prism4® software.

4.3 RESULTS

The presence of non-neuronal cells was examined using immunocytochemical techniques. Small numbers of non-neuronal cells were present within the culture both prior to after stretch injury (Fig. 4.1) as previously reported (Dickson et al., 2000). Substantial glial cell proliferation is inhibited by the culture medium (Dickson et al., 2000; Chuckowree and Vickers, 2003). These cells were largely associated with the neuronal cell clusters and did not come in contact with the axon bundles. Stretch injury resulted in damage to the axon bundle independent to any disruption to the non-neuronal cells.

4.3.1 Delayed cytoskeletal damage and alteration to axolemma permeability.

Axolemma permeability following injury was examined using a low molecular weight tracer, (570Da) Alexa 488 hydrazide dye. With no stretch injury or chemical permeabilization, axons did not take up the Alexa 488 hydrazide dye (i.e. the axolemma remained impermeant to small molecules). Furthermore, there was no dye uptake at 5 min, 30 min, and 1 hr PI. However, chemical permeabilization of unstretched axons with Saponin resulted in a substantial uptake of the tracer (Fig. 4.2). In comparison, at 12 hrs PI only 9% of stretched axons exhibited any uptake of the fluorescent dye. At 24 hrs PI, 22% of stretch-injured axons demonstrated Alexa 488 dye uptake whereas, by 48 hrs PI, 65% of stretch-injured axons demonstrated dye uptake (Fig. 4.2).

The increase in axolemma permeability with increasing PI intervals was associated with disruption to the axonal cytoskeleton. Intra-axonal labelling for NFM, α-internexin and neuronal spectrin was relatively ordered and linear at 24 hrs PI (Fig. 4.3). However, 20% of stretch-injured axons demonstrated dense and compacted NF immunolabelling at this time point. By 48 hrs PI, there was increased disruption of the axonal cytoskeleton, particularly in relation to the loss of linear labelling for spectrin, NFM and α-internexin and the development of localized axonal swellings, the latter also focally labelled for ubiquitin (Fig. 4.3). 48 hrs PI was the peak time point for axon bundles (67%), to demonstrate NF compaction as also detailed in the previous chapter (chapter 3.3). Cytoskeletal alterations

Figure 4.1 Stretch injured axons double immunolabelled for non-neuronal cells. *A, C,* low levels of astrocytes (Red, GFAP) are present in the culture. These non-neuronal cells did not associate with the axonal bundle (Green, NFM) in uninjured cultures (*A*) and were not activated by stretch injury (*C*). *B*, Low levels of microglia (Red, Ferritin) were present in the culture at 24 hrs PI, but were not associated with the injured axon bundle (Green, NFM). *D,E, F*, Nuclear Yellow staining (*D,E*) of an axonal swelling 48 hrs PI, confirms no association of non-neuronal cells with the injured axon bundle (*F*, phase micrograph). Scale bars, 50μm

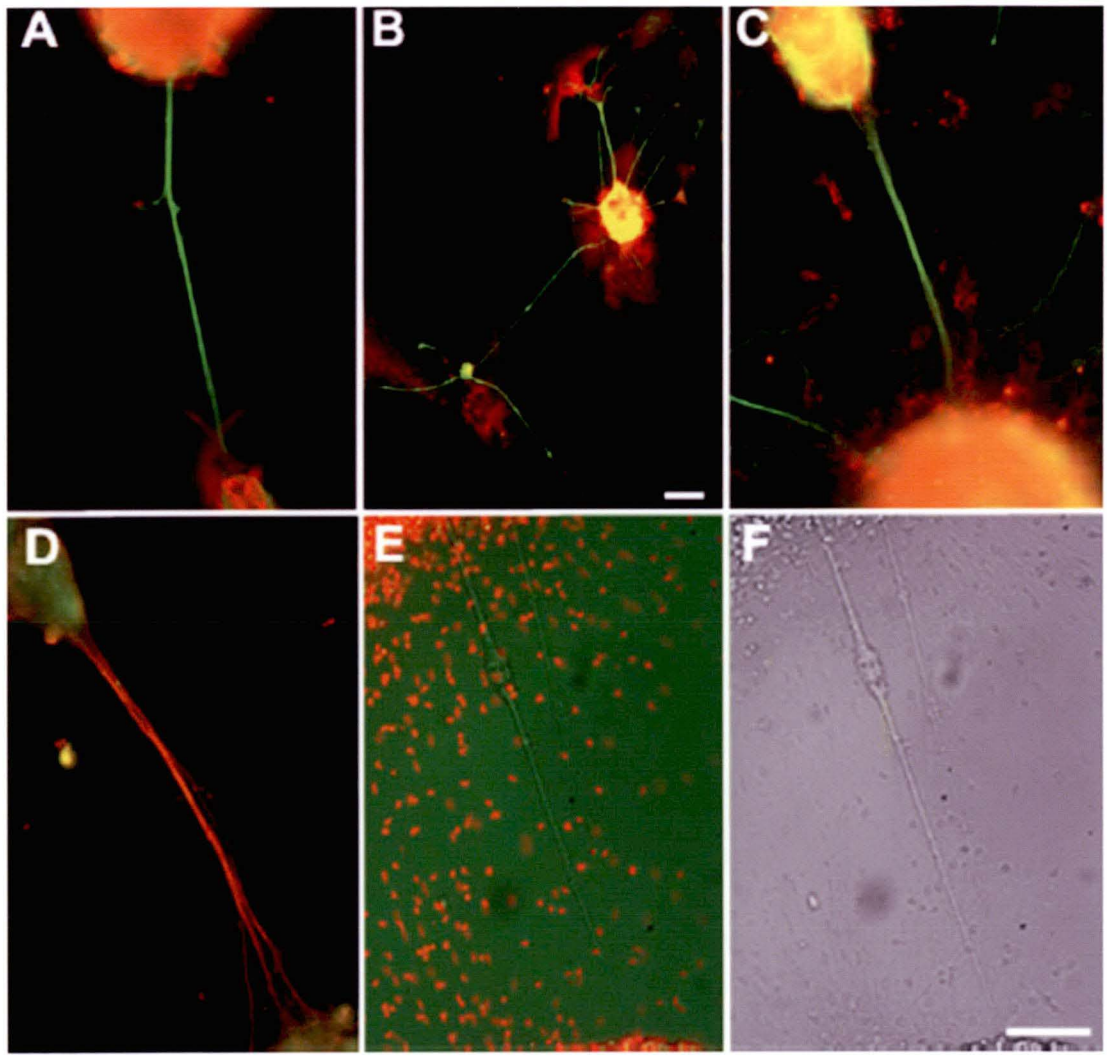
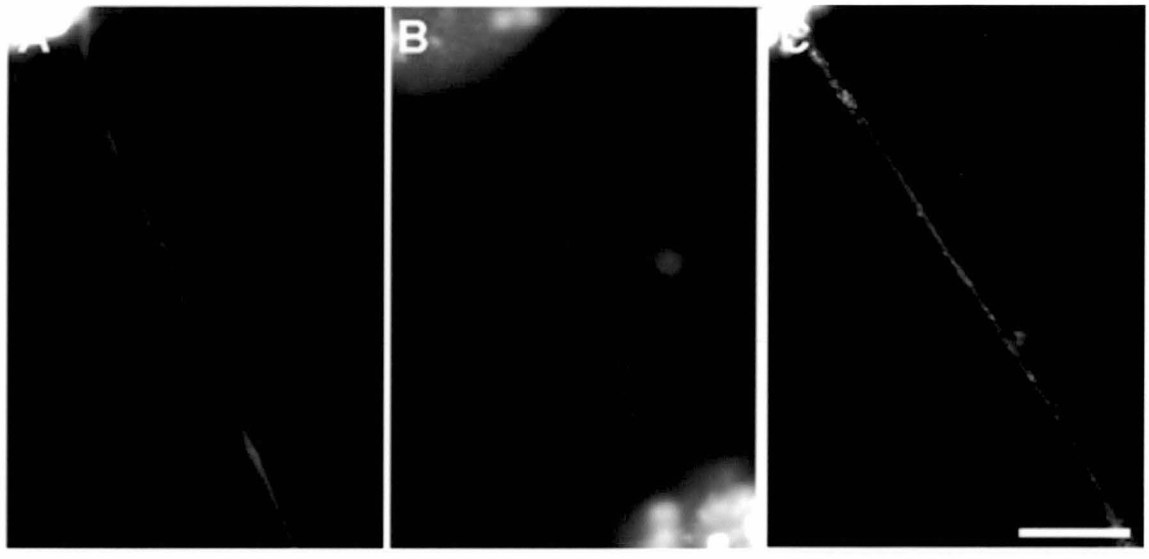

A
B
C
D
E
F

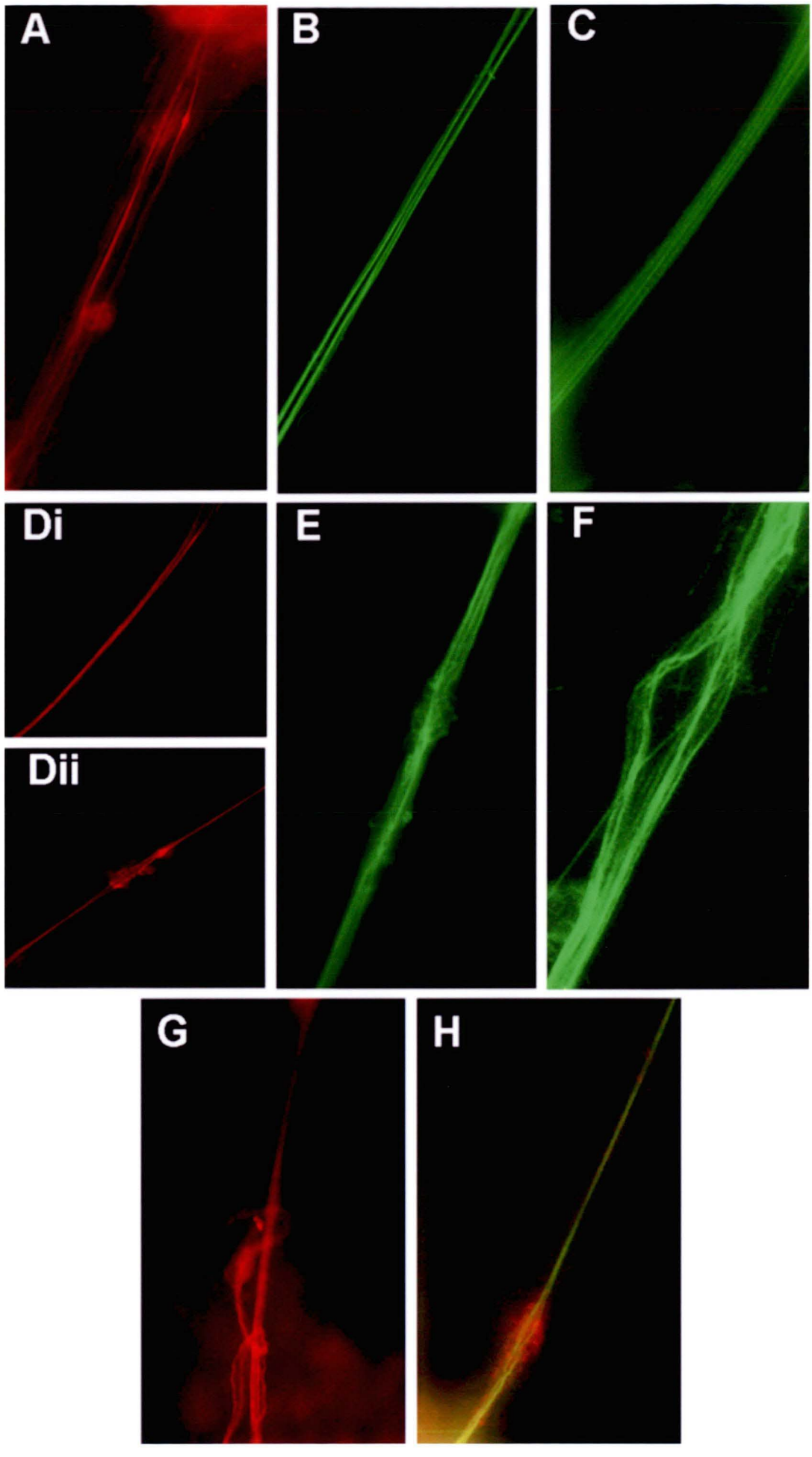
Figure 4.2 Axolemma permeability in stretch injured axons. *A*, Alexa 488 hydrazide dye was readily taken up in uninjured axons following chemical permeabilisation of the axolemma with saponin. *B*, A small but detectable amount of dye was taken up into stretch injured axons 24 hrs PI (this was compared to Alexa 488 labeling in control unstretched axons). *C,* At 48 hrs PI, dye was readily detected within stretch injured axons. Scale bar, 50μm.



Time post injury (PI)	5 min	30 min	1 hr	12 hrs	24 hrs	36 hrs	48 hrs
% permeability change	0 (0/18)	0 (0/14)	0 (0/12)	9 (3/33)	22 (6/27)	67 (18/27)	45 (15/33)

Table 4.1 Table shows the percentage of stretch injured axon bundles with permeability alterations over a period of 48 hrs PI.

Figure 4.3 Cytoskeletal alterations following axonal stretch injury to mature cortical neurons. No alteration to the cytoskeletal arrangement of spectrin *(A)*, NFM *(B)* and α-internexin *(C)* was observed at 24 hrs PI. At 48 hrs PI neurofilament compaction (*Di*) and the disruption of NFM resulting in a 'frayed' morphology (*Dii*) was observed. This abnormal cytoskeletal feature was also present within axons immuno-labeled for α-internexin at 48 hrs PI (*E*). Additionally, there was abnormal, non-linear spectrin immunolabelling at 48 hrs PI (*F*). At 72 hrs PI, there was an increase in axotomy resulting in the frank disruption to the entire axon bundle (*G*). Associated with these cytoskeletal alterations is the accumulation of ubiquitin within a highly localized region at 48 hrs PI, corresponding to an axonal swelling (*H*). Scale bar, 40μm.


A
B
C
Di
E
F
Dii
G
H

were illustrated using an Optiscan F900e krypton/argon confocal scanning system (Fig. 4.4). By 48 hrs PI, 18% of stretch-injured axons had progressed to frank secondary axotomy, increasing to 50% by 72 hrs PI (Fig 4.3).

4.3.2 Post-injury calcium alterations

Acute post axonal stretch injury alterations in free cytosolic calcium ($[Ca^{2+}]_I$) was measured using the fluorescent calcium dye fluo4-AM. Two peak increases in (F-Fo)/Fo were observed following axonal stretch injury. The first peak increase in (F-Fo)/Fo was the largest and occurred immediately after stretch injury (Fig 4.5A). This was followed by a second delayed peak increase in (F-Fo)/Fo, which was significantly less than the first peak increase (Fig 4.5A). All increases in (F-Fo)/Fo were restored back to basal (pre-injury) levels over time.

Calcium was removed from the extracellular ACSF to determine if these post axonal stretch injury increases in $[Ca^{2+}]_I$ were due to influx of calcium from extracellular stores. Stretch injury in calcium-free ACSF resulted in an acute peak increase in (F-Fo)/Fo, however, there was no significant secondary peak increase in (F-Fo)/Fo as observed in injuries conducted in ACSF with calcium (Fig 4.5B). Accordingly, there was no significant difference in the first peak increase in (F-Fo)/Fo between injuries conducted in calcium-containing and calcium-free ACSF. The thapsigargin-induced release of calcium from intracellular stores, conducted in ACSF that contained calcium, resulted in a two peak increase in (F-Fo)/Fo similar in profile to that observed following axonal stretch injury in ACSF that contains calcium (Fig 4.5C). There was no significant difference in peak increases in (F-Fo)/Fo between thapsigargin treated cultures and following axonal stretch injury in calcium-containing ACSF.

4.3.3 Mitochondrial disruption and cytochrome-C release.

Figure 4.4 1.2 μm optical slices, obtained using laser confocal microscopy, through stretch injured axon bundles labeled for NFM at 24-(*A*), 48-(*B*) and 72 hrs PI (*C*). Scale bar. 50μm.

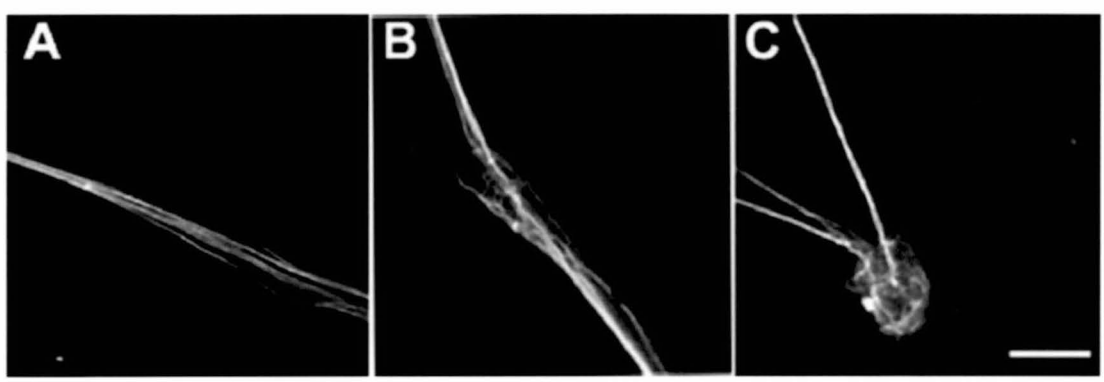
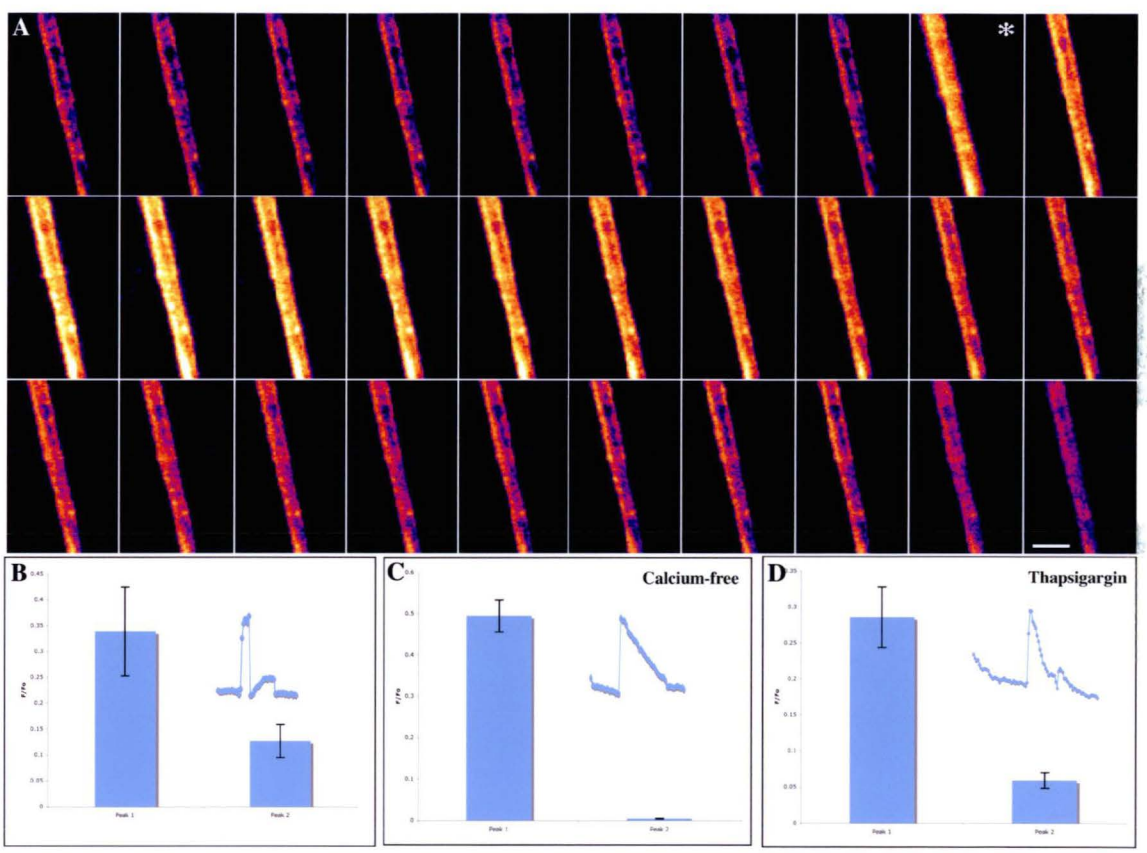

A
B
C

Figure 4.5 Montage of frames (3 sec/frame) showing calcium alterations within Fluo-4AM loaded axon bundle, following axonal stretch injury in calcium-containing artificial cerebrospinal fluid (ACSF) (* denotes time of injury). Images are in Pseudo-colour to emphasize alterations in fluorescence of Fluo-4AM. There is an acute post-injury increase in intracellular calcium levels (illustrated as an increase in fluorescence), prior to calcium returning back to basal levels. *A,B,C*; Bar graphs demonstrate post-injury calcium alterations following axonal stretch injury. A two-peak increase in intracellular levels is observed following axonal stretch-injury (*A*) and thapsigargin treatment (*C*) in calcium-containing ACSF. *B*; Only a single peak increase in intracellular levels is seen following stretch injury in calcium-free ACSF. Scale bar, 20μm.


A
*
B
C
Calcium-free
D
Thapsigargin

Focal accumulation of cytochrome-C occurred at 48 and 72 hrs PI, showing co-localisation to axonal segments demonstrating neurofilamentous compaction and axonal swelling (Fig. 4.6). There was a similar pattern of co-localization of cytochrome-C and MitoTracker™ green (a mitochondrial marker) labelling, in uninjured axons as well as stretch-injured axons at 24 hrs PI (Fig. 4.6C). Separation of MitoTracker™ Green fluorescence and cytochrome-C immunolabelling occurred within stretch injured axons by 48 and 72 hrs PI. This included depletion of cytochrome-C from MitoTracker™-stained mitochondria, the latter also accumulating in focal regions of axonal damage (Fig. 4.6D).

To determine whether disrupting the release of cytochrome-C through the MPT pore attenuates the cytoskeletal damage observed following stretch injury, cyclosporin-A was added to cultures prior to injury. At 24 hrs PI, 0 and 20% of stretch-injured axon bundles demonstrated NF compaction in cyclosporin A-treated and control preparations, respectively. Relatively reduced cytoskeletal and morphological change in cyclosporin A-treated stretched axons was also evident at 48 hrs PI (Fig. 4.7). At this time point, 20% of control preparations demonstrated secondary axotomy, whereas no cyclosporin A-treated cultures had progressed to frank degeneration. At 72 hrs PI, 50% of control preparations and 7% of cyclosporin A-treated bundles had progressed to secondary axotomy. Kaplan-Meier survival analyses demonstrated that there was a significant decrease in axotomy ($p<0.05$) in cyclosporin-A treated cultures in comparison to untreated cultures. In cyclosporin-A treated cultures, there was a reduction in the number of injured axons demonstrating axon swellings in comparison to untreated cultures. At 72 hrs PI, 57% of untreated cultures and 12% of treated injured axon bundles displayed swellings respectively. Furthermore, there was a reduction in the number of injured axon bundles demonstrating release and accumulation of cytochrome-C in treated cultures in comparison to untreated cultures.

4.3.4 FK506 treatment of stretch injured cultures

FK 506, a potent inhibitor of calcineurin (Bavetta et al., 1999), was administered to cultures prior to axonal stretch injury to determine whether the attenuation of secondary axotomy

Figure 4.6 Mitochondrial dysfunction and accumulation of cytochrome-C within an axon swelling. *A, -B*, Double immunofluorescence labelling for NFM (green) and cytochrome-C (red) of a stretch injured axon at 48 hrs and 72 hrs PI, respectively. The accumulation of cytochrome-C was highly localized to regions of maximal cytoskeletal damage. *C, D*, Mitochondria fluorescently labeled with MitoTracker™ green show co-localization with cytochrome-C (red) in a stretch injured axon at 24 hrs PI (*C*). At 48 hrs PI, there was accumulation of cytochrome-C within the centre of the axon swelling and loss of co-localization with MitoTracker™ green on the periphery of the axon swelling (*D*). Inset images demonstrate cytochrome-C immunolabelling (*i*) to sub-regions of the axonal swelling that are not co-localized to mitochondrial elements labeled with MitoTracker™ green *(ii)*. Scale bars, 40μm.

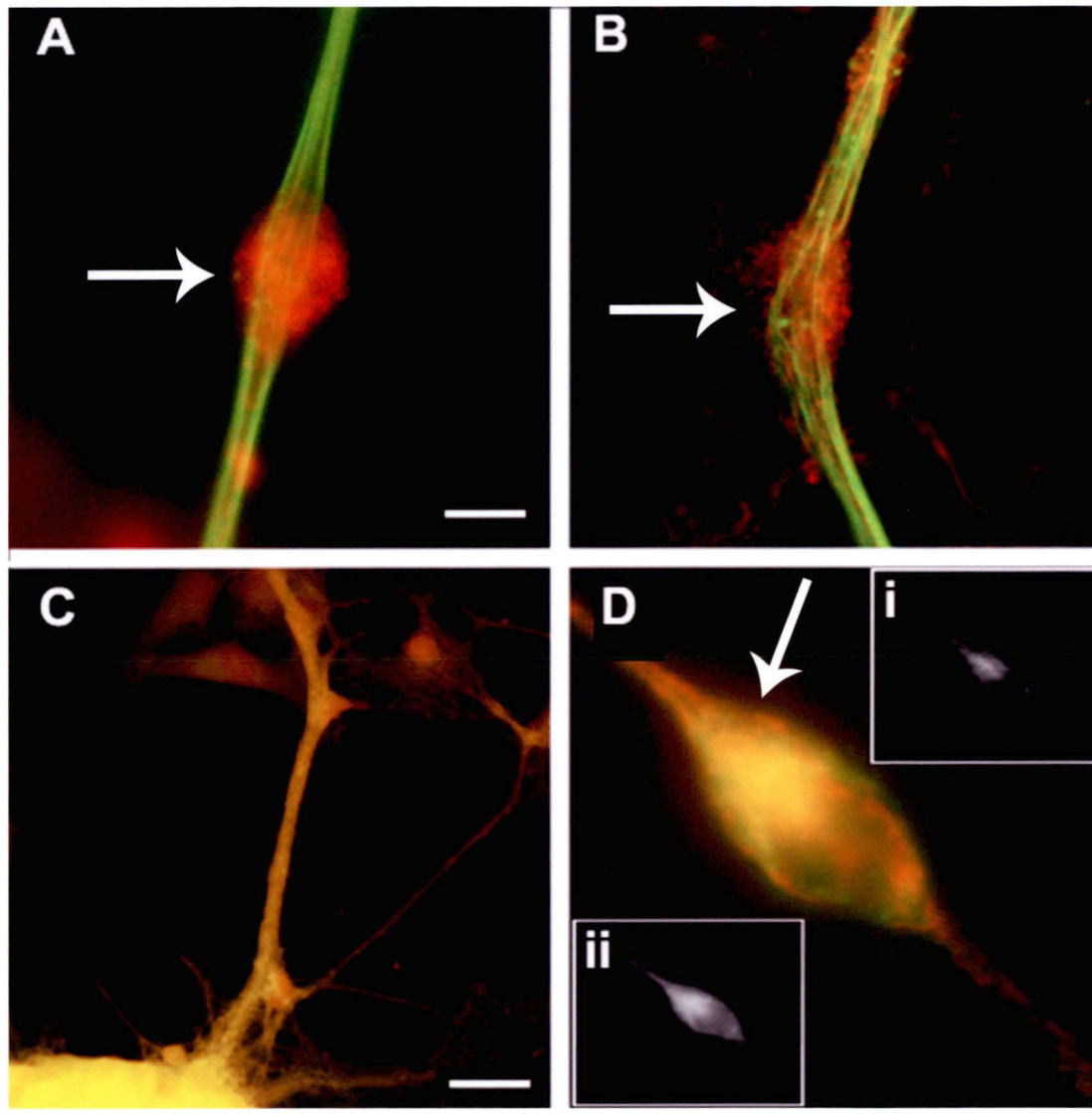
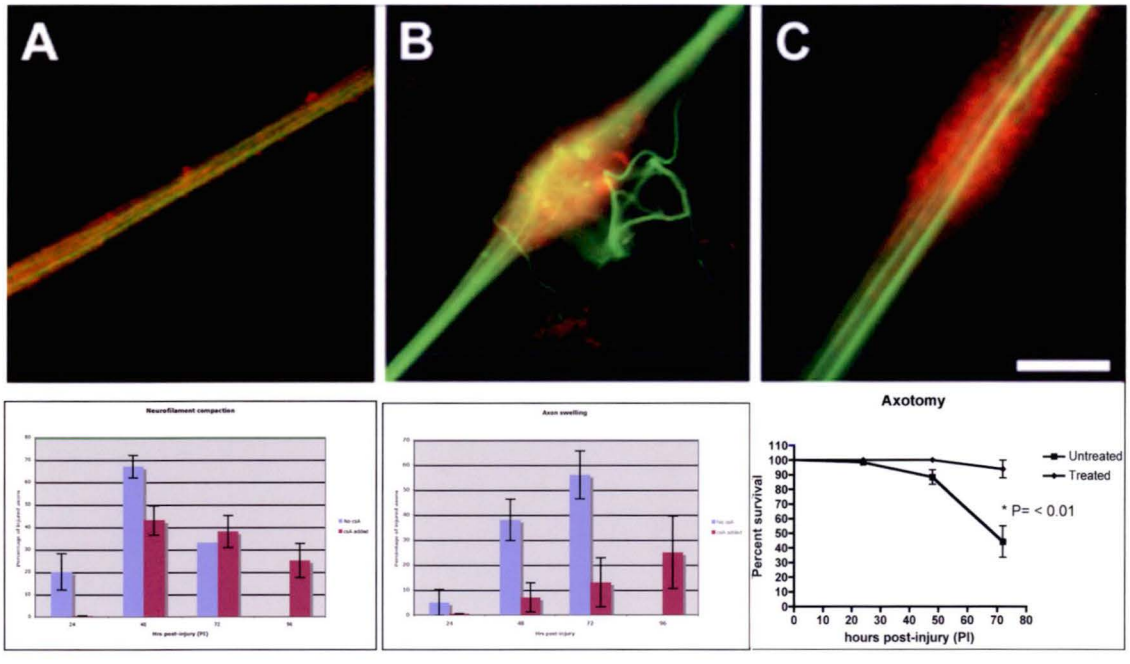
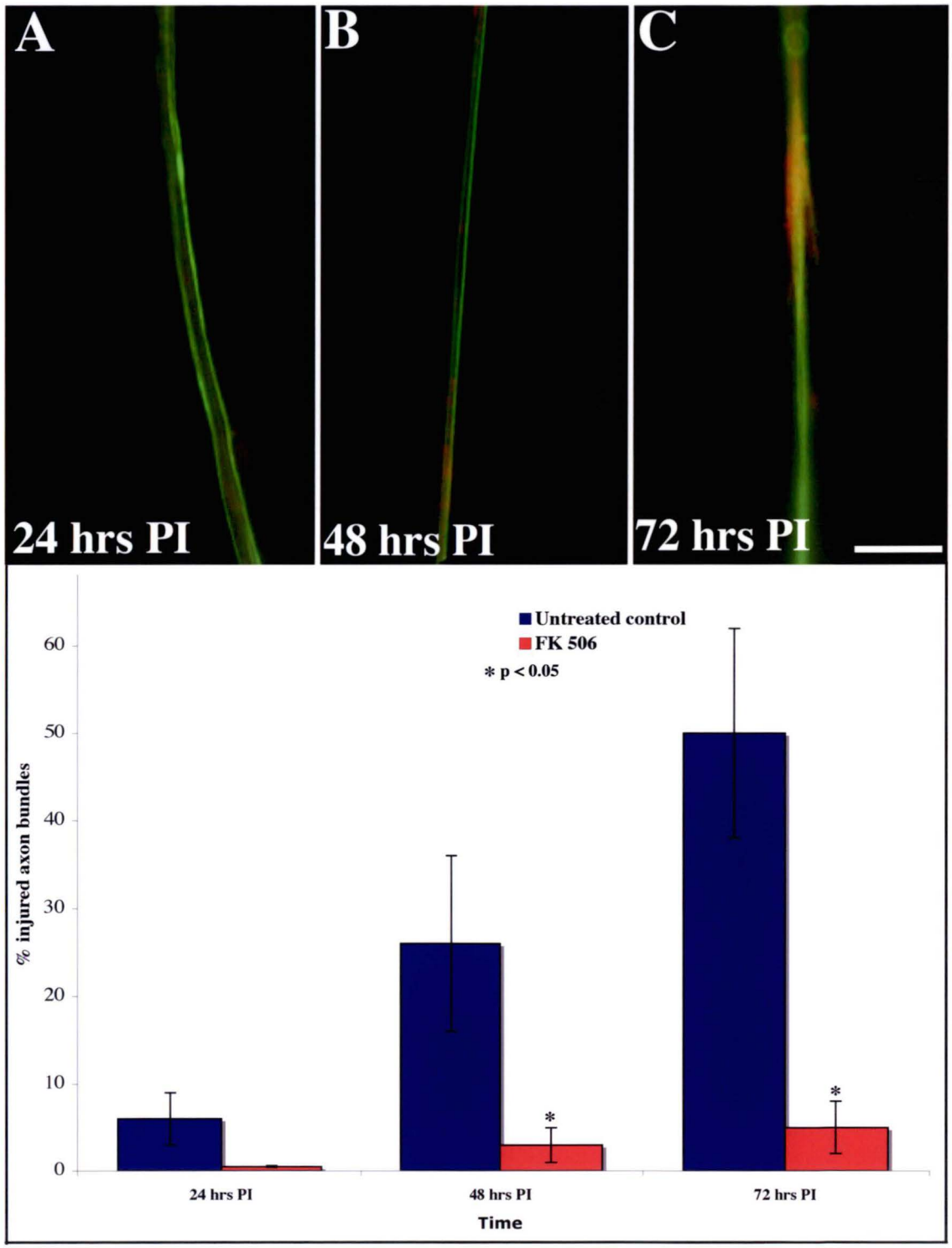

A
B
C
D
i
ii

Figure 4.7 The therapeutic effect of cyclosporin-A treatment on stretch injured axons. *A*, There is no alteration to the cytoskeletal organization of NFM (green) and to the immunoreactive profile of cytochrome-C (red) at 24 hrs PI in cyclosporin-A treated cultures. *B,* In untreated cultures at 48 hrs PI, NFM-labeled axons are disrupted and there is an accumulation of cytochrome-C highly localized within axon swellings. *C,* However, in cyclosporin-A treated cultures, accumulation of cytochrome-C spatially associated with minor cytoskeletal damage was present maximally at 96 hrs PI. The bar graphs compare the percentage of injured axon bundles with cytoskeletal alterations between cyclosporin-A treated and untreated cultures from 24 hrs to 96 hrs PI. Only cyclosporin-A treated cultures examined at 96 hrs PI as most untreated bundles had progressed to secondary axotomy. The line graph is a Kaplan-Meier survival curve, illustrating a reduction in axotomy between cyclosporin-A treated and untreated cultures. Scale bar, 50μm.


A
B
C
Neurofilament compaction
Axon swelling
Axotomy
Untreated
Treated
* P= < 0.01
Percent survival
hours post-injury (PI)

following cyclosporin-A treatment was due to inhibition of the MPT pore or calcineurin. At 24 hrs PI, no stretch injured axon bundles treated with FK506 had progressed to secondary axotomy. 3% of treated stretch injured axon bundles progressed to secondary axotomy, significantly less than untreated control injured bundles (Fig. 4.8). At 72 hrs PI only 5% of FK506 treated cultures had progressed to secondary axotomy which was significantly less that in untreated control cultures (50%) (Fig. 4.8).

Figure 4.8 Stretch injured axon bundles, pre-treated with the calcineurin inhibitor FK506. *A,B,C*; There is no alteration to the cytoskeletal organization of NFM (green) and to the immunoreactive profile of cytochrome-C (red) at 24 hrs PI (*A*) and 48 hrs PI (*B*). Only 5% of FK506 treated injured axon bundles had progressed to secondary axotomy (*C*). The bar graphs compare the percentage of injured axon bundles progressing to secondary axotomy between FK506 treated and untreated cultures from 24 hrs to 72 hrs PI. Significantly ($P<0.05$) more untreated stretch injured axon bundles progressed to secondary axotomy at 48 hrs and 72 hrs PI compared to FK506. Scale bar, 25μm.


A
B
C
24 hrs PI
48 hrs PI
72 hrs PI
Untreated control
FK 506
* p < 0.05
% injured axon bundles
60
50
40
30
20
10
0
*
*
24 hrs PI
48 hrs PI
72 hrs PI
Time

4.4 DISCUSSION

This study has further investigated the series of stereotypical cellular changes leading to delayed axotomy. Cellular changes included compaction of NFs, derangement of other cytoskeletal proteins, cytoplasmic accumulation of cytochrome-C and axon swelling. Axolemmal permeability alterations were also demonstrated, but developed subsequent to the injury. Acute alterations to intracellular calcium were also illustrated immediately following stretch injury. Mitochondrial disruption and inhibition of calcineurin may be a key pathological alteration leading to progressive axonal degeneration, as blocking of the MPT pore and/or calcineurin with cyclosporin-A and FK506 attenuated many of these cellular changes, as well as secondary axotomy.

Cytoskeletal changes in stretch-injured neurons in the current *in vitro* model included NF compaction, the loss of linear organization of filamentous proteins and accumulation in axonal swellings. NF compaction within axons has been described in *in vivo* models of brain injury (Gallyas et al., 2002; Marmarou et al., 2005). It is unclear whether the current data indicate that NF compaction following axonal stretch injury necessarily predates secondary axotomy. However, it may not be that all axons showing such post-injury cytoskeletal alterations will inevitably progress to secondary degeneration (Stone et al., 2001). Indeed, secondary degeneration is more complex than previously thought, and is possibly initiated via multiple pathways, as highlighted by gene expression analyses (Morrison et al., 2000).

Shear strain injury to axons has been proposed to lead to transient, focal disruption of the axonal membrane, concomitant with an overall increase in intra-axonal calcium (Tomei et al., 1990; Povlishock, 1993; Maxwell et al., 1995; Povlishock and Pettus, 1996; Wolf et al., 2001; Stone et al., 2004). Following axonal stretch injury to NT2 cells, Smith et al (1999) reported initial lack of membrane permeability to dyes. In further work with the same model (Wolf et al., 2001), it was demonstrated that stretch-induced strain on the axonal membrane lead to acute and abnormal Na^+ influx through mechanosensitive channels, triggering a reverse of the Na^+/Ca^{2+} exchanger, activation of voltage-gated Ca^{2+} channels

and subsequent pathological influx of Ca^{2+}. Geddes-Klein et al. (2006) demonstrated that biaxial rather than axial stretch of the axons of cultured cortical neurons was more effective at causing acute axolemmal permeability and Ca^{2+} influx. The current study involved transient axonal stretch in cultures maintained for longer periods of time and we have shown in this model that there were no axolemmal permeability alterations immediately, and up to 12 hrs, following injury. Rather, axolemma permeability alterations were delayed and closely associated with the disruption of the axon cytoskeleton following stretch injury. These results indicate that the relatively mild stretch injury utilized in this thesis is not sufficient to cause immediate perturbation of the axolemmal membrane. Interestingly, the current study shows two significant increases in $[Ca^{2+}]_I$ following axonal stretch injury, the first and largest increase occurring immediately after injury followed by a smaller delayed increase. The first and more remarkable increase in $[Ca^{2+}]_I$ appears to be due to release of calcium from intracellular stores, as removal of calcium from extracellular stores does not prevent this increase. Conversely, the second $[Ca^{2+}]_I$ increase is significantly lower when extracellular calcium is removed. The release of calcium from intracellular stores following injury has been illustrated in other *in vitro* models of axonal injury (Weber et al., 2001; Lusardi et al., 2004) and may also result in calcium mediated calcium entry via membrane receptors such as N-Methyl-D-aspartic acid (NMDA) and non-NMDA receptors and L- and N-type calcium channels (Weber et al., 2001; Mobley et al., 2003). This may provide an explanation for the secondary increase in $[Ca^{2+}]_I$, which is from extracellular stores, as the thapsigargin-induced release of calcium from only intracellular stores also results in a secondary increase in calcium. Other axonal stretch injury studies have demonstrated structural changes and calcium influx modulated by TTX-sensitive voltage-gated channels (Iwata et al., 2004). In this regard, increased $[Ca^{2+}]_I$ leads to proteolytic cleavage of voltage-gated sodium channels which may in turn promote persistent elevations of $[Ca^{2+}]_I$. Although persistent increased $[Ca^{2+}]_I$ levels were not demonstrated in this study, the significant increase in $[Ca^{2+}]_I$ might contribute to the secondary degeneration of the injured axon via the release of cytochrome-C and the activation of calcineurin and caspases.

In this thesis, mild axonal stretch injury also resulted in the delayed accumulation of cytochrome-C within highly localized regions of the injured axon, in association with regions of cytoskeletal disruption. Cytochrome-C release within traumatically injured axons has been proposed to have a role in stimulating apoptosis and neuronal degeneration (Büki et al., 2000). The delayed release and accumulation of cytochrome-C has also been reported in ischemic and TBI models (Fujimura et al., 1998; Pike et al., 1998), as well as following excitotoxic injury (Luetjens et al., 2000). This study demonstrates that cytochrome-C accumulation in the axon represents abnormal release into the cytosol rather than simply indicating the accumulation of mitochondria containing this protein. Release of cytochrome-C from mitochondria may have several detrimental effects, for example, disruption of normal mitochondrial respiration and/or activation of the caspase cascade resulting in apoptosis (Liu et al., 1996; Li et al., 1997). With respect to traumatic brain injury, Büki et al. (2000) have suggested that localized release of cytochrome-C in damaged axons results in the activation of caspases (eg caspase 3) that may act directly on cytoskeletal elements such as spectrin. However, calpain activity has also been reported to contribute to progressive intra-axonal cytoskeletal damage and secondary axotomy in an experimental optic nerve stretch injury model (Saatman et al., 2003). As previously stated, cytoskeletal damage and secondary apoptosis may be initiated via multiple pathways, however, it is clear that mitochondrial damage is likely to play an important role (Morrison et al., 2000). Mitochondrial damage may result in disruption to mitochondrial respiration (Vink et al., 1990), release of reactive oxidative species (Lifshitz et al, 2003, 2004) and disruption to calcium homeostasis (Xiong et al., 1997). Cyclosporin-A has been found to attenuate mitochondrial dysfunction in a cortical impact model of TBI (Sullivan et al., 1999). Additionally, cyclosporin-A pre-treatment before impact-acceleration brain injury of rats reduced subsequent immunohistochemically identifiable axonal damage (Okonkwo et al., 1999). Likewise, the current study shows that a single pre-treatment of neuronal cultures with cyclosporin-A reduced the progression of axonal injury into neurite swellings and secondary axotomy. This investigation with neurons isolated in culture demonstrates that the neuroprotective effect is likely to be axon-specific, potentially reducing damage to mitochondrial membrane integrity and the release of cytochrome-C into the cytosol. Interestingly, cyclosporin-A did not have an effect on the development of specific

cytoskeletal changes such as NF compaction. Although previous *in vivo* experiments report attenuation of cytoskeletal damage following constant injections with cyclosporin-A (Riess et al., 2001), it is difficult to translate the therapeutic time window and dosage into *in vitro* models, particularly as constant addition of cyclosporin-A or changes in media may have a further toxic effect on the neurons. Hence, a single pre-treatment of cyclosporin-A was given to attenuate acute, and possibly chronic, axonal disruption.

Another immunophilin ligand, FK506, was found to attenuate secondary axotomy, similarly to cyclosporin-A. In other studies FK506 was reported to reduce axonal swelling but not neurofilamentous compaction following impact-acceleration injury in rats (Marmarou and Povlishock, 2006). These data indicate that NF compaction, which likely follows cleavage of filament side-arms, occurs through the activation of intracellular processes unrelated to the site of action of the immunophilin ligands, and also strongly supports the proposal that NF compaction may not be a critical pathological change leading to axonal swelling, axon transport deficits and degeneration (eg Stone et al., 2001; Marmarou et al., 2005). FK506 itself does not appear to inhibit the MPT pore (Friberg et al., 1998), indicating the intriguing possibility that both FK506 and cyclosporin-A may be acting through inhibition of calcineurin activity to attenuate axonal pathology following injury. Indeed, calcineurin inhibition with either cyclosporin-A or FK506 attenuates axonal degeneration following calcium-induced hypoxic damage to white matter in rats as addition of rapamycin, which binds to the same immunophilin as FK506, reverses this neuroprotective effect (Mobley et al., 2003). Calcineurin inhibition is also shown to protect mitochondria from losing their membranous structure following injury, therefore preventing the detrimental release of intracellular calcium into the cytosol (Mobley et al., 2003). As this study has already shown the significant release of calcium form intracellular stores following stretch injury, this may provide a possible mechanism of protection. An interesting extension to this study would be to determine the effect of FK506 treatment on the calcium transients observed following stretch injury. This extension would provide valuable insight into whether the acute calcium transients triggered at the moment of injury are linked to the delayed progression to secondary axotomy. Furthermore, the therapeutic potential of FK506 after injury (rather than pre-exposure) can be determined.

In summary, although limitations of a cell culture model are an important caveat for direct translation to human TBI, the interpretation of these results in conjunction with previous *in vivo* and *in vitro* studies, provides evidence that calcineurin inhibition and the targeting of specific post-injury cellular changes that develop within axons may be a useful approach for reducing DAI and secondary axotomy following brain trauma. This chapter demonstrates the post-injury activation of cytoskeletal degradative pathways, however, it does not investigate the activation of other pro-apoptotic pathways, such as the UPS, which have also been demonstrated to be initiated following TBI (Schweitzer et al., 1993).

5 INHIBITION OF THE UBIQUITIN PROTEASOME SYSTEM FOLLOWING AXONAL INJURY ACCELERATES PROGRESSION TO SECONDARY AXOTOMY.

5.1 INTRODUCTION

The accumulation of ubiquitin conjugates and inclusion bodies containing ubiquitin is associated with the lesions of several chronic neurodegenerative diseases including the neurofibrillary tangles of Alzheimer's disease and the Lewy bodies in Parkinson's disease (Alves-Rodrigues, 1998; Glickman and Ciechanover, 2002. Indeed, a recent study of human TBI cases has reported the rapid axonal accumulation of proteins implicated in neurodegenerative diseases, including Alzheimer's disease and synucleinopathies (Uryu et al., 2007). In regard to TBI, although previous studies have provided significant evidence linking cytoskeletal alterations to disrupted axonal transport (Maxwell et al, 1997; Graham et al., 2000; Povlishock and Katz, 2005), the specific sequence of events leading to transport interruption, protein accumulation and degeneration have not been fully established.

The molecular mechanisms underlying delayed axonal degeneration are not fully understood, but a crucial role has been ascribed to the activity of the UPS (Korhonen and Lindholm, 2004). Altered UPS function, characterized by ubiquitin inclusions, has been reported in *in vivo* models of TBI (Schweitzer et al., 1993; Li and Farooque, 1996) and neurodegenerative diseases (e.g. Ciechanover and Brundin, 2003; Mackenzie and Feldman, 2005; Forman et al., 2006; Josephs et al., 2007). In both animal models and human TBI cases, ubiquitin-immunolabelled inclusions have been identified in axon swellings (Schweitzer et al., 1993; Ahlgren et al., 1996). As ubiquitin inclusions were found in both axon swellings and the terminal bulb of severed axons following injury, it has been proposed that ubiquitin accumulation contributes to the eventual axotomy and neuronal degeneration (Schweitzer et al., 1993). Accordingly, animal models using transection of

cultured neurons or crush lesions of the optic nerve *in vivo* reported UPS involvement only at the early stages of Wallerian degeneration, and this could be delayed following pharmacological inhibition of the UPS (Zhai et al., 2003). These results provide important information on the role of the UPS in the rapid degeneration of axons within a few hours of injury and in Wallerian degeneration following primary axotomy. However, there is little known about the role of the UPS in the delayed (secondary) axotomy of axons exposed to mild to moderate mechanical trauma, such as the transient stretch/shear injury that leads to diffuse axonal injury following closed brain trauma.

In this study, the role of the UPS in the *in vitro* model of axonal stretch injury was examined, including both immunocytochemical analysis of ubiquitin accumulation and pharmacological inhibition of the UPS.

5.2 MATERIALS AND METHODS

5.2.1 Cortical neuron culture and ubiquitin proteasome inhibition

Cortical neuron cultures were prepared as outlined in chapter 2.1 (Materials and Methods). Cortical cell cultures were pre-treated with either 5 μM MG132 (Zhai et al., 2003), 0.5μM Lactacystin, or vehicle control 30 mins prior to injury. Previous studies suggested pre-treatment of neuronal cultures with MG132, a peptide-based reversible proteasome inhibitor, in neuronal cultures delayed degeneration with UPS involvement only at the early stages of degeneration (Zhai et al., 2003). Lactacystin, a selective inhibitor of the 20 S proteasome, has also been found to be neuroprotective in injured neuronal cultures (Yew et al., 2005; Meller et al., 2008). Additionally, the MG132 and Lactacystin concentration used in this study was shown not to be detrimental to neuronal health in culture over an extended period of time (Dent and Kalil, 2001; MacInnis and Campenot, 2005).

5.2.2 Experimental stretch injury and immunocytochemistry

Axonal stretch injury was conducted according to the procedures presented in chapter 2.2 (Materials and Methods). As detailed in the previous chapter, stretch injured axon bundles were fixed at 24 hrs, 48 hrs, and 72 hrs PI and immunolabelled with antibodies targeting ubiquitin, and phosphorylated neurofilament (SMI312). The cells were then washed and incubated with the species appropriate goat anti-mouse Alexa Fluor 488 or goat anti-rabbit Alexa Fluor 594 secondary antibody (Molecular Probes, Eugene, OR), according to the procedures in chapter 2.3 (Materials and Methods). Immunolabelled preparations were visualized under an upright fluorescing microscope (Leica DMLB2) with images taken using an Optronics Magnafire digital camera as outlined in chapter 2.4 (Materials and Methods). Secondary axotomy was defined as complete breakage of the axonal bundle, and these were counted and presented as a percentage of the total number of injured axon bundles. Three axonal bundles were stretch-injured per coverslip with two uninjured axon bundles on the same coverslip used as controls. For quantative analysis, all injured axonal bundles were assessed under 40X magnification. . Forty-two axon bundles were injured per

time-point, and to ensure reliability, three sets of injuries were conducted at each time-point in different cultures. Percentages were calculated as the total number of injured axon bundles per time-point across the three sets of different culture, and means are reported ± standard error of the mean (SEM). Two way ANOVA statistical analyses were performed using Graphpad Prism4® software, with statistical significance identified as having $p < 0.05$.

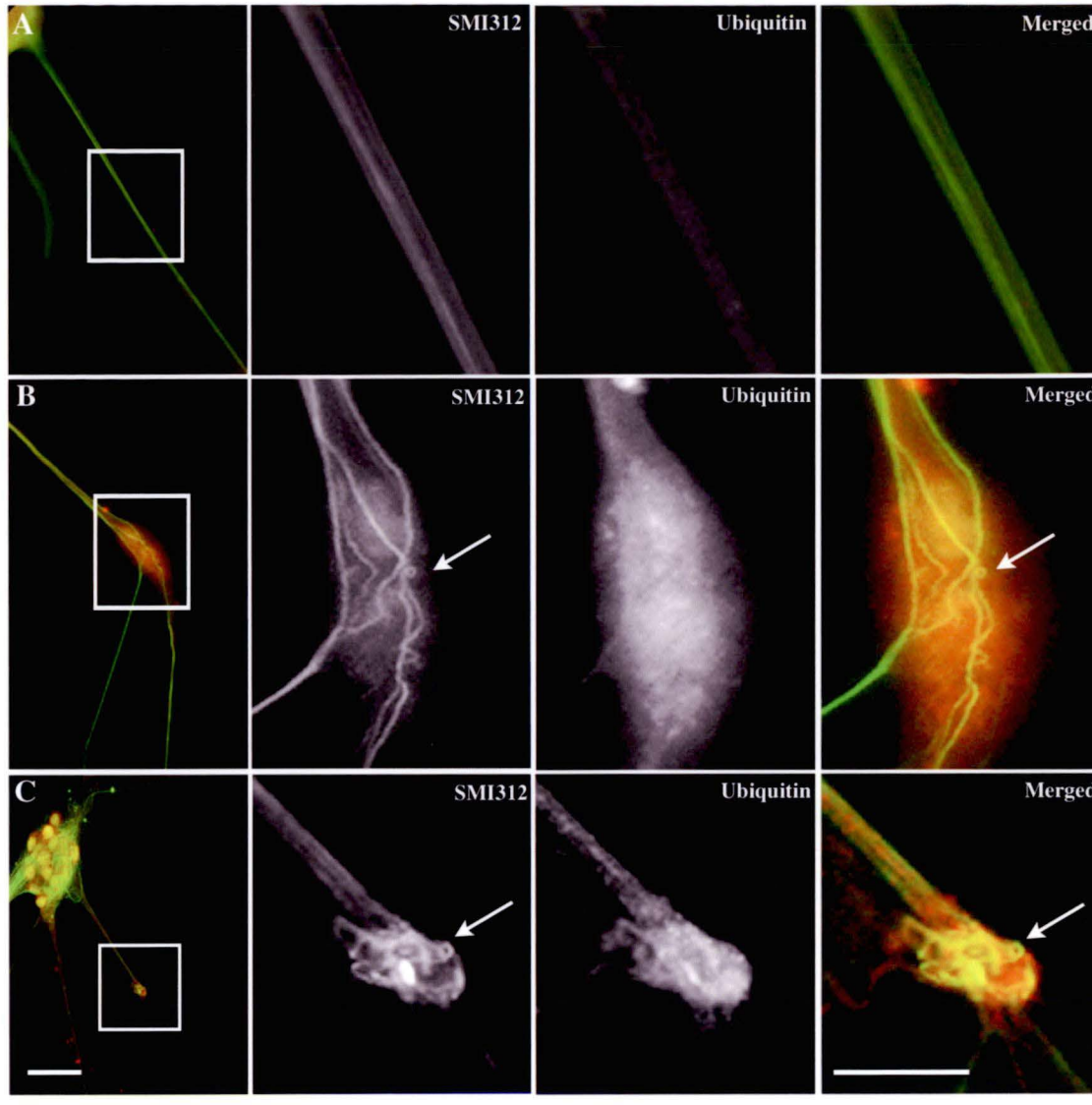
5.3 RESULTS

To examine the role of the UPS in mild to moderate axonal injury, stretch injured axons were immunolabelled with antibodies against ubiquitin at 24, 48 and 72 hrs PI in addition to neurofilament to confirm the cytoskeletal changes described in the previous chapters. At 24 hrs PI, ubiquitin immunolabelling within the injured axon remained diffuse and evenly distributed, with no significant foci of accumulation. At 48 hrs PI, an accumulation of ubiquitin occurred and was localized to swollen regions of the axon bundle. Neurofilament alterations within these axonal swellings included ring-like structures, 'tangles' and loss of ordered arrangement as described in the previous chapter. Axonal injury resulted in injured axons progressing to secondary axotomy at 72 hrs PI as also demonstrated previously (Fig. 3.1B). Ubiquitin immunoreactivity at this time point was increased and concentrated at the terminal region of injured axons following secondary axotomy (Fig. 5.1C).

5.3.1 MG132 and Lactacystin administration

To determine whether pharmacological inhibition of the UPS could perturb delayed axotomy due to stretch injury, neuronal cultures were pre-treated with MG132 and lactacystin. Initial experiments indicated no significant alterations in cell survival and morphology over 72 hrs in uninjured cultures following addition of the proteasomal inhibitors (Fig. 5.2A,B). Ubiquitin immunoreactivity was minimal in MG132- and lactacystin-treated cultures. There was no statistically significant increase ($p=<0.05$) in axotomy between MG132/lactacystin-treated and untreated injured cultures at 24 hrs PI (Fig. 5.3). However, at 48 hrs PI, there was a significant increase ($p=<0.05$) in the number of axon bundles progressing to axotomy following MG132 (59.3%+/- 12%) and lactacystin (43.6% +/- 4.5%) treatment as compared to 26.4% (+/- 8%) of untreated cultures (Fig. 5.3B). Additionally, there was decreased ubiquitin immunoreactivity within injured axon swellings at 48 hrs PI as compared to untreated culture preparations. In treated cultures axonal swellings were primarily associated with increased cytoskeletal damage (Fig. 5.2D). By 72 hrs PI, axonal stretch injury had induced axotomy in 94.7% (+/- 4%) of MG132 and

Figure 5.1 Alterations to neurofilament and ubiquitin immunolabelling following axonal stretch injury. *A*, Phosphorylated neurofilament (SMI312) and ubiquitin alterations were minimal in untreated cultures at 24 hrs PI. *B*, At 48 hrs PI, neurofilaments became disrupted, displaying a highly dystrophic morphology within axon swellings of untreated cultures. Ubiquitin accumulation within axon swellings was evident in untreated cultures at 48 hrs PI, localized to regions of neurofilament damage and neurofilament ring-like structures (arrows). *C*, At 72 hrs PI, untreated stretch injured axons had progressed to secondary axotomy with increased neurofilament ring-like structures around the stretch-injured axon bundles. Ubiquitin accumulation was localized within the terminal bulb of the severed axon (arrows). *D*, Graph showing the percentage of untreated injured axon bundles displaying cytoskeletal damage over 72 hrs PI. Error bars are representative of standard error margins. Scale bars, 50μm.


A
SMI312
Ubiquitin
Merged
B
SMI312
Ubiquitin
Merged
C
SMI312
Ubiquitin
Merged

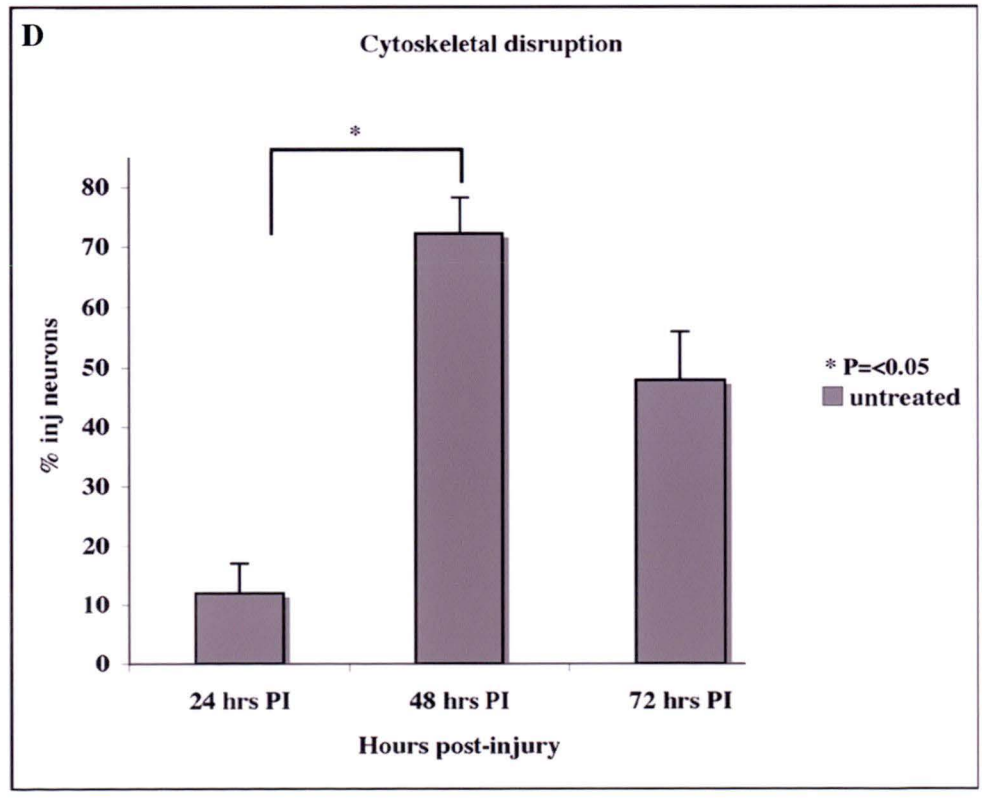

D
Cytoskeletal disruption
*
80
70
60
50
40
30
20
10
0
% inj neurons
24 hrs PI
48 hrs PI
72 hrs PI
Hours post-injury
* P=<0.05
untreated

Figure 5.2 Protease inhibition in mature neurons. Uninjured neuronal cultures treated with 5μM MG132 (*A*) and 0.5μM lactacystin (*B*) did not exhibit any major alterations to cytoskeletal organization up to 72 hrs PI. Increased neurofilament damage and axotomy was evident within lactacystin (*C*) and MG132 (*D*) treated injured axons (arrows indicate sites of axotomy). Scale bars, 50μm.

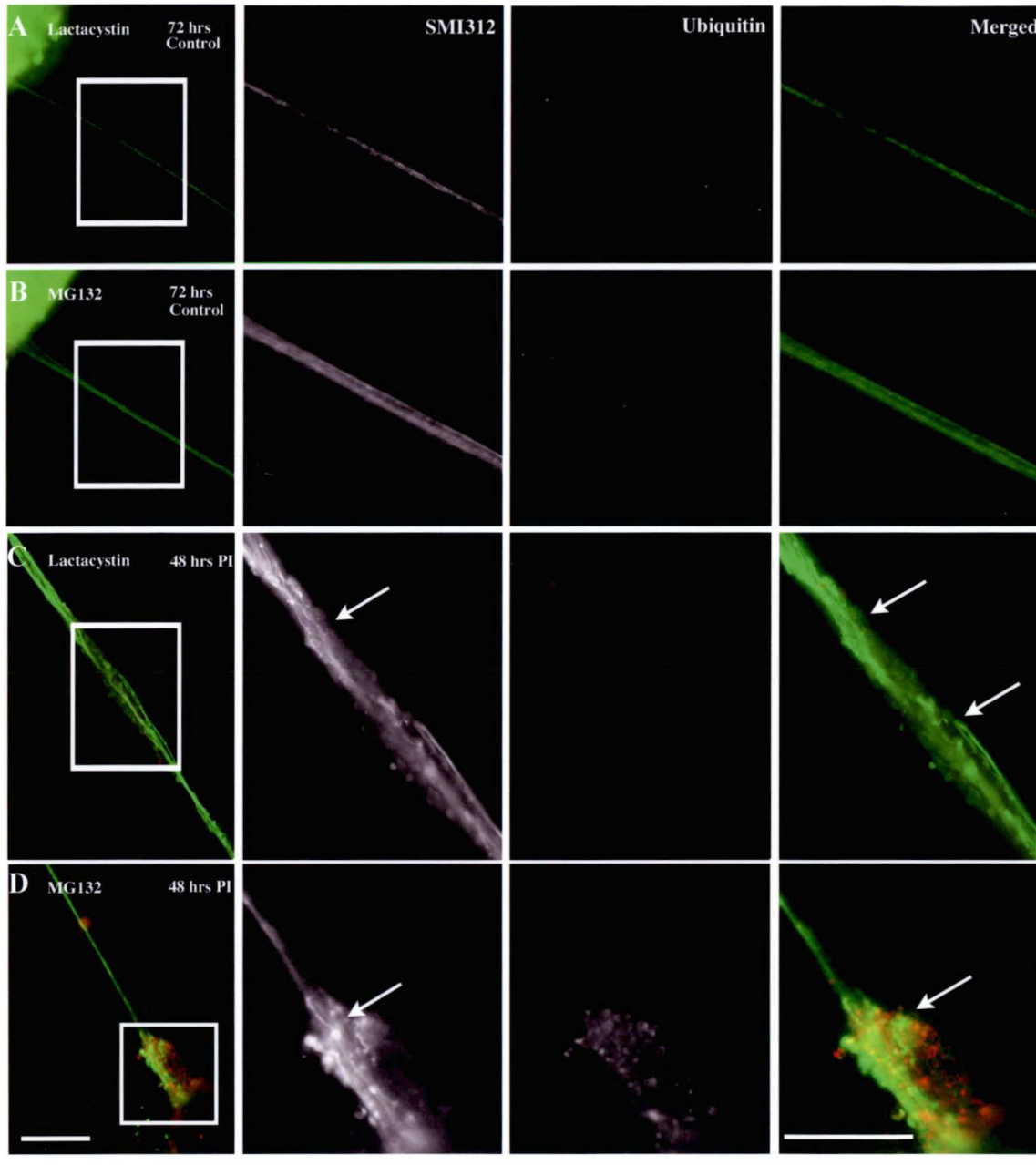
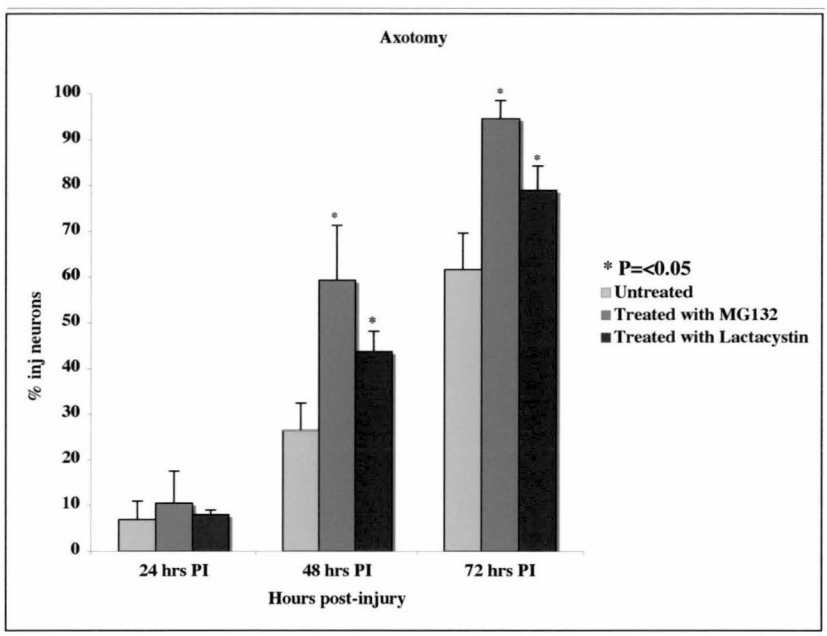

A
Lactacystin
72 hrs
Control
SMI312
Ubiquitin
Merged
B
MG132
72 hrs
Control
C
Lactacystin
48 hrs PI
D
MG132
48 hrs PI

Figure 5.3 Graph showing the percentage of MG132, lactacystin treated and untreated injured axon bundles progressing to secondary axotomy over 72 hrs PI. Error bars are representative of standard error margins.


Axotomy
100
90
80
70
60
50
40
30
20
10
0
% inj neurons
24 hrs PI
48 hrs PI
72 hrs PI
Hours post-injury
* P=<0.05
Untreated
Treated with MG132
Treated with Lactacystin

79% (+/- 5.3%) of Lactacystin treated cultures, which was significantly ($p<0.05$) higher than in untreated cultures (Fig. 5.3).

5.4 DISCUSSION

Reactive axonal alterations resulting in the rapid accumulation of proteins within axon swellings play a pivotal role in the evolution of axonal changes, such as DAI, following TBI (Buki and Povlishock, 2006). In the present study, we have demonstrated that mild to moderate axonal stretch injury induced the delayed accumulation of ubiquitin within focally swollen axon bundles. This delayed accumulation of ubiquitin within swellings, evident from only 48 hrs PI onwards, was also highly localized to regions of maximal cytoskeletal damage. Ubiquitin accumulation within axon swellings is a common feature of human brain and spinal cord trauma (e.g. Martin et al., 1990; Gultekin and Smith, 1994; Sherriff et al, 1994). Early animal TBI models reported the rapid accumulation of ubiquitin within axon swellings immediately prior to secondary axotomy (Schweitzer et al., 1993). However, these animal models involved more severe injuries that resulted in rapid secondary axotomy, unlike the model used in this study where secondary axotomy is maximal at 72 hours PI. Further studies of human and animal spinal cord trauma also reported accumulation of ubiquitin within axon swellings (Li and Farooque, 1996; Ahlgren et al., 1996). Initially, this ubiquitin accumulation was proposed to be involved in the degradation of proteins accumulating in axonal swellings (Li and Farooque, 1996). Over recent years, the accumulation of ubiquitin within axonal swellings was considered to be indicative of UPS dysfunction, reflected in an inability to remove ubiquitinated proteins from axon swellings subsequently contributing to the neuronal degeneration (Ehlers, 2004; Korhonen and Lindholm, 2004). Evidence of axonal swellings containing ubiquitinated material in neurodegenerative diseases supports this hypothesis (Guo et al., 2000; Lye and Shores, 2000; Plassman et al., 2000). However, it is not clear whether the PI accumulation of ubiquitin within swellings directly contributes to susceptibility to secondary axotomy.

To determine the involvement of ubiquitin in relation to secondary axotomy, we pharmacologically inhibited the UPS using MG132 and Lactacystin. This resulted in a significant increase in the number of injured axon bundles progressing to secondary axotomy when compared to untreated cultures. Coupled with the focal accumulation of ubiquitin immunoreactivity by 48 hrs PI, this data indicates that the UPS has a potentially protective role with respect to damaged axon bundles progressing to secondary axotomy.

Proteasome inhibition by Lactacystin in uninjured primary neuronal cells was found to induce both potentially neuroprotective and pro-apoptotic transcriptional responses (Yew et al., 2005). In the current study, the application of both proteasomal inhibitors did not result in alterations to morphology or apoptosis in uninjured cultures over 72 hrs PI. Interestingly, using transection models of the rat optic nerve and of cultured rat neurons, MG132 was found to delay the onset of Wallerian degeneration, suggesting local degeneration of the severed distal axon segment via the UPS (Zhai et al., 2003; MacInnis and Campenot, 2005). In contrast, the current model of transient axonal stretch injury is associated with gradual cellular changes and cytoskeletal re-arrangements within intact axon bundles (refer to chapter 3) that interact to determine the susceptibility of the axonal bundle for secondary axotomy. The dynamic role of the UPS in protecting axon bundles from secondary axotomy within the current model is not fully known and it is possible that ubiquitin accumulation in stretch-injured axon bundles may be caused by increased local ubiquitin activity, reduced ubiquitin C-terminal hydrolase activity (Bizzi et al., 1991), accumulation in lysosomes and/or disruption of axonal transport (Li and Farooque, 1996). It is likely that the UPS changes in axon bundles post-injury are critically associated with alterations to, and degradation of, the cytoskeleton in axon swellings (chapter 4; Uryu et al., 2007). In this regard, inhibition of UPS activity in injured axon bundles accelerated secondary axotomy and also increased cytoskeletal abnormalities within axon swelling. This suggests the possible neuroprotective role of the UPS in degrading abnormal cytoskeletal elements and accumulated proteins results in delayed secondary axotomy. It is important to note that other cell death mechanisms are also activated following mild to moderate axonal injury, which may ultimately initiate secondary axotomy (Iwata et al., 2004; Pfister et al., 2004; Serbest et al., 2007).

In summary, UPS activity may have a neuroprotective function by inhibiting cytoskeletal disruption and degeneration, thus decreasing the likelihood of damaged axon bundles progressing to secondary axotomy.

6 ACUTE AXONAL STRETCH INJURY INDUCES NEURITE SPROUTING.

6.1 INTRODUCTION

Experimental severance of axons has been shown to induce regenerative sprouting in spinal and cortical neurons (Carmichael, 2003), which has been suggested as a possible substrate for abnormal synaptic connections implicated in post-traumatic epilepsy (McKinney et al., 1997). Furthermore, collateral sprouting of 'upstream' segments of long axons that are damaged in lower segments of the spinal cord has been proposed to contribute to some degree of functional recovery (Hagg, 2006). However, mild to moderate non-penetrative forms of brain trauma do not result in substantial primary axotomy at the time of injury. Instead, a cascade of intra-axonal biochemical and structural changes leads to axonal swelling and secondary axotomy over the days and weeks following injury (Stone et al., 2001). This evolving axonal pathology, also known as DAI, relates to a sequelae of focal cellular alterations set in train from the initial transient stretch and shear forces at the instant of injury. *In vivo* studies in related experimental models have indicated that axonal swellings associated with DAI may also give rise to axonal sprouts, although the cellular dynamics underlying this response are unknown, as well as the potential contribution to the development of post-traumatic epileptiform activity.

During development, calcium signalling pathways and cytoskeletal dynamics are an important regulator of initial axon growth and guidance (Henley and Poo, 2004; Wen et al., 2004) as well as the formation of collateral branches (Gallo and Letourneau, 1998; Hutchins and Kalil, 2008). This thesis will sick to investigate whether post-injury sequelae is associated with axonal sprouting, and to determine if such sprouting relies on actin dynamics and calcium-mediated enzyme activation.

6.2 MATERIALS AND METHODS

6.2.1 Cortical cell culture and experimental axonal stretch injury

Cortical neuron cultures were prepared as outlined in chapter 2.1 (Materials and Methods) and in vitro stretch injuries were performed as described in chapter 2.2 (Materials and Methods. 0.5μM tacrolimus (FK506), a known inhibitor of calcineurin, and latrunculin-A, an inhibitor of actin polymerisation, were added to injured and sham cultures one prior to axonal stretch injury. At 24 hrs, 48 hrs, and 72 hrs PI stretch injured cells were fixed and immunolabelled with antibodies targeting growth-associated protein-43 (GAP43) or NFM according to chapter 2.3 (Materials and Methods). Actin filaments (F-actin) were stained with 4μl/ml phalloidin conjugated to AlexaFluor 488 (Molecular Probes, Eugene, OR). Immunolabelled preparations were visualized under an upright fluorescing microscope (Leica DMLB2) with images taken using an Optronics Magnafire digital camera as outlined in chapter 2.4 (Materials and Methods).

6.2.2 Live cell imaging

At 48 hrs PI, 1μl of Cell Tracker CM-DiI (DiI) (Molecular Probes, Oregon) was added to culture and incubated for 15 min. The injured cells were rinsed three times and placed in "imaging buffer" (in mM: NaCl, 5 KCl, 0.2 $CaCl_2$, 1 $MgCl_2$, 30 dextrose, and 25 HEPES, pH 7.3) (Zhang and Benson, 2001) for imaging. A time series of images were captured from a fluorescence (Leica DM IRB) inverted microscope (40X/0.80), equipped with a heated stage. Digital images were captured with a Hamamatsu ORCA-ER digital camera using OpenLab^{TM} software.

6.2.3 Scanning electron microscopy

Stretch injured and sham cultures were prepared for scanning electron microscopy as previously described (Chuckowree and Vickers, 2003). Briefly, cells were fixed in 2.5% glutaraldehyde/0.1% phosphate buffer (pH 7.4) and post-fixed with 0.1% osmium

tetroxide. Specimens were dehydrated by progression through an ethanol series, critical point dried and sputter coated with gold before examination under a Jeol-840 scanning electron microscope. Digital images were captured using ImageSlave software.

6.2.4 Real-time polymerase chain reaction analysis

Real time quantitative RT-PCR reactions were carried out using the Quantitect® SYBR® Green RT-PCR kit (Qiagen; Applied Biosystems Inc. Foster City, CA) in a 10 μl volume on a Rotor-gene 2000 (Corbett Research, Mortlake, NSW, Australia). A standard curve was prepared using PCR products derived from cDNA for the primer set to ensure that the reaction was efficient over the range of the RNA samples. A standard curve was also prepared for the primer set using serial dilutions of brain RNA to ensure that the amount of RNA added to each RT-PCR reaction was not inhibiting the efficiency of the RT-PCR reaction. GAPDH was used as the housekeeping gene. The CT values of all samples were normalized to GAPDH by dividing the CT value of the gene of interest by the CT value of GAPDH for each sample. The GAPDH normalized CT values were then divided by the average CT value of the control samples and expressed as a percentage of the average control value.

6.3 RESULTS

6.3.1 Delayed cytoskeletal alterations follow axonal stretch injury.

As previously described (chapter 3) the axonal stretch injury model did not result in primary axotomy but induced a progressive series of cytoskeletal alterations, maximal at 48 hrs post-injury (PI), ultimately leading to delayed axotomy of the majority (65%) of stretch-injured axonal bundles by 72 hrs PI. Additionally, injured axon bundle shafts displayed a 'frayed' morphology with neurofilament immunolabelling, which was maximal at 48 hrs PI (30% of injured axon bundles). Many of the surviving axon bundles at 72 hours PI also demonstrated this disordered appearance (Fig. 6.1C). The protruding processes from the 'frayed' region of the axon bundle did not have the same morphology as the 'undulating' axons that had already progressed to secondary axotomy seen at 72 hrs PI (Fig. 6.1D). It was difficult to assess the length of these protrusions immunocytochemically, due to their short length (50μm>).

6.3.2 Live imaging of injured axon bundle

Swollen and frayed regions of the injured axon bundle were examined with live imaging at 48 hrs PI for 15 minutes (Fig. 6.2) using the lipophilic dye, DiI. Highly motile filopodia-like sprout protrusions were identified in these regions of axonal swelling (Fig. 6.3; supplementary movie 3). These protrusions were identifiable using NIH Image J (Fig. 6.3) and the movements of the protrusions were in independent directions illustrating non-Brownian movement.

6.3.3 Scanning electron microscopy and immunohistochemistry

Scanning electron microscopy at 24, 48 and 72 hrs PI was utilised to determine the morphological features of injury-related sprouting (Fig. 6.3A-C). At 24 hrs PI, the axon bundle shaft was smooth and ordered with no significant protrusions. At 48 hrs PI, fine protrusions that resembled extended filopodia were observed from the axonal bundle shaft

Figure 6.1 Mild to moderate axonal stretch injury induced a delayed series of neuritic alterations - ultimately resulting in secondary axotomy. *A*, Utilising NFM immunolabelling, at 24 hrs PI, there was no significant cytoskeletal disruption to stretch-injured mature axon bundles, however, at 48 hrs PI, a significant percentage ($P<0.05$) (*D*) of stretch injured axons displayed a 'frayed' morphology (*B*, arrow). *C*, At 72 hrs PI, a significant ($P<0.05$) number of stretch-injured axon bundles had progressed to secondary axotomy (arrows). *E*, The processes in the frayed region of the axon bundle did not have the dystrophic morphology seen after axotomy at 72 hrs PI (arrow). Error bars are representative of standard error margins and P values<0.05 are considered significant (indicated by *). Scale bars, *A,B,C* 50μm; *E*, 15μm.

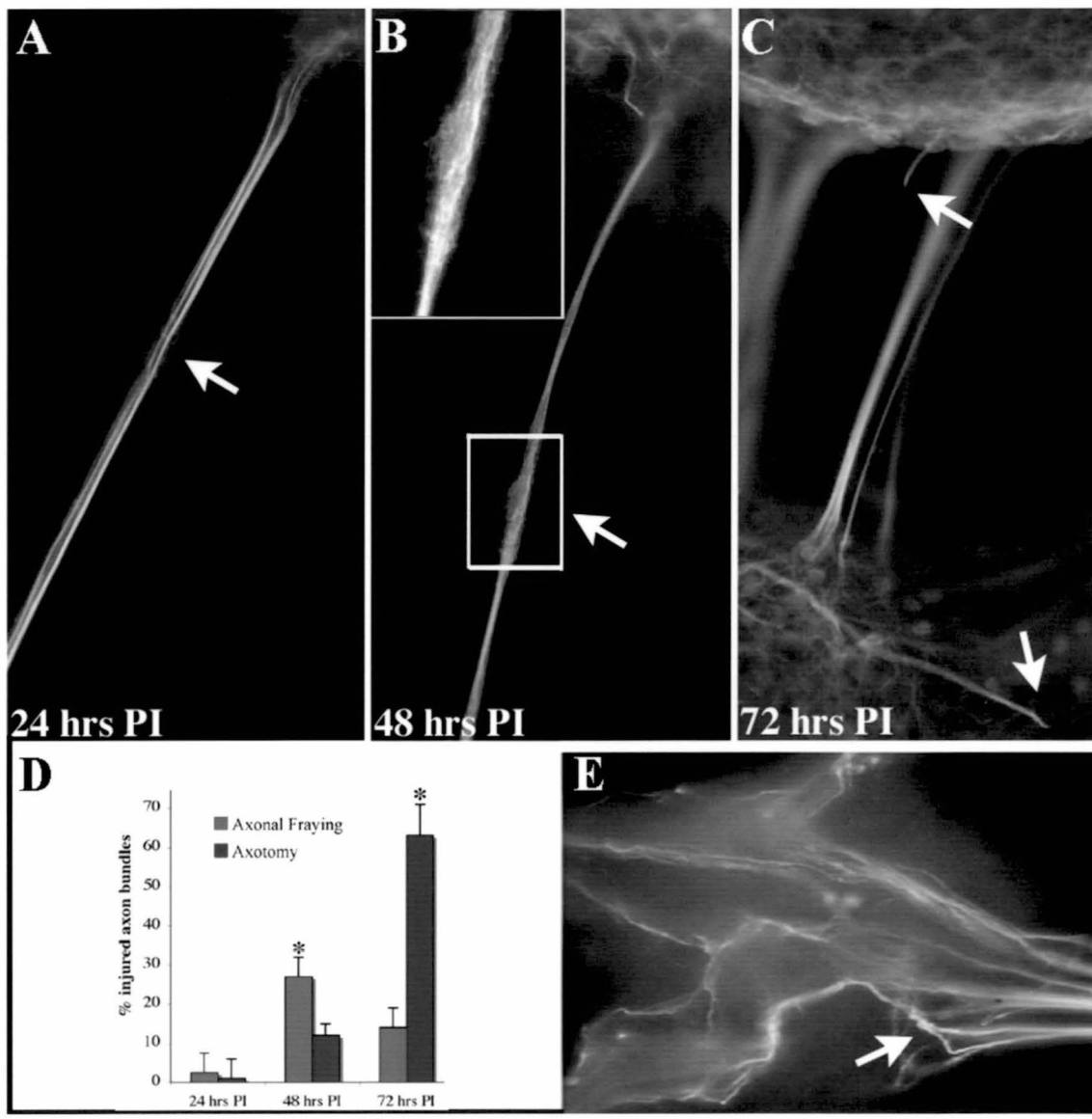

A
B
C
24 hrs PI
48 hrs PI
72 hrs PI
D
E
% injured axon bundles
70
60
50
40
30
20
10
0
Axonal Fraying
Axotomy
*
*
24 hrs PI
48 hrs PI
72 hrs PI

Figure 6.2 Axonal stretch injury induced localized highly motile filopodia-like sprouting. Video stills from live imaging of a DiI labeled stretch-injured axon bundle at 48 hrs PI (Refer to supplementary video 3). *A*, Axonal stretch injury induced the formation of highly motile filopodial sprouts (arrows) at 48 hrs PI. *B*, To increase clarity the video stills were adjusted using the shadows filter from Image J (NIH) software. Scale bar, 25μm.

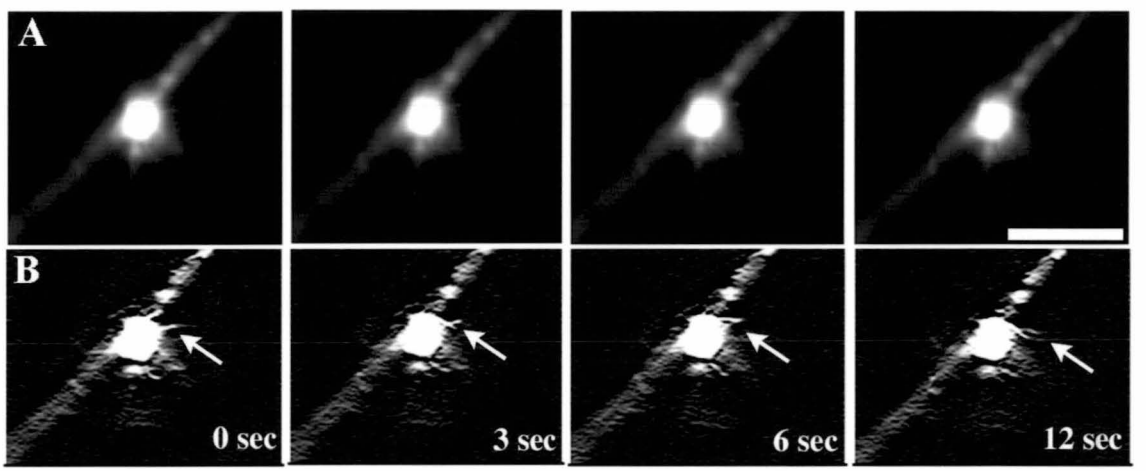
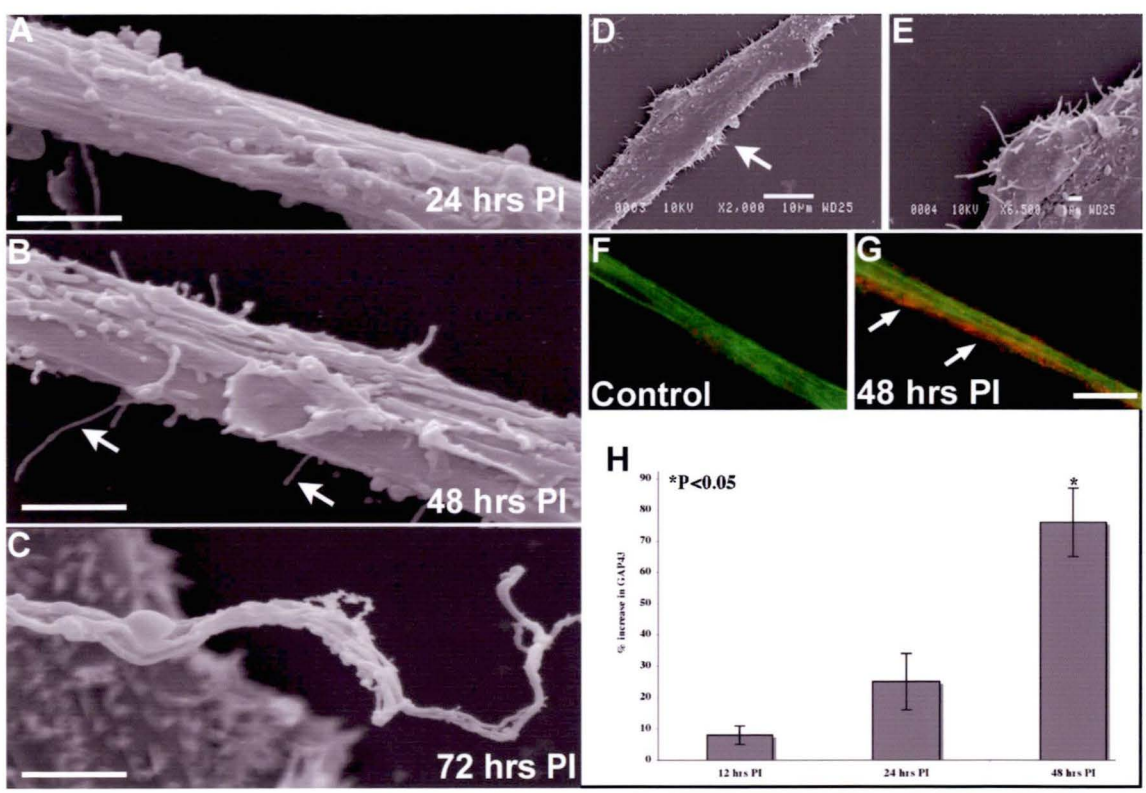

A
B
0 sec
3 sec
6 sec
12 sec

Figure 6.3 Injury-induced axonal sprouts do not have classical growth cone structures but express growth associated protein- GAP43. *A,B,C*, Stretch injured axon bundles exhibit filopodial-like sprouts at 48 hrs PI (*B*, arrows). *D,E*, Scanning electron micrograph of filopodial sprouts (arrow) at 48 hrs PI. F, Immunolabelling for GAP43 (red) and staining for F-actin (green) in an uninjured axon bundle. G, There was an accumulation of GAP43 (arrows) along the injured axon shaft at 48 hrs PI. *H*, Real-time PCR determined that there was an increase in GAP43 mRNA expression in stretch-injured axons compared to uninjured controls. Error bars are representative of standard error margins. Scale bars, 25μm (A-C, F,G).


A
24 hrs PI
D
E
B
48 hrs PI
F
Control
G
48 hrs PI
C
72 hrs PI
H
*P<0.05
% increase in GAPDH
90
80
70
60
50
40
30
20
10
0
12 hrs PI
24 hrs PI
48 hrs PI

at the site of maximal stretch (Fig. 6.3B). These sprouts did not have the classical 'fan shaped' growth cone morphology (Fig. 6.3D&E) commonly seen in development (Chuckowree and Vickers, 2003). At 72 hrs PI, 65% of the stretch-injured axons had progressed to secondary axotomy and significantly less (13%) filopodial sprouts were observed. The loss of filopodial sprouts at 72 hrs PI was not associated with the formation of new axonal branches.

Immunocytochemical labelling of the injured axon bundles, at 48 hrs PI, demonstrated an increased immunoreactivity for GAP43 (Fig. 6.3G) within the frayed region of the axon bundle. Specifically, increased GAP43 immunoreactivity was associated with the periphery of the stretch injured axon bundle. No alterations or increases in GAP43 immunoreactivity were seen in the uninjured control axon bundles at any time point (Fig. 6.3F). Real-time PCR studies also demonstrated a significantly ($P<0.05$) increased expression of GAP43 in stretch injured cultures at 48 hrs PI (Fig. 6.3H).

6.3.4 Inhibition of calcineurin and actin polymerisation

Calcium/calmodulin-dependant kinase II and calcineurin are important regulators of axon collateral growth (Wen et al., 2004). FK506, a potent inhibitor of calcineurin (Bavetta et al., 1999), was utilized to determine whether post-injury axonal sprout formation is modulated by calcium signalling pathways. Pre-injury treatment of stretch injured axons with FK506 resulted in increased length of axonal sprouts (>50μm) at 48 hrs PI (Fig. 6.4).

Actin dynamics play an important role in the formation of collateral filopodial sprouts (Gallo and Letourneau, 1998) and initial growth cone behaviour and neurite development (Dent and Kalil, 2001). In this study, 1μg/ml latrunculin-A, an inhibitor of actin polymerisation, added to cultures prior to axonal stretch injury, significantly ($P<0.05$) inhibited filopodial sprout formation following injury (Fig. 6.5).

Figure 6.4 Calcineurin inhibition was associated with increased axonal sprout length following stretch injury. A,B, Stretch-injured axon bundles (48 hrs PI), pretreated with 0.5μM FK506 and immunolabelled for phosphorylated neurofilaments (A, SMI312) and stained for F-actin (*B*). FK506 results in increased numbers of sprouts (*A*, arrows, inset: additional treated stretch-injured axon bundle) and filopodial length (arrows) at 48 hrs PI. Scale bar, 50μm.

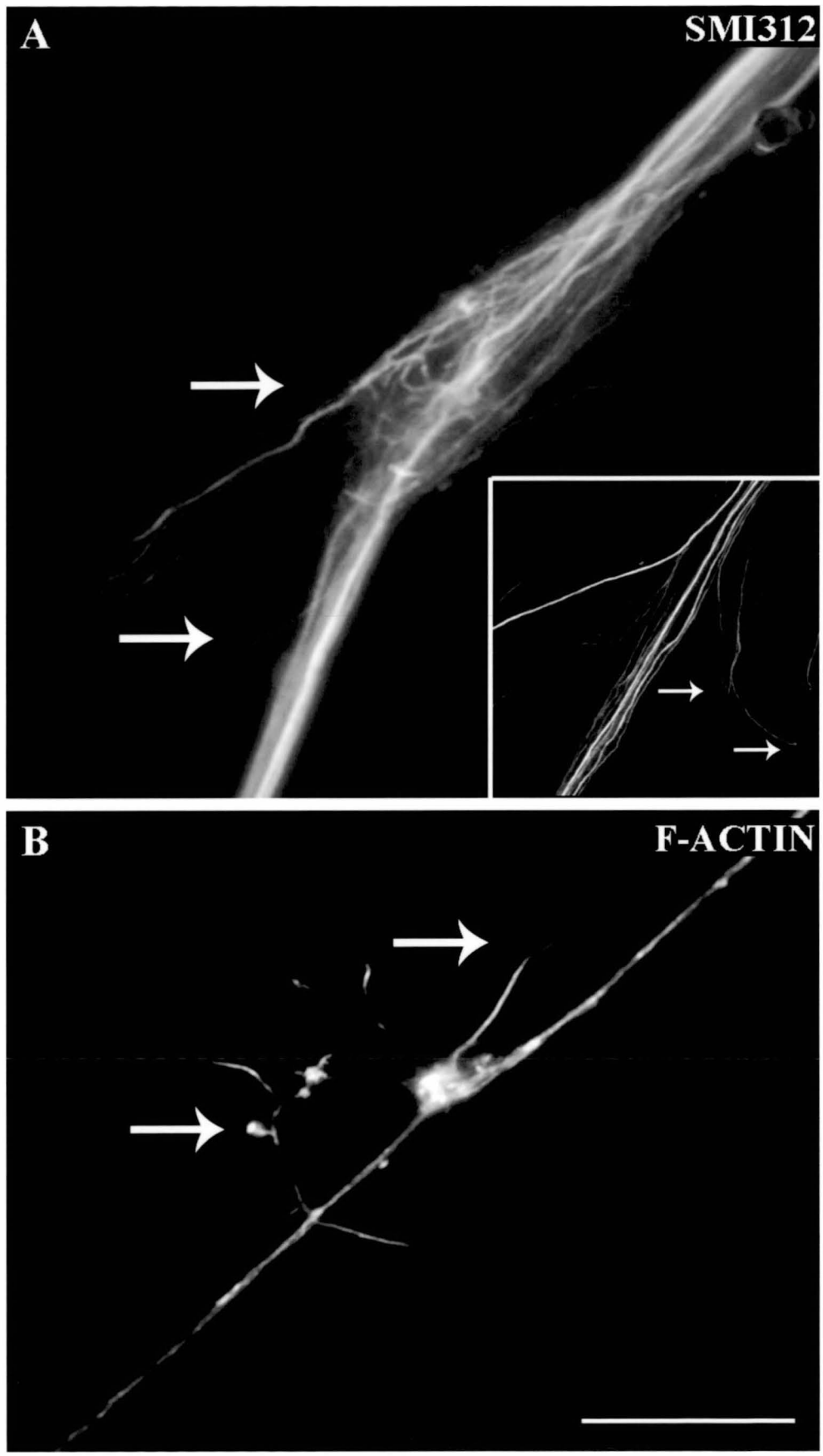
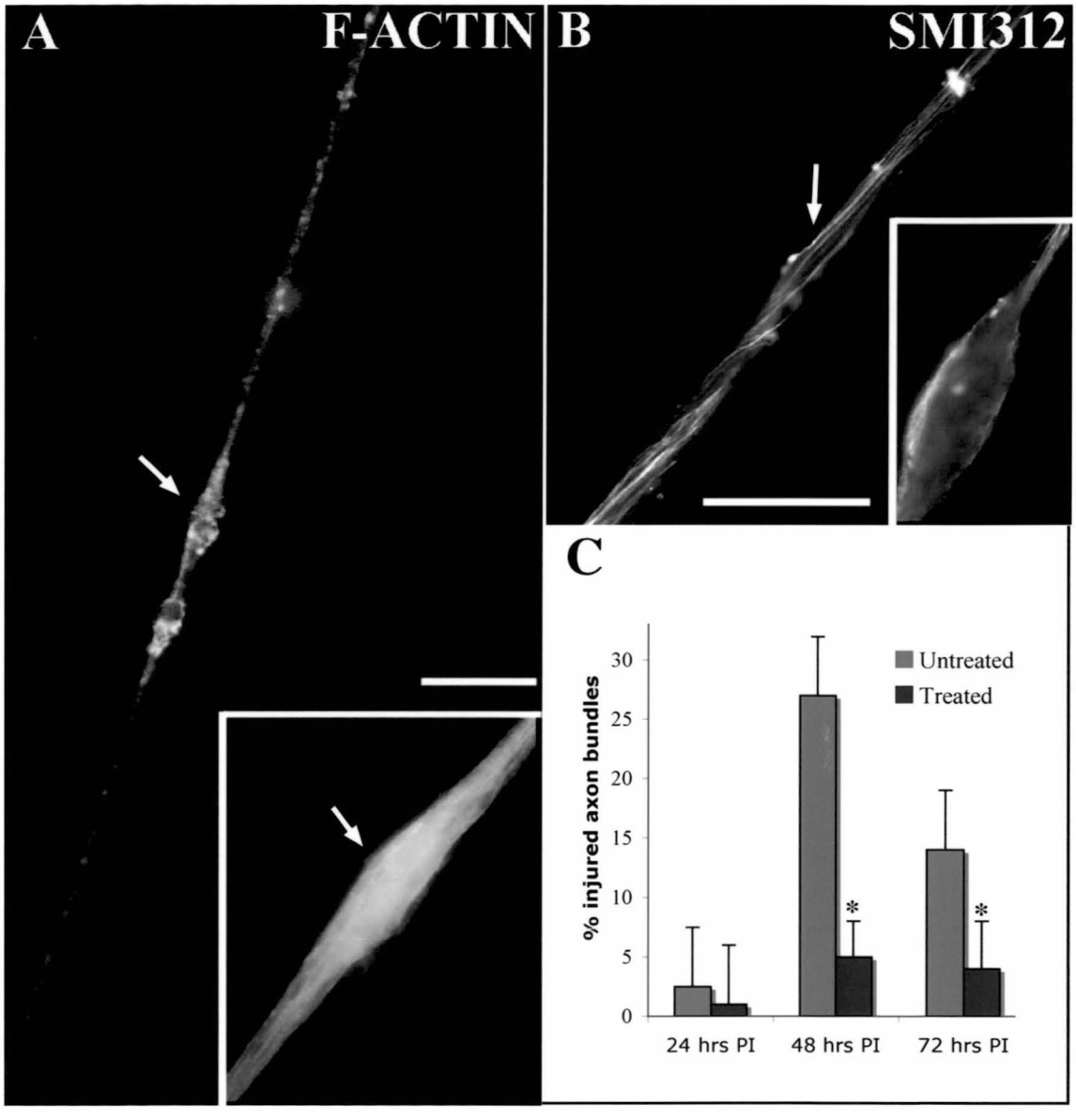

A
SMI312
B
F-ACTIN

Figure 6.5 Inhibition of actin polymerization with latrunculin-A abolished post axonal stretch injury sprouting. *A,B* Stretch-injured axon bundles treated with 1μg/ml latrunculin-A, an inhibitor of actin polymerization, did not exhibit axonal sprouts at 48 hrs PI as demonstrated by immunolabelling for phosphorylated neurofilament (SMI312) and staining for F-actin (For reference, insets in *A* and *B* are of additional separate injured axon bundles). In addition, few latrunculin-treated axon bundles displayed the 'frayed' morphology at regions of maximal strain (arrows) as seen in time-matched untreated control preparations. *C*, Graph indicates the percentage of treated and untreated axon bundles with filopodial sprout formation. Pre-injury latrunculin-A treatment of stretch injured axons significantly ($P<0.05$) reduces axonal sprouting at 48 and 72 hrs PI. Scale bar, 50μm.


A
F-ACTIN
B
SMI312
C
% injured axon bundles
30
25
20
15
10
5
0
Untreated
Treated
*
*
24 hrs PI
48 hrs PI
72 hrs PI

6.4 DISCUSSION

In this chapter, the regenerative capacity of stretch injured axons was assessed. At 48 hrs PI, stretch injured axon bundles display a 'frayed' morphology with NF immunolabelling (chapter 3) (Fig. 6.1B) as well as focal axonal swelling. Live imaging of the swollen and frayed regions of the injured axon bundle demonstrated highly motile filopodia-like sprout protrusions at this time-point. These filopodial protrusions bear close resemblance to the fine sprouts emerging from bulbous, reactive axons some hours following direct transection (Dickson et al., 2007). Similarly, significant cytoskeletal reorganization also observed following stretch injury (Chapters 3 and 4; Fig. 6.1), including MT debundling, underlies the formation of collateral axonal sprouts in cultured spinal sensory neurons induced by contact with beads coated with growth factors (Gallo and Letourneau, 1998). Likewise, Ozaki and Snider (1997) have also reported that peripheral sensory axon collateral formation in the developing spinal cord is preceded by a localized swelling of the axon. These data combined with the current study indicate that sprout formation may follow localised disruption of normal cytoskeletal arrangements and axonal swelling.

During development and following axon transection, *de novo* axon collateral branches are guided by growth cone structures (Castellani et al., 1998; Chuckowree and Vickers, 2003), which contain the growth-associated protein GAP43 (Avwenagha et al., 2003). In this chapter, scanning electron microscopy illustrated that the fine protrusions that resembled extended filopodia, evident at 48 hrs PI, did not have the classical 'fan shaped' growth cone morphology (Fig. 6.3D&E) commonly seen in development (Chuckowree and Vickers, 2003). Furthermore, these motile sprouts failed to develop into axonal branches, suggesting that this injury-induced regenerative sprouting response is deficient. In this regard, the lack of growth cone structures may underlie this abortive sprouting response. This is further supported by previous studies, both *in vitro* and *in vivo*, that show that axon transection in mature neurons results in aberrant sprouting. For example, regenerative sprouts following axonal transection *in vitro* have 'club-like' endings, rather than classic growth cones, which may contribute to an impaired regenerative capacity (King et al., 2001; Chuckowree and Vickers, 2003). Furthermore, axonal sprouting following direct neurite damage lacks

directionality and is unresponsive to a number of growth factors (Blizzard et al., 2007). The lack of growth guidance structures in this study may suggest that the post injury axonal sprouting response is primarily regulated by intrinsic cellular changes that may be related to cytoskeletal de-stabilization and re-organization precipitated by stretch injury.

Pre-injury treatment of stretch injured axons with FK506, a calcineurin inhibitor, resulted in an increased length of axonal sprouts. FK506 has been shown to promote neurite outgrowth in cultures of PC12 cells, sensory ganglia, and developing dopamine neurons *in vitro* (Lyons et al., 1994; Costantini and Isacson, 2000). FK506 acts by binding to receptor proteins, FK506-binding proteins, which in turn regulate a calcium-dependent phosphatase, calcineurin, and a calcium release channel, the ryanodine receptor (Lyons et al., 1994). Interestingly, in experimental models, levels of FK506-binding proteins during neural regeneration parallel those of growth-associated protein GAP43, which is also a calcineurin substrate (Lyons et al., 1994). Stretch injury of primary rat cortical axons induces proteolytic cleavage of the voltage-gated sodium channels resulting in increased intracellular calcium (Iwata et al., 2004), which may lead to activation of calcineurin. Treatment of injured axons with tetrodotoxin prevented this calcium influx (Iwata et al., 2004), and may also prevent calcium transients in developing neurons leading to inhibition of collateral sprouting (Hutchins and Kalil, 2008). The specific growth promoting effects of FK506 remains unclear. In developing dopaminergic neurons, FK506 induced elongation, but not axonal branching, and was similar to the effect of glial-derived neurotrophic factor (GDNF) (Costantini et al., 2000). Previous studies have suggested that inhibition of calcineurin increases the phosphorylation of GAP43, which is mandatory for axon regeneration (Rosenstiel et al., 2003). The FK506-mediated increase in GAP43 following axotomy to rat retinal ganglion cells and the optic nerve may reflect an increased regenerative potency of this compound (Rosentstiel et al., 2003). Additionally, this may also explain the improved axonal sprouting response following administration of FK506, illustrated in this study.

Pre-injury application of latrunculin-A, an inhibitor of actin polymerisation, abolished the formation of axonal sprouts. During development, axon branching is modulated by

interactions between MTs and actin filaments (Dent and Kalil, 2001). In growth cones and at axon branch points, splaying of looped or bundled MTs is accompanied by focal accumulation of F-actin (Dent et al., 1999; Kalil et al., 2000). The pharmacological inhibition of actin polymerisation with cytochalasin D blocked collateral filopodial sprout formation in developing axons (Gallo and Letourneau, 1998). Further studies have shown that inhibition of F-actin dynamics prevents axon branching but not axon elongation (Dent and Kalil, 2001). Similarly, the current results indicate that actin dynamics play an important role in driving the post-injury filopodial sprouting.

In summary, mild to moderate acute axonal stretch injury induces localized delayed filopodial activity, closely resembling that which is known to precede collateral branch formation in developmental models. These fine protrusions resembled extended filopodia more than genuine axonal branches, and contained growth associated proteins. However, these induced neuritic sprouts lacked the necessary growth guidance structures associated with initial neurite development, such as authentic growth cone structure and behaviour. Additionally, calcium signalling pathways and actin dynamics had a role in the formation of these axonal sprouts. These data indicate that damaged axons respond in a dynamic fashion to focal axon perturbation, and may provide insight into abnormal axonal sprouting that has been linked to post-traumatic epileptiform activity.

7 GENERAL DISCUSSION

Traumatic brain injury evokes widespread DAI, which significantly contributes to both morbidity and mortality (Povlishock et al., 1992; Smith et al., 2003; Povlishock and Katz, 2005; Buki and Povlishock, 2006). Although initial reports suggested instantaneous axon severance at the moment of injury, many studies have not supported this premise (Buki and Povlishock, 2006). Rather, the majority of studies have illustrated the evolving pathogenesis of DAI, which results in progressive neuronal alterations ultimately leading to secondary axotomy and degeneration. This thesis has utilised a novel *in vitro* axonal stretch injury model, which leads to pathological alterations associated with DAI, to investigate the cellular and molecular mechanisms initiating and contributing to the delayed degeneration of injured neurons.

The *in vitro* axonal stretch injury model developed in this thesis utilises a well-defined primary cortical cell culture protocol (Dickson et al., 2000; Chuckowree and Vickers, 2003; Haas et al., 2004; Blizzard et al., 2007). This protocol involves the long–term culturing of rat embryonic cortical neurons until relative maturity, and is characterised by the presence of discrete and suspended axonal bundles between cell body clusters (Dickson et al., 2005). These axon bundles are injured using a brief (20 psi over 13-20 msec) fluid pressure pulse, which causes the axonal bundles to rapidly stretch and twist, before returning to its original orientation. Importantly, this form of stretch injury does not cause primary axotomy and is a relatively mild form of injury.

7.1 AXONAL STRETCH INJURY AND THE NEURONAL CYTOSKELETON

Consistent with other models of DAI (Graham et al., 2000; Stone et al., 2001; Lifshitz et al., 2003; Marmarou et al., 2005), this thesis illustrates that mechanical trauma to axons induces a multi-faceted, heterogeneous, response. This is highlighted by the altered NF response to stretch injury, which was shown in some cases to precede secondary axotomy (Fig 7.1). In this regard, stretch injured axons demonstrated either increased SMI312 (labels phosphorylated NFH and NFM) immunoreactivity, or distorted NF organization resulting

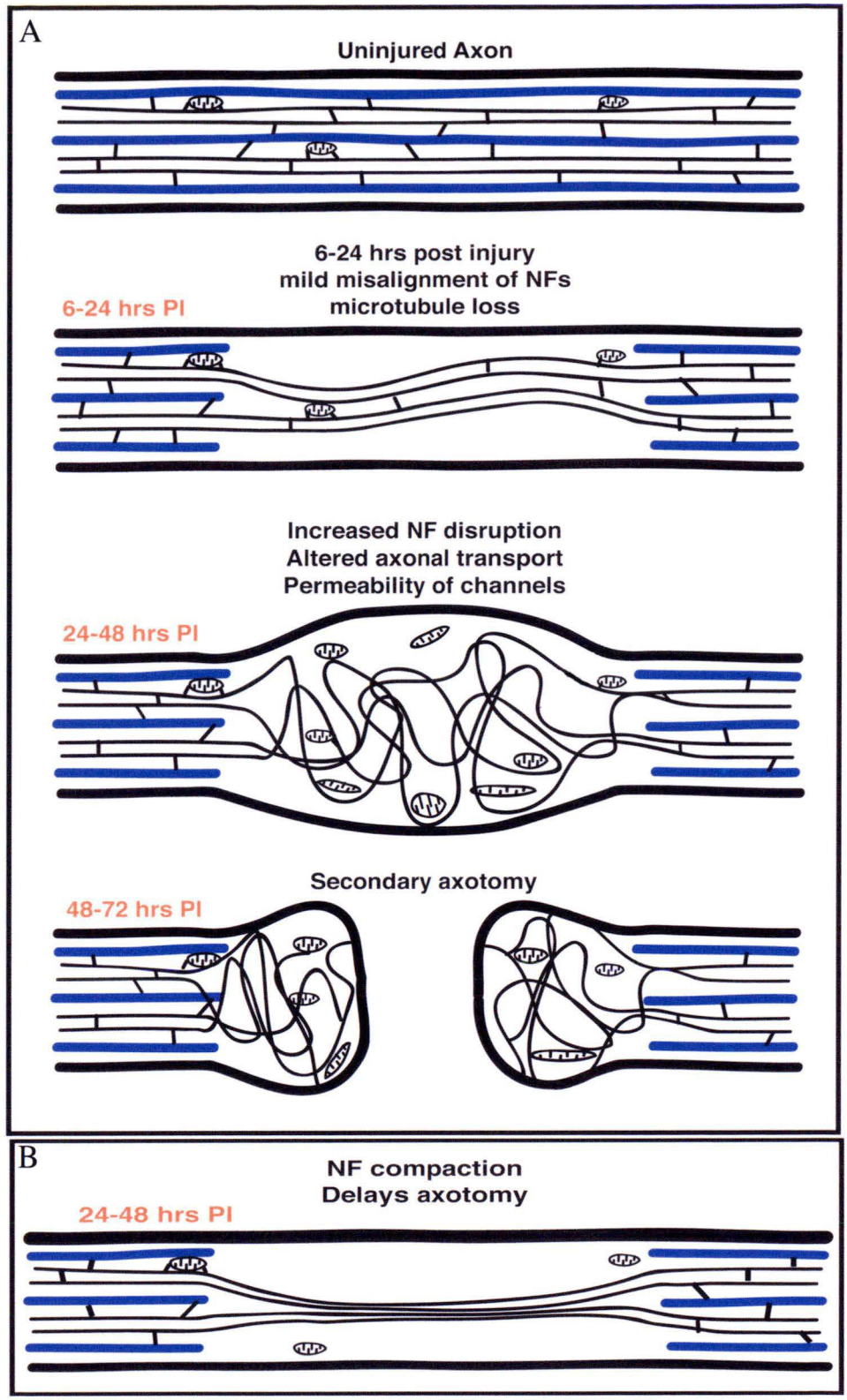


Figure 7.1 Post-injury sequelae involves progressive cytoskeletal disruption ultimately resulting in secondary axotomy at 72 hrs PI (MTs in blue and NFs in black). Axonal swellings are evident at 48 hrs PI and may be due to calcium induced cytoskeletal degradation. Additionally, NF can occur in a subpopulation of stretch injured axons (B).

in segregated and undulated NF fibers localized to regions of axonal swellings. Increased SMI312 immunoreactivity, indicative of NF compaction, occurred early in the post-injury sequelae, first evident at 24 hrs PI and maximal at 48 hrs PI. A similar heterogenous response was illustrated following impact acceleration injury in rats (Stone et al., 2001). Using double-labelling immunocytochemical techniques, Stone and colleagues further illustrated that NF compaction occurs earlier and is independent of altered axonal transport. Interestingly, it is also possible that this increased SMI312 immunoreactivity reflects an alteration in phosphorylation state of some of the NFs present within the injured axon. Indeed, changes in the phosphorylation state of the NF side-arms have long been associated with NF compaction (Povlishock, 1993). The results presented in this thesis also indicate a significant loss of injured axons with NF compaction at 72 hrs PI. It is unclear whether these injured axons have progressed to secondary axotomy. Recent electron microscopy studies have reported significant axonal recovery from NF compaction following brain trauma in rats (Gallyas et al., 2006). Conversely, non-recovering compacted axons progressed to secondary axotomy via a non-Wallerian form of degeneration, which is mostly reversible (Gallyas et al., 2006).

Stretch-injured axon bundles with abnormal rearrangements of NF structure were typically evident at later time-points in comparison to NF compaction. Immunocytochemical labelling of these injured axons in chapter 3 demonstrated that this cytoskeletal disruption is localized to regions of axonal swelling. Furthermore, these regions were the sites of secondary axotomy at 72 hrs PI. Similar cytoskeletal abnormalities have been reported following *in vivo* mechanical head trauma, with NFs withdrawing from the axolemma to display an abnormal alignment oblique to the longitudinal axis of the axon (Povlishock, 1992, Yaghmai and Povlishock, 1992; Fitzpatrick et al., 1998). Additionally, these NF alterations also became worse over time and were followed by axonal expansion and the accumulation of organelles (Fitzpatrick et al., 1998). This sequence of events, with a distinct progression of NF alterations, was also observed in of the CNS brain from fatally injured individuals (Grady et al., 1993; Christman et al., 1994). Altogether, this thesis illustrates that following mechanical trauma axons do not respond in a stereotypical manner, rather variable degrees of shear/strain initiate different responses. In this regard,

NF disruption, particularly resulting in the formation of axonal swellings, plays an important role in the eventual progression to secondary axotomy.

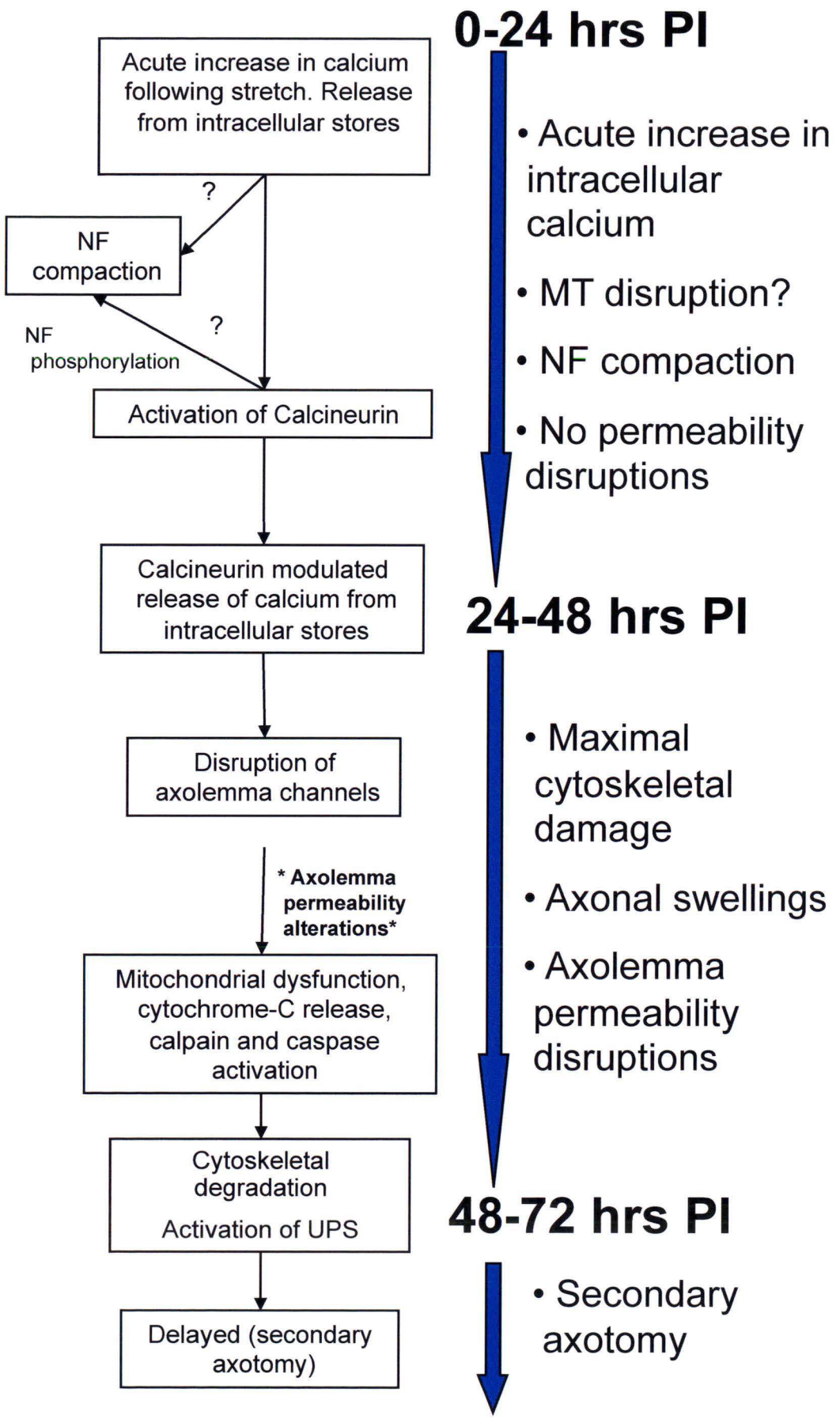
7.2 AXOLEMMA PERMEABILITY ALTERATIONS

Previous studies have focused on the direct consequences of shear and stretch forces on axolemma permeability. Early studies reported focal microscopic mechanoporation of the axolemma, reflected in the influx of normally excluded tracers such as horse-radish peroxidase (Pettus and Povlishock, 1996). The potential consequence of this mechanoporation is the detrimental influx of calcium, which has been linked to the degradation of the cytoskeleton via activation of proteases and phosphatases. However, less severe, non-severing, models of axonal injury such as presented in this thesis do not provide evidence of immediate axolemma permeability alterations. Results from a similar *in vitro* axon stretch injury model support this finding with altered permeability only seen following primary axotomy (Smith et al., 1999). Importantly, the results from this thesis demonstrate delayed alterations to axolemma permeability associated with cytoskeletal damage. It is clear from other studies involving axonal stretch injury that increased intracellular calcium plays an important role in the degradation of the axon cytoskeleton as seen by the calcium mediated activation of calpains and caspases (Saatman et al., 2003; Serbest et al., 2007). Accordingly, cytochrome-C release from mitochondrial stores has also been illustrated in this study and is reported to be induced by increased intracellular transients (Buki et al., 1999). In similar stretch injury models, this increase in intracellular calcium is reported to be due to altered activation of Na^+ channels, with the demonstration that the subsequent axonal depolymerisation triggers the activation of voltage sensitive Ca^{2+} channels (Iwata et al., 2004). Further studies illustrated limited or no increase in intracellular calcium when stretch injuries were conducted in calcium free media, which suggests calcium dysregulation is due to influx of extracellular calcium. In this thesis, axonal stretch injury resulted in two distinct intracellular calcium increases. The thapsigargin-mediated release of calcium from endoplasmic stores induces a similar calcium flux profile as seen following stretch injury. Interestingly, axonal stretch injury in calcium-free ACSF resulted in only a single peak increase in intracellular calcium. Indeed,

this single peak increase in intracellular calcium was similar in magnitude and temporal profile to the initial calcium flux observed in both injuries conducted in calcium-containing ACSF and following thapsigargin-mediated release of calcium from endoplasmic stores. Thus, these results support the possibility that the initial peak increase in intracellular calcium following stretch injury is due to release from intracellular stores. The second peak calcium influx following stretch injury may be due to influx from extracellular stores as this intracellular calcium increase is abolished in calcium-free ACSF. Traumatic injury of cortical neurons has been shown to alter intracellular calcium stores, which is linked to subsequent capacitative (store-operated) calcium influx (Weber et al., 2001). Additionally, this calcium induced extracellular calcium entry has been reported following ischemic spinal cord white matter damage (Ouardouz et al., 2003). Other studies have illustrated the pathological influx of calcium through activated Na-channels, which may be an ideal candidate for further investigation as a possible mechanism associated with the secondary peak increase in intracellular calcium (Wolf et al., 2001; 2005). The results from this study suggest that both the release of calcium from intracellular stores and the subsequent influx of extracellular calcium play an important role in the post-traumatic increase in intracellular calcium (Fig 7.2).

Calcium-mediated proteolysis of Na- channels has been associated with the progressive and sustained increase in intracellular calcium levels in more severely stretch injured axons (Iwata et al., 2004). Additionally, pre-injury and post-injury inhibition of calcium-activated proteases attenuated long-term increases in intracellular calcium as well as mitigating degradation of the sodium channels (Iwata et al., 2004). In summary, the immediate increase in intracellular calcium may activate proteases which in-turn progressively degrades Na-channels resulting in a unique "feed-forward" process, perpetuating pathological calcium influx. This influx of calcium over the post-injury period may result in the activation of apoptotic mechanisms and cytoskeletal degradation, which will be discussed further later in this chapter.

7.3 MITOCHONDRIAL DYSFUNCTION


Acute increase in calcium following stretch. Release from intracellular stores
?
NF compaction
NF phosphorylation
?
Activation of Calcineurin
Calcineurin modulated release of calcium from intracellular stores
Disruption of axolemma channels
* Axolemma permeability alterations*
Mitochondrial dysfunction, cytochrome-C release, calpain and caspase activation
Cytoskeletal degradation
Activation of UPS
Delayed (secondary axotomy)
0-24 hrs PI
• Acute increase in intracellular calcium
• MT disruption?
• NF compaction
• No permeability disruptions
24-48 hrs PI
• Maximal cytoskeletal damage
• Axonal swellings
• Axolemma permeability disruptions
48-72 hrs PI
• Secondary axotomy

Mitochondrial dysfunction is closely associated with DAI following head trauma (Buki et al., 1999, 2000; Arnoult et al., 2002). The consequence of increased intracellular calcium, as described above, is the evoked calcium-induced opening of the MPT pore (Hirsch et al., 1998; Arnoult et al., 2002). The current study illustrates the release and accumulation of cytochrome-C into the cytoplasm within axon swellings. The release of cytochrome-C is indicative of the opening of the MPT pore, which also results in the release and activation of different members of the caspase enzyme family (Mancini et al., 1998; Krajewski et al., 1999; Susin et al., 1999). Although the release of cytochrome-C could be illustrated with co-localisation immunolabelling with mitochondrial markers and cytochrome-C, it is not clear whether there is further mitochondrial damage concomitant with the opening of the MPT, which include mitochondrial swelling due to uptake of water (Hirsch et al., 1998; Buki and Povlishock, 2006). The mitochondrial injury in itself is detrimental to the viability of the neuron as it provides a serious energetic/homeostatic challenge. In this study, the release and accumulation of cytochrome-C was localised to the site of maximal cytoskeletal damage and associated with the eventual loci of axotomy. Similar studies involving *in vivo* rat brain injury models have also supported this finding by illustrating that the foci of cytochrome-C release is localised to regions of calpain-mediated spectrin proteolysis (Buki et al., 1999, 2000). Other immunocytochemical studies have further demonstrated that caspase-3 activation and spectrin breakdown product, is also consistently co-localised with cytochrome-C and calpain mediated spectrin proteolysis (Buki et al., 2000; Farkas et al., 2005; Buki and Povlishock, 2006; Serbest et al., 2007). These results, including evidence of calpain mediated spectrin proteolysis leading to the collapse of the subaxolemmal membrane cytoskeleton, support this thesis findings of cytoskeletal damage associated with cytochrome-C release and delayed permeability alterations (Wang et al., 1998; Buki and Povlishock, 2006). However it is not clear whether both caspases and calpains are associated with the full spectrum of post-injury sequelae or just the later sequence of events immediately prior to secondary axotomy. Current reports, including the results of this study, tend to demonstrate cytochrome-C release at later stages of the post-injury sequelae, typically associated with mitochondrial damage (Buki and Povlishock, 2006). Accordingly, in this thesis, cytoskeletal damage is occurred prior to, and in some circumstances, independent to, axonal swellings and cytochrome-C release. This may indicate that other

cytoskeletal degradative pathways are initiated, particularly at the earlier stages of the post-injury sequelae. Indeed, in this thesis, inhibition of calcineurin with FK 506, which does not inhibit the MPT pore and release of cytochrome-C as with cyclosporin-A, resulted in an attenuation of cytoskeletal damage and secondary axotomy. These results provide further evidence that cyclosporin-A's therapeutic effect may principally be due to calcineurin activity rather than inhibition of the MPT pore. This is further supported by other *in vivo* rat brain injury studies that demonstrated attenuation of impaired axonal transport and cytoskeletal damage resulting in improved neuronal survival following administration of FK 506 (Marmarou and Povlishock, 2005; Reeves et al., 2007). However, these studies in conjunction with the results of this thesis suggest that FK 506 does not attenuate the full spectrum of cytoskeletal damage, specifically, NF compaction.

7.4 CALCINEURIN ACTIVITY

Calcineurin, a calcium/calmodulin-dependant protein phosphatase, is known to dephosphorylate a number of substrates including the cytoskeleton (Morioka et al., 1998). Due to the diversity of substrates dephosphorylated by calcineurin, FK 506 may exert a neuroprotective effect via multiple pathways. In addition to modulating the phosphorylation state of cytoskeletal proteins resulting in abnormal reorganisation, calcineurin may also operate on other cellular targets involved in the axonal response to injury. The drug FK 506 binds to FK 506 binding protein-12, an integral part of the IP3 and ryanodine receptor complexes, and appears to alter cytosolic calcium released through these channels (Brillantes et al., 1994; Cameron et al., 1995; Reeves et al., 2007). This calcineurin-modulated calcium release may further be involved with the later activation of calcium-modulated degradative cascades. As discussed above and illustrated by this thesis, inhibition of calcineurin attenuates cytoskeletal damage commonly associated with activation of caspases and calpains (Marmarou and Povlishock, 2005; Reeves et al., 2007). This suggests the initial activity of calcineurin is linked to the activation of these calcium-modulated proteases. In this regard, the subtle calcium alterations, linked primarily to release from internal calcium stores, may only be adequate to trigger high-affinity calcium-calmodulin targets such as calcineurin, which require only nanomolar concentrations

(Shields et al., 1997; Stys and Jiang, 2002;). This would preclude calpains, as activity of this enzyme is dependant on millimolar calcium concentrations (Marmarou et al., 2006). Further and persistent calcium dysregulation demonstrated later in the post-injury sequelae might be associated with the activation of calpain and caspases. However, as shown in this study, inhibition of calcineurin with FK 506 did not attenuate NF compaction.

There are emerging studies that demonstrate increased levels of NFM phosphorylation following inhibition of calcineurin (Fiumelli et al., 2008). This thesis illustrated increased NF phosphorylation following axonal stretch injury, which was also associated with NF compaction. Indeed, alterations in NF phosphorylation would be expected to alter NF-side arm interactions resulting in compaction (Okonkwo et al., 1998). As previously discussed (section 7.1), much controversy remains on the mechanisms underlying NF compaction, however, it is clear that NF compaction occurs at the earliest stages of the post-injury sequelae and in human TBI cases remains stable for at least up to six hours (Maxwell et al., 1997; Jafari et al., 1998;). It remains to be determined whether NF compaction confers neuronal death exhibited by secondary axotomy. As discussed, a significant number of injured neurons can recover from NF compaction in the axon, conversely, a subset of neurons progressed to secondary axotomy via a mostly reversible non-Wallerian form of degeneration (Gallyas et al., 2006). Interestingly, phosphorylation of NFH reduces the rate and extent of proteolysis by calpain (Greenwood et al., 1993), and preferential proteolysis of dephosphorylated compared to phosphorylated NFH has been shown in spinal cord injuries (Schumacher et al., 1999). This may suggest that phosphorylation of NF's, which is increased following injury and inhibition of calcineurin, results reduced proteolysis by calpains and NF compaction. The spontaneous recovery of NF compacted axons may explain in humans one of the causes leading to the eventual improvement of physical and mental capabilities, that were lost at the moment of injury (Gallyas et al., 2006). In summary, this thesis highlights the multiple cytoskeletal initiated by calcium dysregulation. In regard to mild to moderate axonal trauma, which results in immediate subtle calcium dysregulation without overt axolemma alterations, the high affinity calcium/calmodulin targets such as calcineurin may be the initial modulator of eventual cytoskeletal damage.

7.5 UPS INVOVLEMENT FOLLOWING AXONAL INJURY

The delayed accumulation of Ub within axonal swellings was associated with the release and accumulation of cytochrome-C. The accumulation of Ub within axon swellings has also been illustrated using the fluid percussion model of closed head injury in the cat (Schweitzer et al., 1993). As seen in this thesis, the rapid accumulation of Ub immunoreactivity is linked to alterations in NF structure (Schweitzer et al., 1993). Furthermore, the accumulation of Ub conjugates and inclusion bodies as long been illustrated in lesions associated with many chronic neurodegenerative diseases, including the neurofibrillary tangles of Alzheimer's disease, Lewy bodies in Parkinson's disease and recently in other neurological syndromes including gracile axonal dystrophy (Alves-Rodrigues et al., 1998; Saigoh et al., 1999; Glickman and Ciechanover, 2002). Inhibition of the UPS, with MG132 and lactacystin, profoundly delayed degeneration following *in vitro* axon transection of cortical neurons (Zhai et al., 2003). The pre-injury administration of these protease inhibitors in this study's *in vitro* model of axonal injury, however, accelerated the progression to secondary axotomy. These results suggested the involvement of ubiquitination in the survival of injured axons following stretch injury. It is important too note that axonal stretch injury and axon transection, may initiate different mechanisms of axonal degeneration. Indeed, axon transection typically results in the delayed Wallerian degeneration (Zhai et al., 2003). In this regard, UPS involvement in Wallerian degeneration is proposed to be detrimental to the survival of the transected neuron (Wang et al., 2001; Ehlers, 2004). In contrast, few studies have investigated the role of the UPS in a non-transectional model of axonal injury. As a result, the UPS has been implicated as having a similar, pathological, role in both injury paradigms. However, early studies reporting a rise in cytoplasmic ubiquitin levels following nerve crush injury, have suggested Ub-mediated degradation of damaged proteins in peripheral motor neurons favouring survival of the damaged neuron (Shyne-Athwal, 1986; Savedia and Kierman, 1994; Wang and Ingoglia, 1997; De Stefano et al., 1998). Following axonal stretch injury, it is possible that the UPS is involved in the degradation of products from calpain-mediated cytoskeletal damage (Ehlers, 2004). This is supported by evidence of increased ubiquitin immunoreactivity localized to regions of cytochrome-C release and maximal cytoskeletal degradation.

Accordingly, calpain inhibitors have also been found to delay axon degeneration following axon transection (Raghupathi, 2004). Interestingly, removal of extracellular calcium, using EGTA, also delayed axonal degeneration following transection, suggesting an upstream calcium-dependant signalling event. As calcium dysregulation resulting in caspase and calpain activation is illustrated following axonal stretch injury (Iwata et al., 2004; Raghupathi, 2004), it is possible that these events could also involve activation of the UPS pathway (Ehlers, 2004). Altogether, these results suggest that alternative techniques aimed at improving UPS function following axonal injury, may be a more effective therapeutic strategy opposed to inhibition of the UPS itself.

7.6 AXONAL SPROUTING

Axonal sprouting following axon transection and during development has been studied extensively (Chuckowree and Vickers, 2003; Dent et al., 2004; Hagg, 2006; Blizzard et al., 2007). There is increasing data that suggest that following mild to moderate TBI, diffuse deafferentation sets the stage for the sprouting of adjacent intact nerve fibres that, in many cases, results in significant recovery of synaptic input to the previously deafferented neuronal domains (Erb and Povlishock, 1991; Povlishock and Katz, 2005; Hagg, 2006). This thesis illustrates that axonal stretch injury induces sprouting of filopodial-like processes along the injured axon shaft. These highly motile processes resemble that which is seen prior to collateral branch formation during development. Specifically, collateral branches are initiated by the appearance of localized filopodial activity along previously quiescent axonal shafts (Gallo and Letourneau, 1998). This rapid sprouting of filopodia is induced in sensory neurons at sites of contact with nerve growth-factor coated polystyrene beads (Gallo and Letourneau, 1999). These axonal sprouts, induced by stretch injury, do not continue to form new axonal branches in this *in vitro* model and this may be due to the lack of classical growth guidance structures illustrated during development (Chuckowree and Vickers, 2003; Blizzard et al., 2007). Abnormal growth guidance structures are also implicated in the deficient regenerative response following axon transection (Blizzard et al., 2007). The axonal sprouting response illustrated in this thesis is modulated by actin dynamics, which is also demonstrated to play a crucial role in the development of collateral

branches in uninjured neurons (Dent and Kalil, 2001). There may also be similarities in the mechanisms initiating this axonal sprouting response. Recent studies of collateral sprouting, suggest that transient intracellular calcium increases may play a pivotal role in the protrusion of axon branches in cortical neurons (Tang and Kalil, 2005). The release of calcium from intracellular stores, as demonstrated following axonal stretch injury, is implicated as a source for these calcium transients (Tang and Kalil, 2005). Interestingly, calcium/calmodulin-dependent pathways, which are also initiated following stretch injury, also modulate axonal branching during development.

There are few studies that have investigated the regenerative sprouting response of mild to moderately injured axons. Indeed, further investigation is required as axonal sprouting following injury may be indicative of an adaptive mechanism for functional recovery after injury, or maladaptive, playing a pivotal role in the generation of epileptiform events due to increased numbers of synaptic contacts between neurons (Hagg, 2006). It is generally accepted that following more severe forms of axonal injury, there is maladaptive change due to inappropriate fibre ingrowth and/or cytoarchitecture modification (Phillips et al., 1994, 2001; Povlishock and Katz, 2005). In this regard, the inappropriate fibre ingrowth is proposed to originate from severed axons as this sprouting response has been well characterised *in vitro* (Chuckowree and Vickers, 2003). This, however, has not been proved and there is possibility that these inappropriate fibres may be originating from injured, but not severed, axons as demonstrated in this thesis. Axon compression has been illustrated to induce axonal sprouting following end-to-side neurorrhaphy (Hayashi et al., 2008). Although this study is based in the PNS it highlights some interesting caveats. Extrinsic nerve compression can increase small diameter unmyelinated axon counts, hence, it is possible that the axonal sprouting response is from unmyelinated sensory axons (Mackinnon et al., 1986; Hayashi et al., 2008). Only recently has it become evident that TBI results in damage to multiple fibre populations including large and fine-calibre myelinated and non-neuropathologically detected unmyelinated fibres. The stretch injured axons in this study are unmyelinated, and there is evidence of increased susceptibility of these axons to TBI, which may significantly contribute to the ensuing morbidity (Reeves et al., 2005; Povlishock and Katz, 2005). It is not inconceivable that reactive axonal sprouting

may be from unmyelinated axons, given the growth inhibitory nature of myelin (Wang et al., 2001). In summary, intracellular calcium dysregulation induced by stretch injury may trigger axonal sprouting via activation of calcium/calmodulin dependant pathways. These aberrant sprouts contain growth-associated proteins, however, lack guidance structures, which may result in inappropriate synaptic formation *in vivo*.

In summary, an advantage of the *in vitro* model presented in the thesis, is that it reliably reproduces the evolving axonopathy, characteristic of human DAI, over an extended period of time. Thus, it provides an ideal experimental platform to investigate important mechanistic insights into the underlying cause of DAI, and also examine novel therapeutic approaches. However, this novel model of axonal stretch injury involves injury to axonal bundles and not single neurites. Another reservation is that the injured axon bundles in this thesis are unmyelinated. Additionally, as only a small population of neurons in culture are injured, opposed to a broad injury to the majority of cultured neurons, it is often difficult to utilise current proteomic techniques. In this regard, the results from this experimental model of stretch injury provide only part of the complex post-DAI response and needs to be addressed in conjunction with other *in vitro* axonal stretch injury models, so as to provide a more clinically relevant understanding. Indeed, *in vitro* models of brain injuries are not capable of surpassing *in vivo* models, but they are able to complement them, and permit the exploration of different aspects of head trauma including insights into the mechanism of cellular change that leads to axonopathy.

7.7 CONCLUSIONS

- **We present a simple, reproducible model of axonal stretch injury that results in progressive cytoskeletal alterations and secondary axotomy, characteristic of DAI.**
- **Axons do not respond in a stereotypical manner to a transient stretch insult, and indeed that variable degrees of stretch injury activate diverse responses within axons, with dramatically different outcomes.**

- **Axonal stretch injury does not result in overt mechanoporation of the axolemma, rather, injury induces intracellular calcium dysregulation due to release of calcium from intracellular stores.**
- **There is the delayed release and accumulation of ubiquitin and pro-apoptotic cytochrome-C within axon swellings, which is associated with maximal cytoskeletal damage.**
- **Inhibition of calcineurin with FK506 attenuates cytoskeletal damage and secondary axotomy following injury.**
- **Inhibition of the UPS accelerates the axonal progression to secondary axotomy.**
- **Axonal stretch injury induces axonal sprouting. Axonal sprouts contain growth proteins, however, lack guidance structures.**

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9 SUPPLEMENTARY MOVIES

Supplementary movie 1 At 21 days in vitro, an individual bundle of axons were targeted with a single pulse of fluid, and the resultant deflection was captured by video camera. In this movie, the axon bundle is rapidly stretched, before recovering to its original position. For reference, the maximal increase in original axon length of this bundle caused by the deflection was 3%.

Supplementary movie 2 Following three-dimensional reconstruction of images captured through the entire axon bundle, the compiled stack was sequentially rotated in 1° intervals in the x-axis and combined into a QuickTime™ movie to allow accurate 3-D spatial representation of immunocytochemically-labelled cytoskeletal neurofilament protein features within the injured axon bundle.

Supplementary movie 3 Live-imaging of an injured mature cortical neuron at 48 hrs PI. The neuron is loaded with DiI prior to imaging. Frames were captured at 1 frame/3 seconds.

10 APPENDIX – SOLUTIONS

10.1 GENERAL SOLUTIONS

All solutions were made up in Milli-Q® water, unless otherwise stated.

0.01M phosphate buffered saline (PBS), pH 7.4

- 100ml NaCl - 90g/L stock solution
- 40ml di-sodium hydrogen orthophosphate ($Na_2HPO_412H_2O$) - 28.4g/L stock solution
- 10ml sodium di-hydrogen orthophosphate ($NaH_2PO_4.H_2O$) - 31.2g/L stock solution
- 850ml Milli-Q® water
- pH 7.4

4% paraformaldehyde (PFA) (100ml)

- 4g paraformaldehyde
- 4g sucrose
- 10ml di-sodium hydrogen orthophosphate ($NaH_2PO_4.H_2O$) - 31.2g/L stock solution
- 40ml sodium di-hydrogen orthophosphate (NaH_2PO_4) - 28.4g/L stock solution
- 50ml Milli-Q® water
- dissolve by heating (~80°C) on stirrer hotplate in fume hood

Borate buffer

- 38.16g/L di-sodium tetraborate ($Na_2B_4O_7.10H_2O$)

- 6.18g/L boric acid (H_3BO_3)
- add disodium tetraborate solution to the boric acid solution until pH 7.4 is reached

10mM HEPES (N-2-hydroxyethyl piperazine-N-2 sulphonic acid) buffer

- make up 10mM solution in 0.01M PBS (2.38g/L), filter sterilise before use

Propridium iodide

- 5mg propridium iodide
- 100mg sodium citrate
- Dissolve in 100ml Milli-Q® water

Imaging buffer (50ml)

- 1.55ml NaCl – 234g/L stock solution
- 250µl KCl – 149.2g/L stock solution
- 5µl $CaCl_2$ – 294g/L stock solution
- 25µl $MgCl_2$ – 406.6g/L stock solution
- 750µl Dextrose – 360.4g/L stock solution
- 625µl HEPES – 520.6g/L stock solution

Diluent

- 0.3% Triton X-100 in 0.01M PBS

0.1M Phosphate buffer

- 216ml dibasic sodium phosphate ($Na_2HPO_42H_2O$) – 35.6g/L

- 84ml monobasic sodium phosphate ($NaH_2PO_42H_2O$) – 20.76g/L
- 300ml Milli-Q® water
- pH 7.4

Supplementary movie 1 At 21 days in vitro, an individual bundle of axons were targeted with a single pulse of fluid, and the resultant deflection was captured by video camera. In this movie, the axon bundle is rapidly stretched, before recovering to its original position. For reference, the maximal increase in original axon length of this bundle caused by the deflection was 3%.

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