Molecular and Cellular Events Underlying the Response of CNS Neurons to Structural Injury

By

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Declaration

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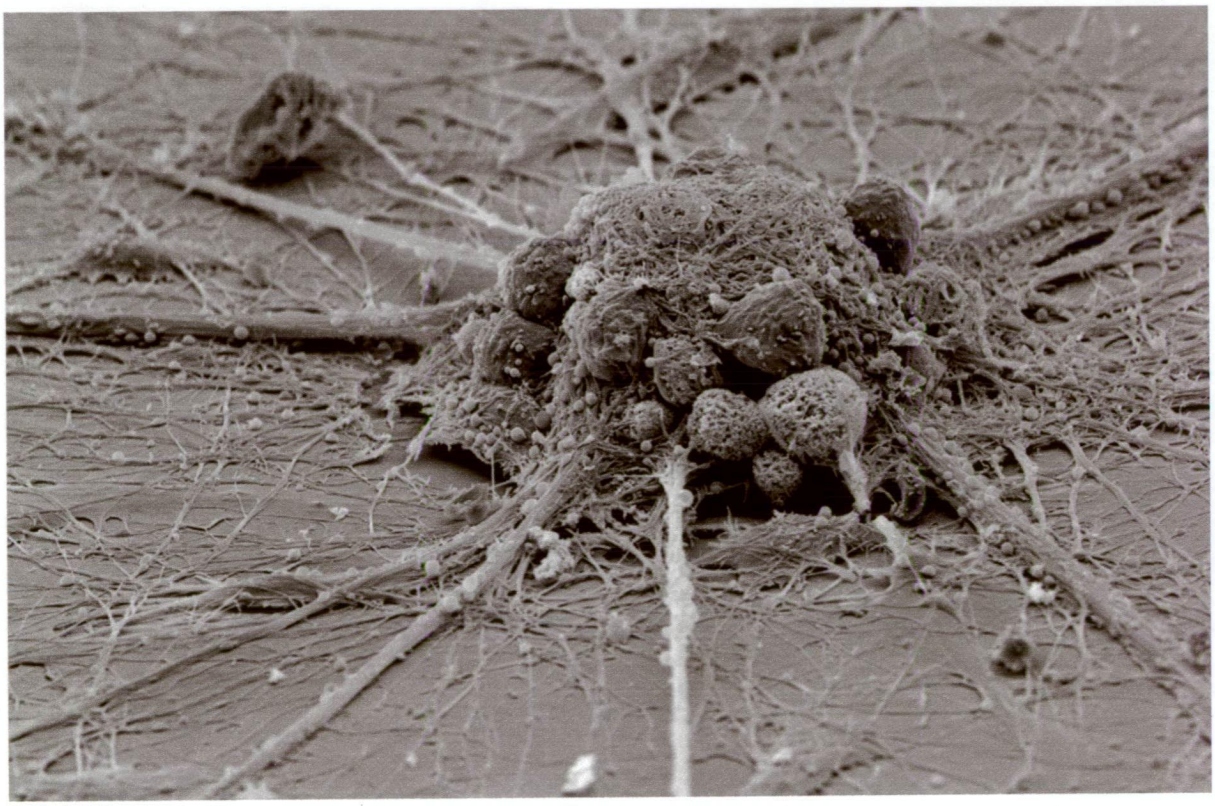
Scanning electron microscope image of an aggregate of neocortical neurons grown to relative maturity. Following axotomy these neurons demonstrate a remarkable sustained regenerative attempt.



Publications

Part of the work contained in this thesis has been published or submitted for publication as detailed below:

1. **Chuckowree J.A.**, Dickson T.C., Chopin S., and Vickers J.C. (2005) Proliferating and progenitor cells contribute to brain healing following localised structural injury to the rodent neocortex. *Neuroscience* - Submitted.

2. Haas M.H., **Chuckowree J.A.**, Chung R.S., Vickers J.C., and Dickson T.C. (2005) A role for ezrin-radixin-moesin (ERM) proteins in neuron translocation and migration. *Experimental Neurology* – submitted.

3. **Chuckowree J.A.,** Dickson T.C., Chuah M.I., West A.K., and Vickers J.C. (2005) α-Internexin immunoreactivity reflects variable neuronal vulnerability in Alzheimer's disease and supports the role of the ß-amyloid plaques in inducing neuronal injury. *Neurobiology of Disease*, 18 (2): 286-295.

4. Woodhouse A, West A.K., **Chuckowree J.A.**, Vickers J.C., Dickson T.C. (2005) Does beta-amyloid plaque formation cause structural injury to neuronal processes? *Journal of Neurotoxicity Research*, 7(1-2): 5-15.

5. Chung R.S., Staal J.A., McCormack G.H., Dickson T.C., Cozens M.A., **Chuckowree J.A.**, Quilty M.C., and Vickers J.C. (2005) Mild axonal stretch injury *in vitro* induces a progressive series of neurofilament alterations ultimately leading to delayed axotomy. *Journal of Neurotrauma*, 22(10): 1081-1091.

6. **Chuckowree J.A.**, Dickson T.C., and Vickers J.C. (2004) Intrinsic regenerative ability of mature CNS neurons. *The Neuroscientist*, 10(4): 280-285.

7. **Chuckowree J.A.,** and Vickers J.C. (2003) Cytoskeletal and morphological alterations underlying axonal sprouting after localized transection of cortical neuron axons *in vitro*. *Journal of Neuroscience*, 23(9): 3715-3725.

Abstract

The extent of effective regeneration in the adult mammalian brain following structural injury has been an issue of contention for the last several decades. Historical views proposed that the adult brain was set in a state of stasis, preventing effective regenerative alterations and functional recovery following brain trauma. The irreversible loss of function following injury to the adult mammalian brain has been attributed to an intrinsic inability of damaged neurons to re-initiate growth following injury, which is further compounded by a non-facilitative environment. However, accumulating evidence, based on diverse models of experimental neuronal and brain lesion, have challenged two major dogmas in neuro-repair research, namely, that neurons from the adult mammalian brain are incapable of intrinsically driven regeneration, and that neurogenesis is strictly restricted to developmental periods. Collectively, these studies have provided compelling evidence indicating that the adult brain may possess a vast intrinsic capacity for repair following injury. Furthermore, strategies aimed at facilitating neuronal replacement or re-establishing lost neuronal connections have made remarkable inroads into understanding the potential for injury-induced plasticity in the adult brain.

Despite these advances, however, no effective treatment currently exists to target the full repertoire of pathological alterations that contribute to permanent loss of function following acquired brain injury. A broader understanding of the specific molecular and cellular events responsible for the limit in brain repair is still required, particularly for therapeutic interventions to effectively complement and enhance endogenous brain repair mechanisms. This thesis, therefore, sought to address three

specific aspects associated with the intrinsic capacity for brain and neuronal repair following structural injury. Initially, the neurogenic potential of the injured adult brain was evaluated in an experimental model of structural brain injury by examining populations of proliferating and progenitor cells, to determine whether these cell populations have the capacity to undergo neuronal differentiation and contribute to neuronal replacement in injured brain tissue. Secondly, the reactive and regenerative alterations associated with the neural response to structural brain injury was investigated in a range of neuronal and glial cell populations to determine particular alterations that may be indicative of neuronal regeneration and brain healing. Finally, utilising an *in vitro* model of axonal injury in which neurons can be studied in relative isolation, free of compounding glial responses, the intrinsic regenerative potential of individual mature brain neurons was determined and the mechanisms underlying this response were characterised through comparison with developing neurons and application of agents that specifically disrupt the cytoskeleton.

Results from this study highlighted several important aspects of the neural response to injury indicating that, rather than responding passively, the adult brain mounts an adaptive repair process. These alterations involved the coordinated activation of both neuronal and glial cell populations, which ultimately resulted in the restoration of relatively normal cytoarchitecture. Specifically, adaptive injury-induced alterations included the activation of various cell populations, particularly neural progenitor cells, astrocytes and microglia, which may contribute to brain healing; evidence of re-vascularisation surrounding the lesion site; and regenerative neuronal changes, such as a profuse axonal sprouting response and an up-regulation of regeneration-associated genes. In summary these findings indicate that the adult

mammalian brain possesses a remarkable intrinsic capacity for repair following injury and highlight several aspects of this response that may be therapeutically targeted to enhance brain repair following injury.

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Abbreviations

AD	Alzheimer's disease
APP	amyloid precursor protein
ANOVA	analysis of variance
BDNF	brain derived neurotrophic factor
BrdU	5-Bromo-2'-deoxyuridine
CO_2	carbon dioxide
CERAD	Consortium to Establish a Registry for Alzheimer's disease
°C	degrees Celsius
CNS	central nervous system
CsA	Cyclosporin A
DAI	diffuse axonal injury
DIV	days *in vitro*
DCX	doublecortin
DNs	dystrophic neurites
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
FDA	Federal Drug Administration
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
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
NIFID	neuronal intermediate filament inclusion disease
NMDA	N-methyl-D-aspartate
PBS	phosphate buffered saline
PFA	paraformaldehyde
PI	post-injury
PNS	peripheral nervous system
RT-PCR	reverse transcriptase polymerase chain reaction

Sca	slow component a
Scb	slow component b
SE	standard error of the mean
SVZ	subventricular zone
TBI	traumatic brain injury
VZ	ventricular zone

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General background and introduction

1.1 Overview

Traumatic brain injury (TBI) is the most common cause of death and disability in individuals under the age of 45 years in developed nations, and places an enormous socio-economic and medical burden upon society. A common consequence of trauma to the head is widespread axonal disruption, or diffuse axonal injury (DAI), in which neural circuitry is disrupted or lost throughout the brain. Importantly, the majority of neurons within the mature mammalian brain are present at birth and do not divide. Thus, the loss or disruption of even a small number of neurons may result in the interruption of thousands of neural connections and widespread dysfunction of the circuits into which they integrate.

Head trauma frequently results in both primary and secondary brain damage. Primary damage results from mechanical forces that create deformation of blood vessels, neurons and glia at the time of the injury. Primary damage may subsequently evoke secondary alterations, such as ischemia, hypoxemia, swelling, raised intracranial pressure and infection. These changes generate an evolving pathology. The ultimate outcome of this process is relative the severity of the initial injury. Severe injuries generally result in axonal tearing at the time of trauma, however, more commonly axons are deformed by the initial insult and undergo reactive changes that result in axonal disconnection by a delayed process. Numerous cell populations are activated in response to brain injury. However, whether these cells play a largely inhibitory or facilitative role in the post-injury sequela remains to be fully elucidated.

Historically, regeneration in the mature mammalian brain was believed to be impossible. However, an accumulating body of literature from the last several decades indicates that the damaged brain may instigate active repair processes. Indeed, several recent studies have indicated that the mammalian brain retains remarkable plasticity into adulthood. From a neuronal perspective, regenerative attempts may manifest as the elaboration of new axons or axonal sprouts from pre-existing neurons and/or the generation and integration of new neurons from resident populations of neural progenitor or precursor cells. Whether injury-induced axonal sprouting and neurogenesis result in the restoration of neural connectivity and functionality remains contentious and it is generally agreed that if the repair process is purely reliant on endogenous capacity, regeneration is limited.

Endogenous adaptive and compensatory mechanisms may allow partial restoration of neural tissue. Additionally, various strategies are utilised to slow the progression of detrimental brain alterations following trauma. However, no treatment currently exists that effectively replaces or regenerates damaged neurons, re-establishes neuronal connectivity and restores function. It is likely that such a therapy would be multi-target based, to include appropriate inhibition or enhancement of the various factors that are required for successful regeneration. In this regard, research efforts are now being aimed at unravelling the reasons why endogenous brain repair is limited and ways in which this can be extrinsically manipulated through, for example, manipulation of homeostatic mechanisms, application of growth factors or other therapeutic agents, inhibition of known regeneration inhibitors and transplantation of peripheral nerve grafts or neural progenitor cells.

Whether these experimental approaches will translate into clinical treatments for head trauma remains to be elucidated. However, in the advancement of successful treatments for brain injury it is imperative to consider the possibility of maladaptive responses, which have already been demonstrated following various forms of brain lesion. To this end, this thesis will seek to investigate how the mature mammalian brain responds to focal cortical injury and how individual neocortical neurons undergo potential regenerative adaptation. The findings from this thesis will be discussed in regard to an accumulating literature supporting both adaptive and maladaptive neural responses following neuronal injury.

1.2 The mammalian brain

Comprised of literally hundreds of billions of highly specialised neuronal and glial cells, the brain is the most complex, intricate, and highly organised organ in the human body. Thus, it is intriguing to imagine that the mammalian brain begins as a layer of neuroepithelial cells surrounding a fluid-filled ventricular compartment (Conover and Allen, 2002). The relatively large size and high complexity of the adult human brain, and particularly the cerebral cortex, is responsible for providing distinctively human mental capabilities. In this regard, one of the most remarkable features of the human brain is the convoluted surface of the cerebral hemispheres, which provide the structural basis for higher brain functions that set humans ahead of any other animals in terms of mental capacity (Nolte, 2002). The brain can be broadly divided into grey and white matter. Grey matter consists predominantly of neuronal cell bodies and dendrites and is highly vascularized, whereas white matter is composed primarily of axons, the majority of which are ensheathed by specialised central nervous system (CNS) myelinating glial cells.

1.2.1 Organisation of the adult mammalian neocortex

Although the cerebral cortex is just a few millimetres thick, it is comprised of a phenomenal number of neurons, estimated to be around 25 billion, and comprises up to 80% of the human brain (Nolte, 2002; Markram et al., 2004). As illustrated in Figure 1.1, the neocortex is a six-layered structure, denoted layer I through VI, from the most superficial to deep layer (Nolte 2002; Silberberg et al., 2005). The cortical laminae are generated by variations in cell body size and density throughout the cortical depth (Parnavelas, 2000). Each layer contains a different complement of

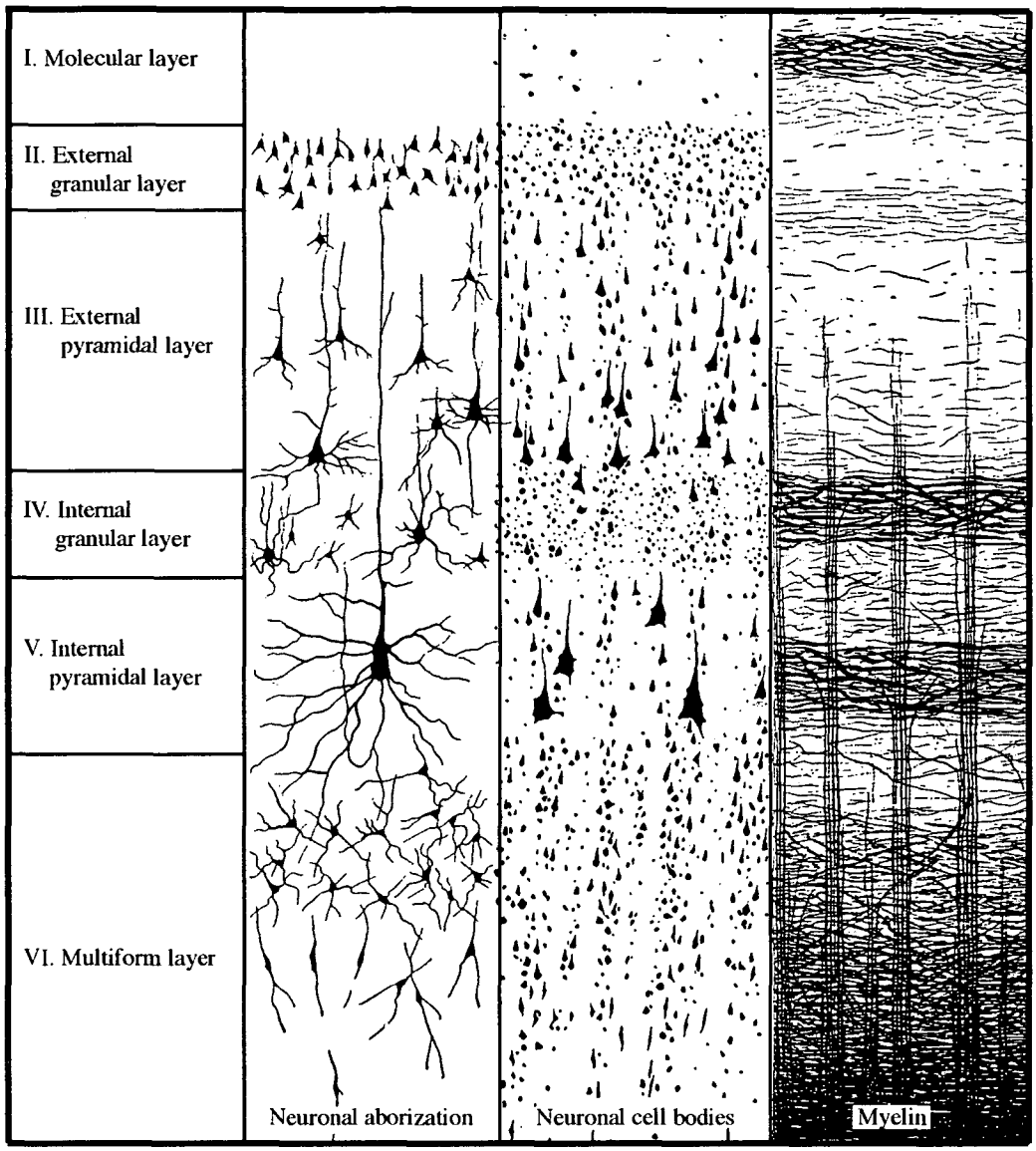


Figure 1.1 The six layers of the mammalian neocortex

The diagram above depicts the six layers of the neocortex, demonstrating neuronal arborisation, packing density of neuronal cell bodies and the distribution of myelin within each of the layers. As illustrated the cortical laminae can be defined based on the predominant cell type within each layer.

Adapted from Nolte J: The Human Brain, New York, 2002, McGraw-Hill

neurons, which can be loosely divided into two groups based primarily on their morphological properties: pyramidal cells and cortical interneurons, or non-pyramidal cells (Peters and Jones, 1984). Pyramidal neurons possess a pyramidal-shaped soma, from which a prominent apical dendrite is elaborated and extends up towards the pial surface. Basal dendrites extend laterally from the base of the soma. The axon of pyramidal cells, which is usually myelinated, generally extends from the base of the soma, opposite to the apical dendrite, but may arise from a basal dendrite (Feldman, 1984). Thus, pyramidal cells have extensive dendritic arborisations that elaborate specialised protrusions (dendritic spines), large cell bodies and a long projecting axon. Interneurons incorporate a sub-population of cortical neurons with a vast array of morphological features and molecular identities (Parnavelas, 2000). Interneurons are generally identified by the absence of a prominent apical dendrite and the presence of few, if any, dendritic spines - although specific sub-types of interneurons may have dendritic spines equivalent to pyramidal neurons (Fairen et al., 1984; Markram et al., 2004). Thus, interneurons have less extensive dendritic arborisations, a short, locally projecting axon and a small cell body (Hof et al., 1999; Nolte, 2002; Markram et al., 2004). Pyramidal neurons are the primary glutamatergic excitatory cells of the neocortex, whereas interneurons predominantly provide inhibitory input (Fairen et al., 1984; Peters and Jones, 1984; Markram et al., 2004).

The layers of the neocortex can be distinguished based on the predominant cell population (Figure 1.1). Interneurons exist in all layers of the neocortex, although the distribution of a specific interneuron sub-type may vary between cortical laminae

(Hof et al, 1999; Markram et al., 2004). Conversely, pyramidal neurons are primarily contained within layers II to VI and comprise approximately 70-80% of all neocortical neurons (Peters and Jones, 1984; Markram et al., 2004; Voelker et al., 2004; Silberberg et al., 2005). Layer I, the molecular layer, is relatively cell free. Layers II and IV, the external and internal granular layers are comprised of relatively small locally projecting granular or stellate cells, whereas layers III and V contain characteristic large cortical pyramidal cells and are thus designated the external and internal pyramidal layers, respectively. The pyramidal cell layers contain predominantly neurons that project long axons to other regions of the CNS. The axons of pyramidal neurons in layer III terminate on neurons in other regions of the ipsilateral and contralateral hemisphere, termed intra- and inter-hemispheric connections respectively, whereas axons of pyramidal neurons in layer V project to more distant targets, terminating on neurons outside the cerebral cortex or in subcortical areas (Silberberg et al., 2005). The innermost layer, layer VI, is comprised of modified fusiform-shaped pyramidal cells. In addition to the laminar the organisation of the neocortex, the neocortex also has a distinct columnar organisation with several structures, including the apical dendrites of pyramidal cells, cortical afferents, axons of some intra-cortical cells and the cell bodies of cortical neurons, arranged perpendicular to the cortical surface.

Synaptic transmission within the neocortex follows a distinct and highly complex pattern, with integration both within and between the cortical laminae (reviewed by Silberberg et al., 2005). Briefly, thalamic input primarily enters layer IV and is passed onto layer III pyramidal cells that, in turn, project to layer II. Layers II and

III also receive input from association brain regions and provide descending input to layer V pyramidal cells. Depending on their type, layer V pyramidal neurons either project to subcortical regions, comprising the major source of neocortical output, or to the contralateral hemisphere. Layer V pyramidal cells also project to layer VI, forming both cortico-cortical interconnections and corticothalamic feedback circuits. A majority of neocortical pyramidal cells also project their dendrites into layer I of the neocortex, forming an additional level of cortical processing and integration. Interactions between cortical pyramidal cells and interneurons facilitate highly integrated cortical processing.

1.2.2 Development of the mammalian neocortex

Extensive and tightly regulated patterns of neuronal proliferation and migration are responsible for transforming the dorsal embryonic forebrain into the adult cerebral cortex (Noctor et al., 2004). Corticogenesis, the process of development of the cortex, has been widely described (for example see reviews by Frisén et al., 1998; Parnavelas, 2000; Nadarajah and Parnavelas, 2002; Honda et al., 2003; Kubo and Nakajima, 2003; Marshall et al., 2003).

1.2.2.1 Origin, source and division of neocortical destined neurons

The cortex develops from a highly proliferative neuroepithelium during embryonic development. Depending on their type, cortical neurons are generated in two proliferative regions of the embryonic cortical telencephalon. As embryonic development proceeds, a pseudo-stratified region in the neuroepithelium lining the lateral ventricles, known as the ventricular zone (VZ), primarily gives rise to

excitatory cortical projection neurons (Noctor et al., 2002). Additional neurons, as well as postnatally derived glial cells, are generated in the subventricular zone (SVZ), located adjacent to the VZ (Noctor et al., 2002). Conversely, cortical interneurons are predominantly generated in a specialised region in the ventral telencephalon of the developing brain, the ganglionic eminence, which forms the basal ganglia of the adult brain (Parnavelas, 2000).

All cortical neurons differentiate from precursor cells within specific regions of the embryonic brain, which are capable of generating astrocytes, oligodendrocytes and neurons (Figure 1.2). Importantly, neural precursor cells may undergo different forms of division depending on developmental stage and the daughter cells to be generated (reviewed by Frisén et al., 1998) (Figure 1.3). In this regard, symmetrical progenitor cell divisions generate new progenitor cells and expand the progenitor population, asymmetrical progenitor cell divisions generate single neurons and symmetrical terminal divisions generate two neurons, but deplete the progenitor population (Noctor et al., 2004).

1.2.2.2 Development of the neocortex proceeds through distinct stages

The ventricular neuroepithelium initially spans the entire cortex and undergoes rapid thickening and differentiation into distinct zones during corticogenesis. As displayed in Figure 1.3, development of the mammalian cerebral cortex occurs in two distinct stages (reviewed by Frisén et al., 1998; Parnavelas, 2000; Nadarajah and Parnavelas, 2002; Honda et al., 2003; Kubo and Nakajima, 2003; Marshall et al., 2003). Initially, the first generation of post-mitotic neurons moves out of the VZ to margin

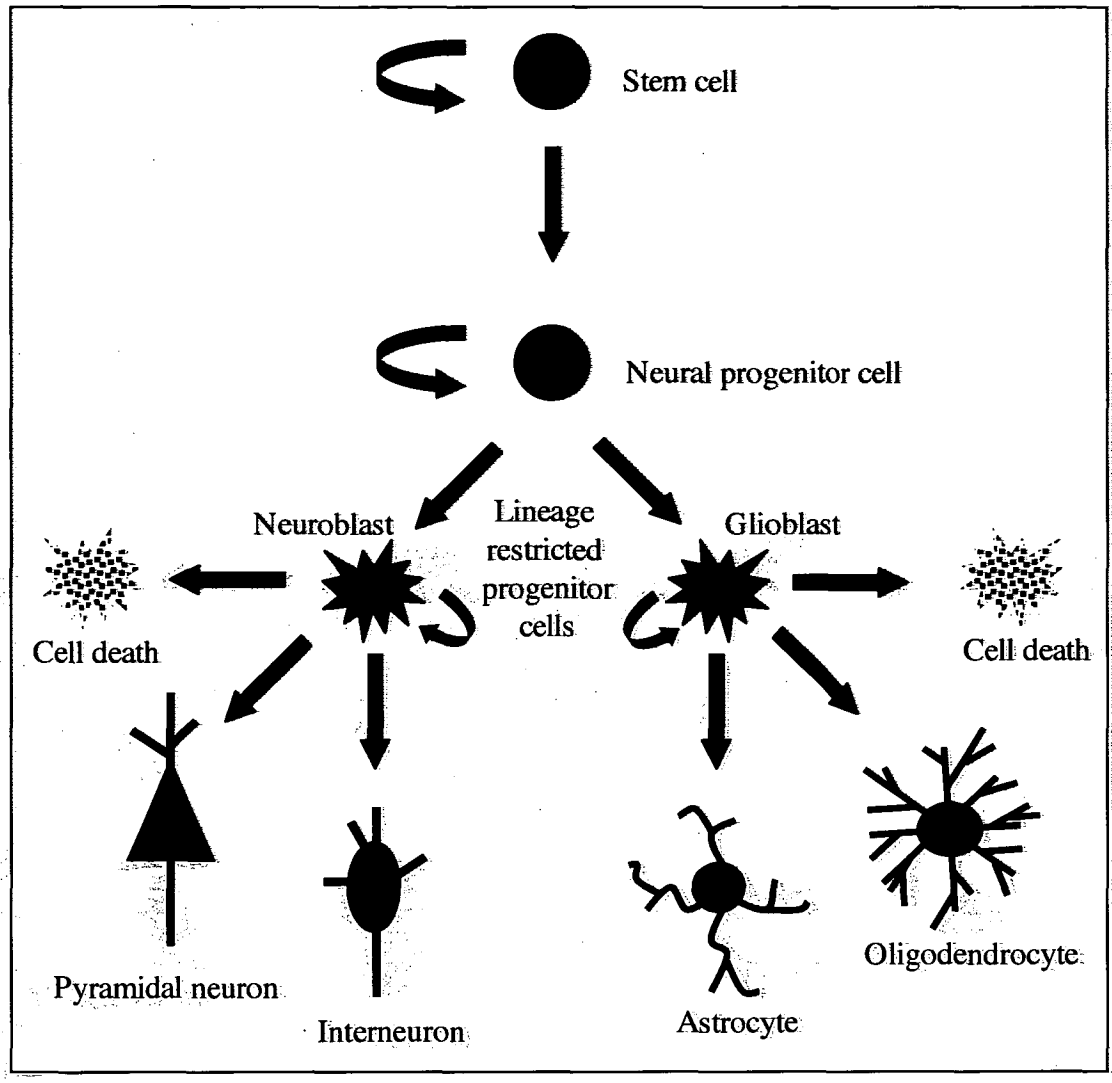


Figure 1.2 Diagrammatic representation of stem cell differentiation in the CNS

Stem cells are multi-potent cells, capable of self-renewal. In defined regions of the embryonic and adult CNS, these cells continually produce neural progenitor cells capable of differentiating into the three primary neural cell types, namely neurons, astrocytes and oligodendrocytes. Stem and progenitor cells contribute to cell turnover throughout life. However, the generation and integration of new neurons is restricted to discrete regions of the adult mammalian brain under normal conditions, but may be influenced by a variety of environmental and other factors, some of which include injury, exercise and stress.

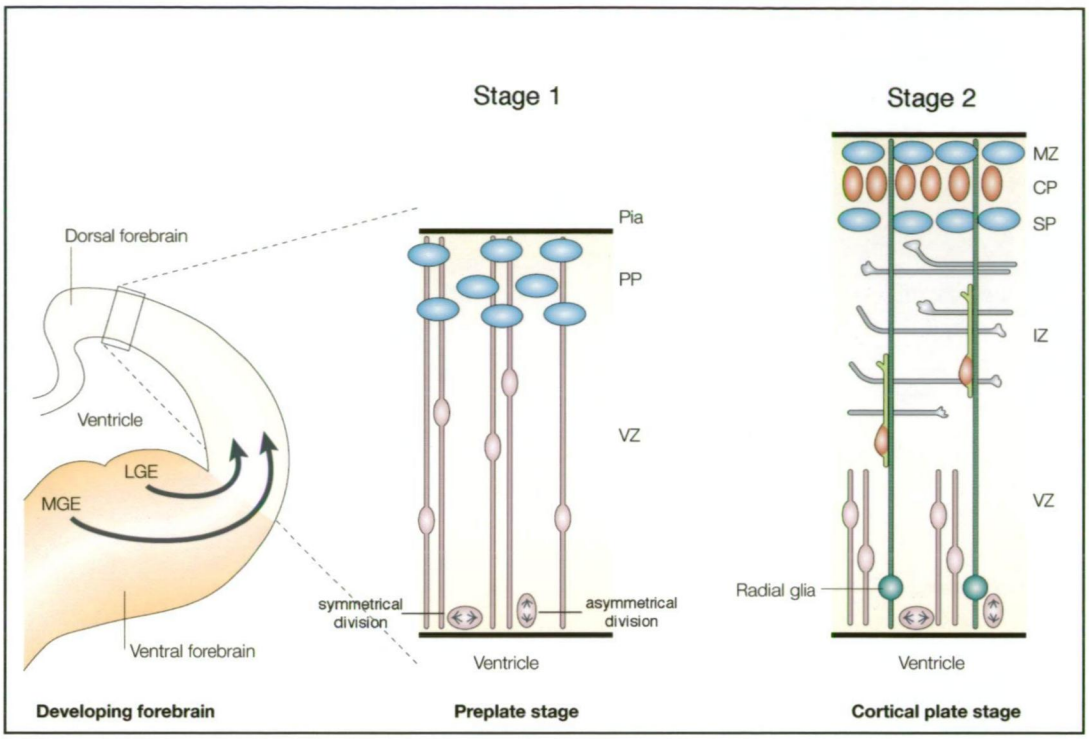


Figure 1.3 Development of the mammalian neocortex

In embryonic development the dorsal forebrain gives rise to the neocortex. Progenitors destined to differentiate into pyramidal neurons are derived from the ventricular zone (VZ), whereas progenitors destined to differentiate into cortical interneurons are predominantly derived from the medial and lateral ganglionic eminences (MGE and LGE, respectively) in the ventral forebrain. Interneuron progenitors migrate tangentially to the dorsal forebrain. The neocortex develops in 2 distinct stages. In stage 1, post-mitotic neurons migrate to the pial margin of the cerebral wall, forming a pre-plate (PP). In stage 2, the pre-plate splits to form the marginal zone (MZ), cortical plate (CP) that will form layers II to VI of the neocortex, and sub-plate. Neurons reach their destination in the cortical plate by radial migration, either by somal translocation or by glial-guided locomation along the fibres of radial glial cells. The cortex is generated in an inside-out fashion.

Adapted from Nadarajah B and Parnavelas J: Modes of neuronal migration in the developing cerebral cortex: Nature Reviews Neuroscience, 2002, 3, 423-432.

of the cerebral wall, to form a pre-plate adjacent to the VZ.

The pre-plate subsequently splits to form the marginal zone, cortical plate and sub-plate. The cortical plate is the primary destination of newly generated migrating neurons and is bordered at its upper and lower margins by the marginal zone and sub-plate, respectively. The marginal zone forms at the pial surface. It contains specialised pioneer neurons, or Cajal-Retzius cells, and will ultimately form layer I of the adult cortex. Below this, the cortical plate is generated, which will eventually constitute cortical layers II through to VI. During corticogenesis, migrating neurons take up their position in the cortical plate in a deep to superficial pattern. In this regard, the cortex is generated in an inside-out fashion. The intermediate zone will ultimately comprise the sub-cortical white matter. Importantly, although the cortical layers are largely established during gestation, neuronal connectivity and myelination continue well beyond birth (Nolte, 2002).

Towards the end of neurogenesis, an additional zone, the SVZ, is generated between the ventricular and intermediate zones. This zone harbours precursor cells that, in the perinatal period, give rise predominantly to glial cells including astrocytes and oligodendrocytes and in adulthood give rise to specific populations of migrating neuronal progenitors, largely destined for the olfactory bulb (Marshall et al., 2003). Expansion of the SVZ in late gestation and early postnatal life is concomitant with the disappearance of the VZ (Nadarajah and Parnavelas, 2002).

1.2.2.3 Modes of neuronal migration in the developing neocortex

Patterns of neuronal migration in the embryonic cortex are an intense area of research. Nadarajah and Parnavelas (2002), in addition to Kriegstein and Noctor (2004) have recently provided excellent reviews summarising these data, as detailed below. Interestingly, the migration of neurons form the VZ to their final position in the cortex is not direct and has been shown to involve four distinct phases (reviewed by Kriegstein and Noctor, 2004). In phase one, cells migrate from their birth-place in the VZ to the SVZ. Cells then become multipolar and pause in the SVZ or lower intermediate zone (phase two). Some neurons subsequently undergo retrograde movement towards the ventricle (phase three), prior to radial migration to their final destination in the cortical plate (phase four). The primary mechanisms of neuronal migration thought to underlie corticogenesis are radial and tangential migration. Radial migration is the migration of neurons from the VZ towards the pial surface, whereas tangential migration involves cell movement that is parallel to the surface of the brain. Thus, radial migration largely dictates columnar cortical organisation, whereas tangential migration may underlie the development of increased cellular and laminar complexity (Park and Rao, 2002).

Radial migration is the primary method of migration in the developing cerebral cortex and can be sub-divided into two broad categories, somal translocation and glial-guided locomotion, which have recently been demonstrated by Nadarajah et al. (2001, 2003). Somal translocation is characterised by a fast rate of migration involving the attachment of a leading process to the pial surface and subsequent shortening and thickening of this process to draw the neuron to its final position in

the cortex (Kubo and Nakajima, 2003). This mode of migration is primarily utilised by early-born neurons (Nadarajah et al., 2001). Glial-guided locomotion predominantly involves the movement of cells destined to differentiate into cortical pyramidal cells. The rate of glial-guided locomotion is slower than that of somal translocation, involving a saltatory pattern of movement of migrating neurons along the radially extending fibres of radial glial cells, which traverse the entire thickness of the cortex (Nadarajah et al., 2001). Neurons migrating along radial glial cells demonstrate distinct bipolar morphology, with a thick leading and thinner trailing process (Kubo and Nakajima, 2003). Somal translocation may occur during early stages of cortical development, when the cortex is relatively thin and glial-guided migration may predominate at later stages, when the cortex is thicker and more difficult to navigate (Nadarajah and Parnavelas, 2002; Kubo and Nakajima, 2003) (Figure 1.3).

Accumulating data indicate that, in addition to their role in guiding migrating neurons, radial glial cells may have much broader functions. For instance, Noctor et al. (2002, 2004) have recently shown that a majority of cycling VZ cells have morphological, molecular and physiological features characteristic of radial glial cells and thus propose that radial glia have a unique role in the VZ, where they initially generate new neurons and subsequently guide their neuronal progeny along radially extending fibres during corticogenesis. This process has been implicated in the development of functional radial units within the neocortex, whereby sustained contact between radial glial cells and their progeny may form local micro-networks spanning the cortical laminae (Noctor et al., 2001). The formation of radial units is

proposed to underlie the increase in cortical surface area without comparable increase in thickness during mammalian evolution (Rakic, 2000).

Importantly, a third form of radial migration, termed multipolar migration has recently been discovered, characterised by net radial migration, interspersed with episodes of tangential migration (Tabata and Nakajima, 2003). This mode of migration might be incorporated into phase two of radial migration (Krigstein and Noctor, 2002). Nadarajah et al. (2003) have also described a sub-population of migrating cells they term 'branching cells'. These cells are characterised by multiple rapidly extending and retracting processes, similar to those described by Tabata and Nakajima (2003), and elaborate a branched leading process that, distinct from the somal locomoting cells does not contact the pial surface, but rather displays exploratory behaviour (Nadarajah et al., 2003). Branching cells may represent a sub-population of cortical interneurons (Nadarajah et al., 2003)

The second major form of neuronal migration in the developing cortex is tangential migration, whereby neurons (generally interneurons) move parallel to the brain surface along axons or other neurons. In this regard, interneurons derived from the ventral telencephalon ganglionic eminence migrate tangentially to the VZ and then radially to their finally resting position in the cortex (Figure 1.3). Collectively, studies of cortical neuron migration indicate that the processes of cortical development and neuronal migration are complex, involve transitions between distinct phases and may involve heterogenous patterns of migration for any single cell type (Tabata and Nakajima, 2003; Kriegstein and Noctor, 2004).

1.2.2.4 Regulation of neurogenesis and neuronal migration during corticogenesis

Proliferation and differentiation within the developing brain must be tightly regulated to ensure correct cell numbers and appropriate cell types are generated (Frisén et al., 1998). The proliferation, differentiation, migration and morphologic development of neural precursors is dependent upon spatio-temporal interactions between endogenous cellular processes and exogenous molecular signals, including a variety of growth factors, signalling molecules, hormones, extracellular matrix molecules, cell adhesion molecules and other cell surface proteins, soluble and membrane-bound factors and neurotransmitters (reviewed by Cameron et al., 1998; Park and Rao, 2002; Sobeih and Corfas, 2002; Honda et al., 2003; Guan and Rao, 2003; Hagg, 2005). Precursors become responsive and unresponsive to specific combinations of molecular signals as they proliferate, differentiate and mature. Exogenous signals may be generated by both the radial glial cells, along which neurons migrate and Cajal-Retzius cells in the marginal zone of the embryonic cortex (Soriano and Del Rio, 2005).

The process of neuronal migration during development is heavily reliant upon interactions between guidance cues in the micro-environment and alterations in the neuronal cytoskeleton. In this regard, migrating and differentiating neurons must appropriately translate guidance cues into cytoskeletal rearrangements. Some of these signals are common to the process of axon outgrowth and pathfinding (Park and Rao, 2002; Sobeih and Corfas, 2002). Involvement of the neuronal cytoskeleton during migration of cortical neurons has recently been summarised by Bielas and Gleeson (2003). Neuronal migration is sensitive to genetic mutations as well as a

variety of chemical, physical and biological factors. Thus, disruption of the various components involved in neuronal migration at different stages of cortical development can result in a variety of developmental malformations and related disorders (Golden, 2001; Bielas and Gleeson, 2003; Kubo and Nakajima, 2003).

1.2.2.5 Identity of neural stem cells and origin of adult-born neurons

The SVZ persists into adulthood as the largest site of neurogenesis in the adult brain (Conver and Allen, 2002). The SVZ is comprised of four major cell types; ependymal cells that line the ventricle, astrocytes, newly generated neuroblasts and transitory amplifying progenitor cells with immature phenotypes (Doetsch et al., 1997). The identity of the SVZ stem cells capable of supporting neurogenesis remains elusive (Barres, 1999; Morshead, 2004), but has been proposed as either the ependymal cell (Johansson et al., 1999) or the SVZ astrocyte (Doetsch et al., 1999). However, stem cells are essentially self-renewing multi-potent immature cells, contradictory to the notion that highly specialised cells such as astrocytes or ependymal cells form the stem cell population in the developing and adult brain.

Although some studies have suggested that ependymal cells may function as stem cells in the adult brain, recent studies have demonstrated that ependymal cells are generated from radial glial cells during embryonic and early postnatal development, are not a reservoir of adult stem cells and do not divide following differentiation (Spassky et al., 2005). Thus, processes of dedifferentiation, reprogramming and transdifferentiation may be involved in the elaboration of other mature cell phenotypes from mature ependymal cells and astrocytes (Wagers and Weissman,

2004; Conover and Allen, 2002), although this notion remains controversial (Wagers and Weissman, 2004). Importantly, neural stem cells may in fact be a sub-population of SVZ derived astrocyte- or ependymal-like cells (Conover and Allen, 2002). Due to the conflicting identification of SVZ stem cells and different methodologies utilised to perform these studies, further investigation is required to resolve this 'identity crisis' (reviewed by Morshead, 2004).

1.2.3 Cell types in the brain

Despite its complexity the brain is composed of just two principal cell types, neurons and glia. Neurons are the basic functional units of the brain, responsible for collecting, processing and conveying information. They are, however, unable to function effectively without a supportive glial milieu.

1.2.3.1 Glial cells

An array of glia cell populations, including astrocytes, oligodendrocytes, microglia, and ependymal cells, are intimately associated with neurons and perform important roles during development, maturation, plasticity and repair of the CNS. During development glial cells provided scaffold and guidance support to growing neurons. In the adult, these cells are responsible for performing a variety of nutritive, physiological, and structural functions. The predominant CNS grey and white matter glial populations, astrocytes and oligodendrocytes respectively, vastly outnumber neurons in a ratio of approximately 10:1 (Nolte, 2002).

1.2.3.1.1 Astrocytes

Astrocytes may be divided into two major populations – protoplasmic astrocytes reside in the grey matter and fibrous astrocytes reside in the white matter – however, both types have essentially the same features. Astrocytes are stellate-shaped cells with a well defined cytoskeleton and small cell body (3-5μm). Astrocytes play important structural and homeostatic roles in the brain (Nolte, 2002). They provide neurons with structural support and contribute to formation of the blood brain barrier. Astrocytes also play crucial roles in CNS metabolism and in homeostatic regulation of the ionic and molecular environment, for example they regulate extracellular potassium concentration, are involved in neurotransmitter uptake and produce neurotrophic factors, which contribute to neuronal development and are implicated in the neural response to injury. Astrocytes perform particular roles following brain injury, where they undergo a stereotypical sequence of reactive alterations referred to as reactive gliosis, culminating in the formation a glial scar at the lesion margin (Maxwell et al., 1997; Raivich et al., 1999). This structure acts to segregate the damaged from non-damaged tissue, but may inhibit neuronal regeneration (Fawcett and Asher, 1999).

1.2.3.1.2 Oligodendrocytes

Oligodendrocytes are the predominant glial cell type within the CNS white matter, where they form myelin sheaths that encase axons and aid in axonal conduction. Oligodendrocytes also have small cell bodies (1-3μm) and elaborate an array of branched projections (Nolte, 2002). Although an individual oligodendrocyte myelinates numerous axons, each axon is only myelinated by a single

oligodendrocyte. This is contrary to the peripheral nervous system (PNS) where several Schwann cells are responsible for the myelination of a single axon (Totora and Grabowski, 1996). CNS myelin, contrary to myelin in the PNS, contains factors inhibitory to axonal growth, which may inhibit axonal regeneration following injury (Fawcett and Asher, 1999; Bandtlow and Schwab, 2000; Watkins and Barres, 2002), but may confine developing neurites to correct trajectories during development (Nagashima et al., 1999; Goldberg and Barres, 2000).

1.2.3.1.3 Microglia

Microglia, or CNS-resident macrophages, are smaller than astrocytes and oligodendrocytes and are viewed as the primary immune cell type of the CNS. The origin of microglia is still disputed, with studies suggesting that they may arise from mesodermal, neuroectodermal or monocytic sources (reviewed by Cuadros and Navascués, 1998; Kaur et al., 2001). Following entry into the CNS, microglial precursors undergo a defined sequence of events involving dispersion and differentiation to generate a brain-resident population of cells (Cuadros and Navascués, 1998; Navascués et al., 2000). Microglia develop a ramified morphology as they settle within the brain and form a population of brain-resident macrophages (Barron, 1995; Navascués et al., 2000). During brain development, microglia function as phagocytic cells that remodel the developing tissue (Barron, 1995), whereas in the adult brain they are believed to participate in dynamic immune-surveillance functions (Nimmerjahn et al., 2005). Microglia play a distinct and crucial role in the neural response to injury where they migrate to sites of injury, multiply and develop into brain macrophages responsible for clearing neuronal

debris and destroying pathogens (Thomas, 1992; Lotan and Schwartz, 1994; Aihara et al., 1995; Barron, 1995; Ravivich et al., 1999). Macrophages may also be extruded from circulation in response to injury (Kaur et al., 2001).

1.2.3.1.4 Ependymal cells

Ependymal cells line the ventricles of the brain and central canal of the spinal cord. Regions of the ventricular epithelium form specialised secretory tissue, which is associated with invaginations of small blood vessels. This tissue is known as the choroid plexus and is responsible for producing cerebrospinal fluid. The ependymal cells lining the ventricles extend cilia into the ventricular cavity, which contribute to cerebrospinal fluid circulation (Nolte, 2002).

1.2.3.2 Neural progenitor cells

In the mature brain, neural progenitor cells are a population of precursor cells that give rise to the restricted cell phenotypes, which are specific to the CNS. Unlike many other organs, the mature mammalian brain is largely regarded as a relatively stable organ, which undergoes limited cell, and particularly neuronal, turnover. Although cell proliferation is widespread in the developing brain, just two primary proliferative zones are retained in the adult brain, the SVZ of the anterior lateral ventricles and the dentate gyrus of the hippocampus (Doetsch and Hen, 2005). The SVZ, in particular, is viewed as the principal germinal region of the adult brain and is comprised of neural progenitor cells capable of differentiating into neurons, astrocytes and oligodendrocytes (Doetsch et al., 1997, 1999; Conover and Allen, 2002; Doetsch, 2003; Marshall et al., 2003; Mignone et al., 2004; Abrous et al.,

2005; Doetsch and Hen, 2005). Additionally, neural progenitor cells capable of extensive migration and integration have been demonstrated in several other brain regions (reviewed by Emsley et al., 2005).

1.2.3.3 Neurons

Neurons are the information processing units of the brain, and nervous system in general. Electrical and chemical signals are used to convey information both within and between individual neurons. The process of information collection, integration, and transmission is reflected by a highly specialised morphological organisation. Thus, neurons are functionally and anatomically polarised to conduct electrical signals in one direction and, although neurons vary vastly in shape and size, all neurons have the same basic morphological features. The soma, varying in size from 5 to 100μm in diameter, supports the metabolic and synthetic requirements of the rest of the neuron (Nolte, 2002). Specialised extensions of the soma, collectively referred to as neurites, constitute neural circuitry. Dendrites are branched neurites, with a distinct proximal to distal taper, which increase the cell surface area for receiving information. Regions of the dendritic membrane, post-synaptic densities, are specialised to receive synaptic input. Some dendrites, particularly, but not exclusively, those of pyramidal cells, possess specialised dynamic membrane protrusions, or dendritic spines, which contain post-synaptic densities (Ethell and Pasquale, 2005). Dendritic spines are variable in both morphology and relative permanence (Knott et al., 2002; Ethell and Pasquale, 2005). Neurons also elaborate a single slender process, the axon, which is responsible for conveying electrical signals to target cells via specialised terminal synaptic boutons.

1.2.3.3.1 The neuronal cytoskeleton

The characteristic and elaborate neuronal morphology is dictated and maintained by the neuronal cytoskeleton, a rigid yet highly dynamic intricate intracellular scaffold (Burgoyne, 1991; Bear et al., 2001). Owing to its dynamic nature the neuronal cytoskeleton is implicated in several fundamental cellular functions such as mitosis, differentiation, motility, intracellular transport and synaptic transmission (Hirokawa, 1991; Maccioni and Cambiazo, 1995). As displayed in Figure 1.4, the neuronal cytoskeleton is comprised of three fibrillar systems, namely microtubules, actin microfilaments and neurofilaments. Although these three systems may be viewed as discrete entities, they are intimately associated and undergo highly regulated interactions to function as a single organelle (Burgoyne, 1991). Immediately underneath the axolemma lies the cortical cytoskeleton, a meshwork of actin filaments and an array of associated proteins. Deeper within the axoplasm is a core cytoskeleton composed of interwoven microtubules and neurofilaments, which are linked by an assortment of cross-linking and other related proteins (Nixon, 1991).

Intriguingly, up to 99% of a neuron's cytoplasm may be contained within the axon (Nixon and Sihag, 1991; Nixon and Shea, 1992; Nixon, 1998). However, the machinery for protein synthesis, Nissl substance, is generally excluded from this neuronal compartment. Axons, therefore, rely heavily on active axonal transport mechanisms to bi-directionally transfer molecules between somal and axonal compartments and within the axoplasm. The axonal cytoskeleton plays a pivotal role in axonal transport and itself must be transported into the axon. Axonally destined core cytoskeletal proteins (such as neurofilaments and tubulin) are predominantly

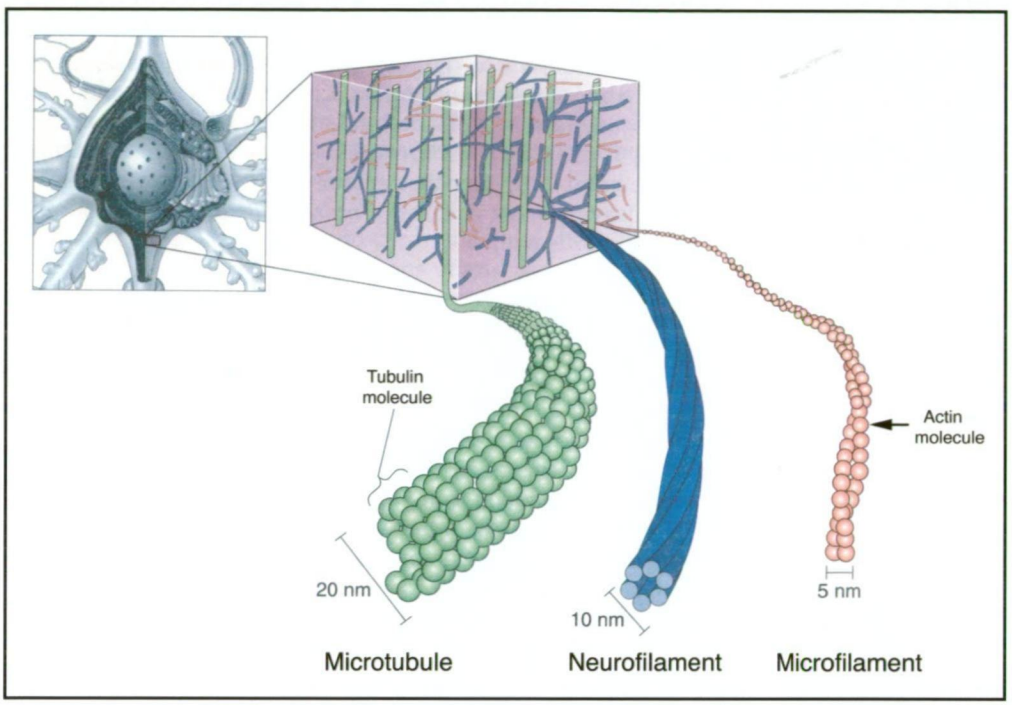


Figure 1.4 Diagrammatic representation of the neuronal cytoskeleton

The neuronal cytoskeleton is composed of three fibrillar systems, microfilaments, neuronal intermediate filaments (neurofilaments) and microtubules. Microfilaments are the smallest diameter cytoskeletal component and are composed of helically intertwined strands of polymerised actin molecules. Microtubules are tube-like structures, composed of tubulin molecules (consisting of α- and β-tubulin sub-units). Microtubules are the largest diameter cytoskeletal component. 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.

synthesised in the neuronal soma and subsequently transported into the axon via slow axonal transport, in a process known as slow component a (Sca) (Nixon, 1991), which relies on active metabolic processes (Galbraith et al., 1999). Slow component b (Scb) is the mode by which other cytoskeletal proteins and certain glycolytic enzymes are transported (Nixon, 1991). Membrane bound vesicles are transported by fast axonal transport mechanisms. Whether neurofilament and tubulin monomers are predominantly transported in a polymerised or un-polymerised form remains controversial (Lariviere and Julien, 2004). The axonal cytoskeleton is regionally specialised and locally regulated and as cytoskeletal proteins and assembled polymers are synthesised and transported into the axon, they continually exchange with those in stationary and slower moving cytoskeletal structures (Nixon, 1991).

1.2.3.3.1.1 Microfilaments

The microfilament cytoskeleton is a highly dynamic network of intermeshed actin filaments. Individual globulin, or G-actin, monomers polymerise to form filamentous, or F-actin. These filaments then form helical structures, referred to as microfilaments (Bear et al., 2001) (Figure 1.4). Actin filaments are capable of rapid assembly and disassembly and are predominantly localised to areas of high cell motility (Bamburg and Bernstein, 1991). The structure of the microfilament cytoskeleton is under the regulation of a variety of actin-associated proteins (Bamburg and Bernstein, 1991; da Silva and Dotti, 2002; Korey and Van Vactor, 2000). Moreover, the actin cytoskeleton and associated proteins link the extracellular matrix to intracellular signalling cascades via membrane-associated complexes, or focal adhesion and adherens junctions (da Silva and Dotti, 2002).

1.2.3.3.1.2 Microtubules

Microtubules are tube-like structures composed of tubulin (Hirokawa, 1991; Bear et al., 2001). Individual tubulin molecules, consisting of α- and β-tubulin, polymerise to form protofilaments. A total of 13 protofilaments aggregate around a hollow core to form a microtubule (Bear et al., 2001; Nolte, 2002). Microtubules are polarised and arranged longitudinally in neurites and radially in the soma. Microtubule positive ends always point to the periphery in the soma and distally in the axon, however, microtubules display mixed orientation within dendrites (Brandt, 1998). Microtubules participate in maintenance of neuronal morphology, motility, intracellular transport, modulation of interactions with cell surface receptors, cell attachment and neuronal plasticity (Burgoyne, 1991). Axonal microtubules have specialised functions in structural support, axonal transport and axonal growth (Nixon, 1991; Hoffman and Cleveland, 1988; Suter and Forscher, 2000).

An extensive network of microtubule-associated proteins (MAPs) coats the surface of microtubules. MAPs link microtubules to cytoskeletal and cellular structures (Tucker, 1990; Burgoyne, 1991; Hirokawa, 1991; Maccioni and Cambiazo, 1995; Mandelkow and Mandelkow, 1995; Drewes et al., 1998; Nixon, 1998) and are considered putative organisers of neuronal morphology (Matus, 1991). Through phosphorylation-dependent interactions, MAPs stabilise microtubules and regulate microtubule spacing (Maccioni and Cambiazo, 1995; Mandelkow and Mandelkow, 1995; Tanaka and Sabry, 1995). Notably, tau and MAP-2 are examples of MAPs, confined to the axonal and somato-dendritic compartments, respectively (Tucker, 1990; Byrgoyne, 1991; Hirokawa, 1991; Mandelkow and Mandelkow, 1995).

1.2.3.3.1.3 Neuronal intermediate filaments

Neurofilament triplet proteins, commonly referred to as neurofilaments, together with α-internexin, comprise the neuronal intermediate filament family of type IV intermediate filament proteins (Lariviere and Julien, 2004) (Figure 1.5A). Unlike the globular sub-units comprising microfilaments and microtubules, all neuronal intermediate filament proteins are comprised of filamentous proteins, which are coiled into a tight spring-like configurations, making them mechanically very strong (Bear et al., 2001). Whereas individual α-internexin filaments self-assemble to form homopolymers, neurofilaments are obligate heteropolymers, comprised of three sub-units designated NF-L, NF-M and NF-H based on their relative molecular masses (Nixon and Sihag, 1991; Shaw, 1991; Lee and Cleveland, 1994, 1996; Lariviere and Julien, 2004).

NF-M and NF-H subunits have distinctive carboxy terminal tail domains (or 'side-arms') that protrude from the core filament (Figure 1.5B), vary in length between subunits and undergo extensive phosphorylation (Steinert and Roop, 1988; Nixon and Sihag, 1991; Chertoff et al., 1995; Lee and Cleveland, 1996), which increases the angle between the core filament and side-arm to increase the spacing between individual neurofilaments (Nixon and Sihag, 1991; Shaw, 1991; Lee and Cleveland, 1996). Thus, neurofilaments are implicated in the control of axonal calibre (Hoffman et al., 1984, 1985, 1987; Sakaguchi et al., 1993; Julien, 1999; Hall et al., 2000; Perrone Capano, 2001), as well as the dynamic function of other cytoskeletal components (Julien and Grosveld, 1991; Julien and Mushynski, 1998). Neurofilaments form the most stable component of the neuronal cytoskeleton, but

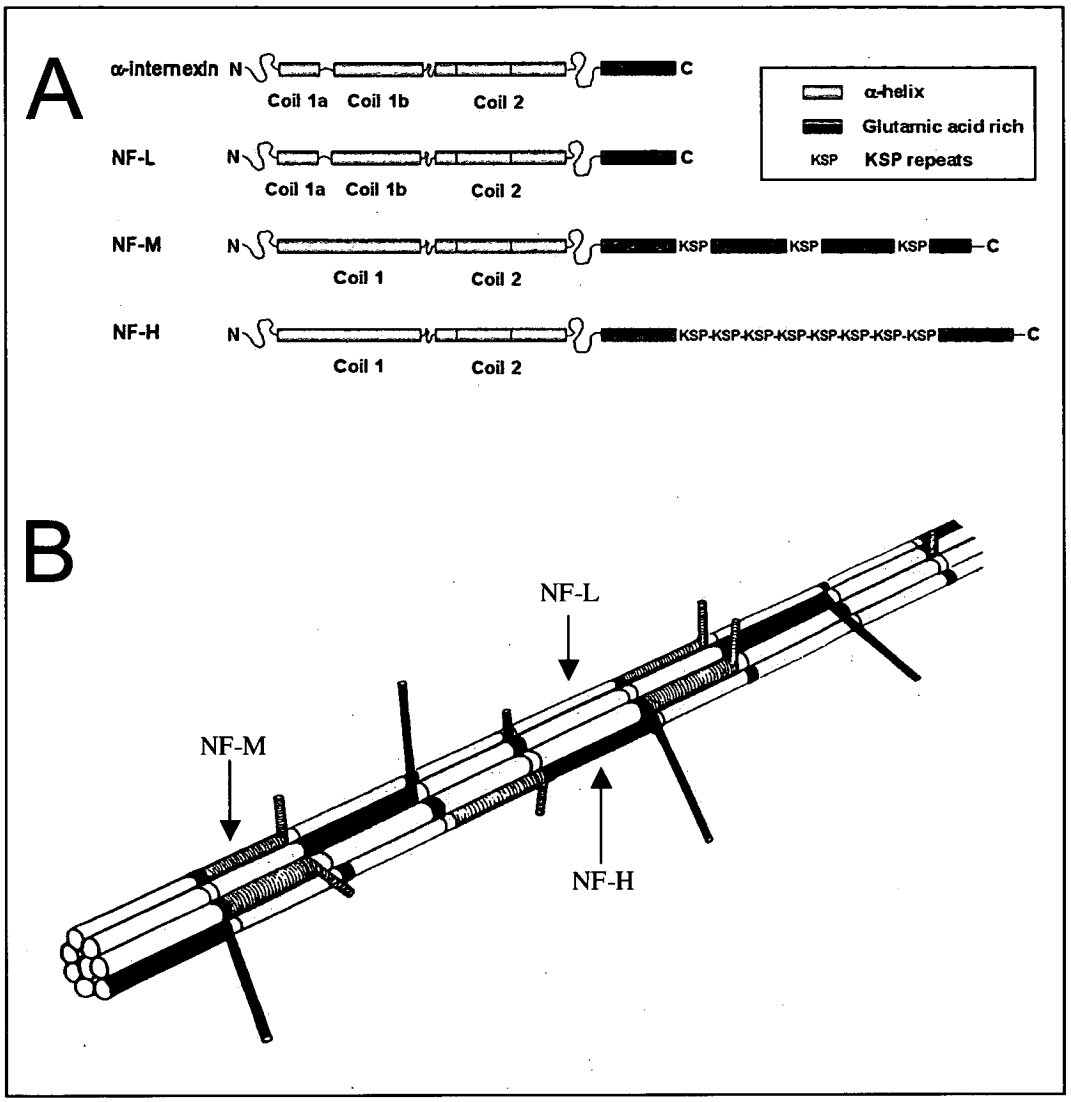


Figure 1.5 Diagrammatic representation of type IV neuronal intermediate filament proteins

(A) Type IV neuronal intermediate filament proteins include the neurofilament triple proteins, NF-L, NF-M and NF-H, 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 NF-L, NF-M and NF-H subunits. NF-M and NF-H 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.

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

possess dynamic properties and can vary in size and composition along the axonal length, where they may undergo local turnover (Nixon and Sihag, 1991; Okabe, 1993; Nixon, 1998; Chan et al., 2003).

Neuronal intermediate filaments are representative of mature neurons and thus expression of these proteins increases concomitant with decreases in other development-associated proteins including nestin and peripherin (Lariviere and Julien, 2004). Importantly, neurofilament expression is dramatically elevated after synapse formation, axonal diameter expansion and myelination (Nixon and Shea, 1992; Lee and Cleveland, 1996). The succession of neuronal intermediate filament expression facilitates the transition of a developing neuron, from a highly plastic mitotic cell, to a post-mitotic highly polar structure with distinct morphological features (Nixon and Sihag, 1991; Nixon and Shea, 1992). Additionally, the distribution of neurofilaments is not uniform; neurofilaments are generally more abundant in axons than dendrites and are more heavily phosphorylated in the axonal than somato-dendritic cellular compartment (Shaw, 1991).

1.2.3.3.2 Neuronal development

The dynamic nature of the neuronal cytoskeleton is crucial to neuronal development and enables developing neurons to migrate to their correct final destination, elaborate processes and form appropriate interconnections. Neuronal development has been extensively documented (for example reviews see Goslin et al., 1988; Tanaka and Sabry, 1995; Tessier-Lavigne and Goodman, 1996; Brandt, 1998; Yamaguchi, 2001; da Silva and Dotti, 2002). Neurons acquire their characteristic morphology through

a stereotypical sequence of events that varies depending on neuronal type and whether growth is occurring *in vivo* or *in vitro* (for review see da Silva and Dotti, 2002). Initially spherical cells, neurons undergo a complex process called neuritogenesis, involving dissolution of cell symmetry, membrane budding and the elaboration of fine protrusions (neurites) from the perikarya (da Silva and Dotti, 2002). *In vivo*, a majority of neuroblasts elaborate a solitary neurite, which serves as a migrational guide and usually forms an axon. Once migration is complete, the somal membrane is again disrupted and neurites, which will form future dendrites, are generated. Conversely, *in vitro* neurons initially extend multiple, morphologically indistinguishable processes (minor neurites) and one minor neurite rapidly elongates, forming an axon. Developing neurites continually extend and retract and may, especially in their infancy, retract altogether.

Growing neurites are tipped by characteristic highly motile "direction sensing" structures, termed growth cones, which determine the directional growth of the neurite and are the sites at which new components are added to the growing plasma membrane (Gungabissoon and Bamburg, 2003). Growth cones, consisting of a microtubule-rich central domain and actin-rich peripheral zone (Figure 1.6), are responsible for translating extracellular guidance signals into the cytoskeletal rearrangements that dictate axonal morphology and the final route of axonal growth (Tanaka and Sabry, 1995; Brandt, 1998). The unique arrangement of the actin cytoskeleton within growth cones in believed to underlie their high motility. The actin-rich peripheral domain of growth cones is composed of remarkably motile protrusions called filopodia and lamellipodia, which are short-lived and continually

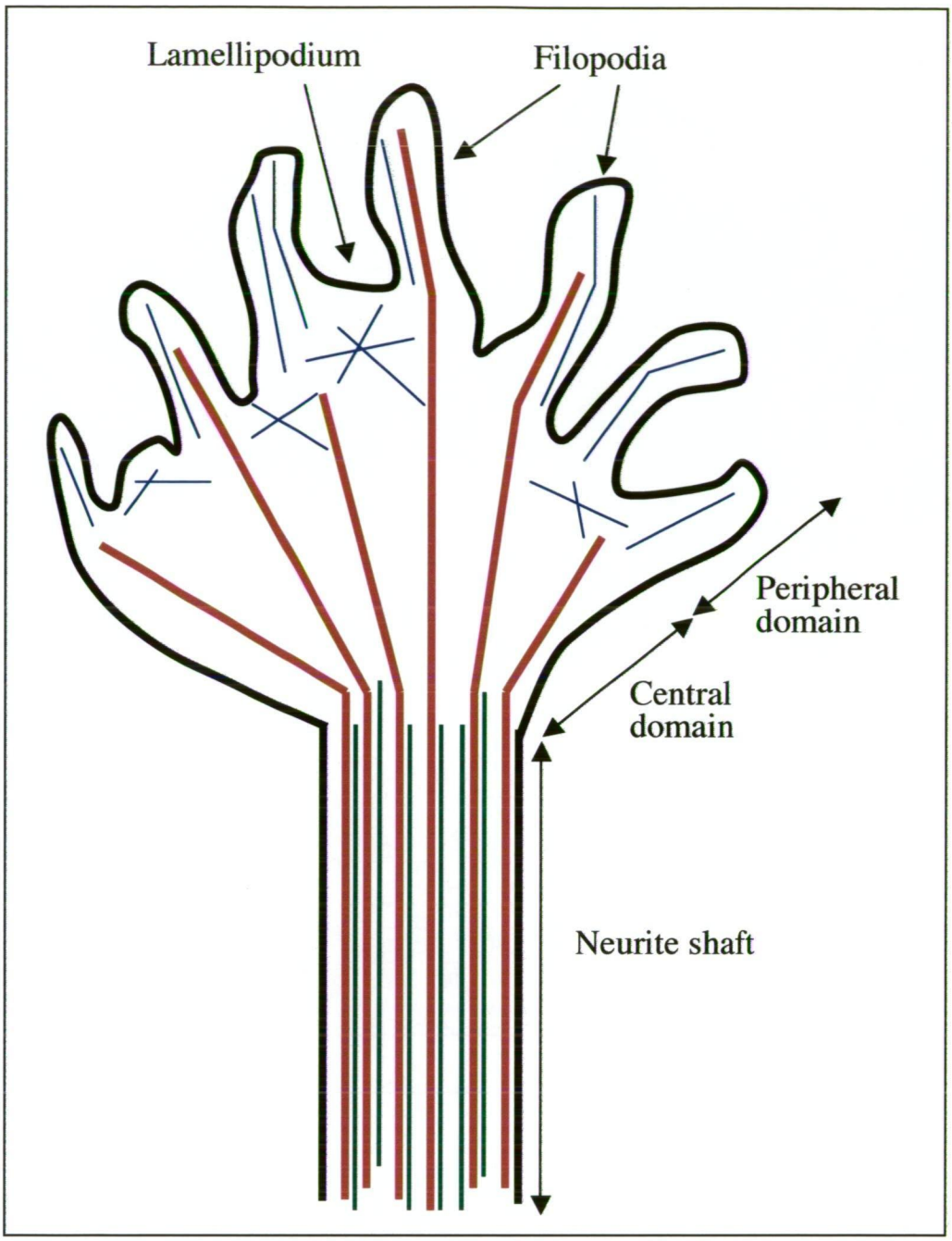


Figure 1.6 Diagrammatic representation of the major components of the developmental growth cone

In the developmental axonal growth cone, neurofilaments are generally restricted to the neurite shaft. Microtubules are prominent in the growth cone central domain and actin microfilaments comprise the primary cytoskeletal component of the highly motile filopodia and lamellipodia, where they are arranged as bundles and veils, respectively.

extend and retract. Filopodia are slender tubular protrusions in which actin filaments are tightly bundled and longitudinally orientated. Lamellipodia are fan-like structures, consisting of dense meshworks of actin filaments, which often form between filopodia (Korey and Van Vactor, 2000; Gallo and Letourneau, 2000). Actin elongation in the growth cone periphery drives filopodial and lamellopodial elongation (Gungabisson and Bamburg, 2003).

Two distinct but related mechanisms regulate axonal growth. Firstly, a fast transient scanning by filopodia and lamellipodia, predicts the direction of future elongation, and secondly, a slower orientation of the proximal growth cone establishes the final orientation of the newly formed section of axonal shaft (Brandt, 1998). Orchestration of actin treadmilling (polymerisation and depolymerisation), and myosin-dependent retrograde transport of actin subunits dictates the behaviour of the actin cytoskeleton in the peripheral domain of the growth cone, enabling exploration of the local environment (Brandt, 1998; Mallavarapu and Mitchison, 1999; Korey and Van Vactor, 2000; Gallo and Letourneau, 2000). Microtubules extend into the actin-rich peripheral domain to explore the internal environment of the growth cone (Tanaka and Sabry, 1995; Gallo and Letourneau, 2000; Suter and Forscher, 2000). Microtubule assembly and bundling in the desired direction of growth with concomitant quiescence and coalescence of remaining growth cone structures, results in the formation of a new portion of axonal shaft, which is remarkably un-dynamic compared to the growth cone (Gallo and Letourneau, 2000).

Both progressive events (neuritogenesis and axonal outgrowth) as well as regressive

events (neuronal death, axonal retraction and neurite pruning) act synergistically to orchestrate the final pattern of neuronal circuitry within the mature brain (Luo and O'Leary, 2005). Ultimately, function of the nervous system depends on the highly specific pattern of neuronal connectivity formed between neurons during development (Bandtlow and Schwab, 2000). This pattern of connectivity is determined by the precise targeting of axons to the cells they will eventually innervate (Gungabisson and Bamburg, 2003). Thus, development of the nervous system, and brain in particular, is magnificently complicated and strictly regulated, involving a multitude of cell types, internal and external guidance cues as well as numerous associated factors. Although the brain may be subject to several developmental defects, the mature brain is by no means resilient to insult. The complexity and intricacy of neural circuitry in the adult brain confers particular vulnerability to a variety of insults including chemical, physiological and physical lesions as well as, or in addition to, disruptions in homeostatic mechanisms generating neurotoxic alterations in biochemical and metabolic pathways. Additionally, the combined influences of environment, genes and/or age may render the brain vulnerable to a variety of neurodegenerative diseases.

1.3 Traumatic brain injury

TBI, any injury to the head that results in brain damage, is a major cause of mortality and morbidity, contributing to the leading cause of death under the age of 45 years in developed nations (Adams, 1992; Egeler-Peerdeman, 1993). Importantly, head injury often affects individual in the most productive years of their lives, causing death or brain injuries ranging from mild to severe (Duff, 2001; Finnie and Blumbergs, 2002). Additionally, head injury has been implicated in the development of neurodegenerative diseases, such as Alzheimer's disease later in life (Smith et al., 1999; Graham et al., 2000; King et al., 2000a; Vickers et al., 2000; Jellinger et al., 2001). Severe head injury often causes overt brain damage resulting in widespread neuronal dysfunction. However, even mild brain trauma can elicit an insidious cascade of cellular changes that culminate in neuropathology. Neuronal damage is particularly serious because neurons in the mature CNS are generally post-mitotic and unable undergo cell division. Thus injury induced neuronal loss results in permanent alterations in the structure of the nervous system, which are often accompanied by long lasting functional changes (Kelly, 1981).

1.3.1 Types of brain injury

Due to the combined influences of injury type, severity and factors intrinsic to the patient, brain injury has a vast array of manifestations. However, the principal mechanisms causing brain injury can be divided into two broad categories: contact and non-contact injury (Finnie and Blumbergs, 2002). Contact injuries may be penetrative or non-penetrative. This form of injury results from an object striking the head, often generating local effects such as scalp laceration, skull fracture and

extradural haematoma. Non-contact injuries are non-penetrative and result from dynamic inertial forces that rapidly rotate the brain within the skull. Both contact and non-contact injuries are capable of generating focal, multifocal and diffuse lesions (Povlishock and Katz, 2005). Focal lesions incorporate focal cortical contusion as well as both deep and extracerebral haemorrhage. In addition, shock waves generated at the site of impact may travel through the skull and brain, possibly causing contusions and intracerebral haemorrhage remote from the site of impact. Differential movement between the brain and the cranium may also generate diffuse injuries in which pressure gradients within the brain tissue create shear, tensile and compressive forces (Adams, 1992; Steward et al, 1999). Figure 1.7 highlights some of the forces involved in TBI.

Common manifestations of diffuse brain injury include diffuse axonal injury (DAI) and diffuse vascular injury (DVI), where axons and microvessels, respectively, are disrupted throughout the brain (Povlishock and Christman, 1995; Maxwell et al., 1997; Smith and Meaney, 2000; Sahuquillo and Poca, 2002; Povlishock and Katz, 2005). Importantly, DAI may occur in the absence of other parenchymal alterations and is therefore common in mild to moderate head trauma (Povlishock and Becker, 1985; Christman et al., 1997; Maxwell et al., 1997; Graham et al., 2000; Smith and Meaney 2003; Povlishock and Katz, 2005). Additionally, brain injury may also evoke a variety of generalised abnormalities such as neuroexcitation and metabolic changes (Povlishock and Katz, 2005). Rotational/angular acceleration of the head, often occurring in falls, car crashes and sports injuries, is a common cause of diffuse brain damage and may cause both acute and chronic neurological symptoms as well

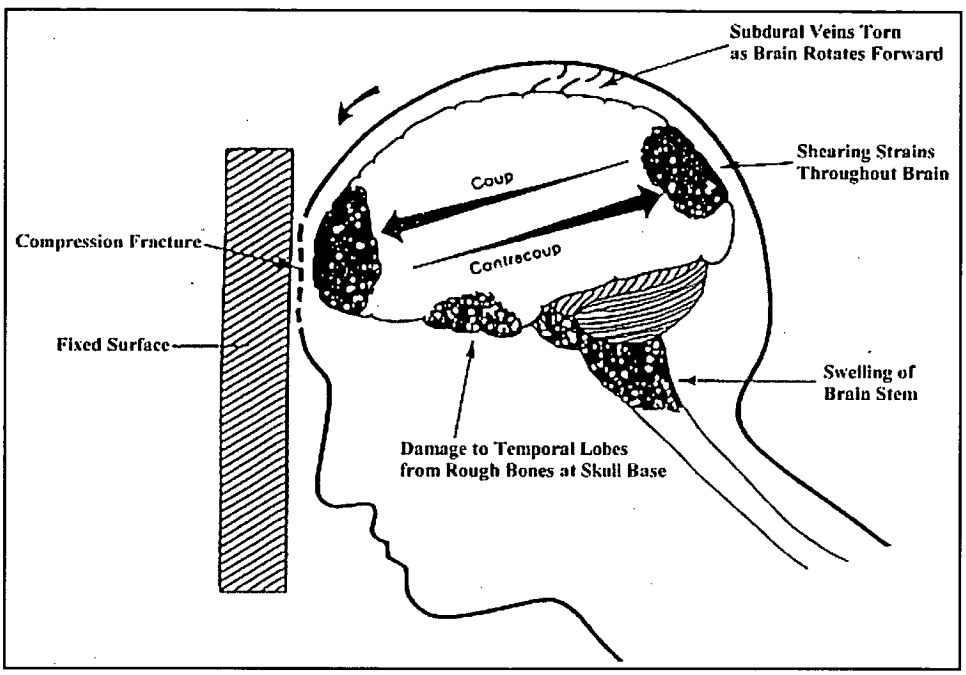


Figure 1.7 Diagrammatic representation of potential forces involved in traumatic brain injury

During head injury, the brain may undergo both focal and diffuse injuries. Focal injures occur when the head strikes and object or vice versa and may involve skull fracture, brain contusion and haemorrhage. Alternatively, or additionally, the brain may move within the skull causing multi-focal injuries. Differential movement between the brain and cranium also results in shearing strains throughout the brain parenchyma, which may generate both diffuse axonal and diffuse vascular injuries.

as psychiatric deficits. The brain regions affected by such damage are generally those important for social behaviour, memory and movement (Hamberger et al., 2003).

1.3.2 Primary and secondary brain damage

Primary damage following brain injury can be classified as any neural disruption that occurs at the time of injury (Maxwell et al., 1997; Graham et al., 2000; Finnie and Blumbergs, 2002). Such damage may involve neuronal and glial cell populations as well as disruption to brain vasculature. In this regard, cells and blood vessels may be sheared, torn or stretched at the time of injury casing haemorrhage, axonal damage and immediate cell death (Finnie and Blumbergs, 2002). However, primary injuries often culminate in a range of secondary alterations. Secondary events develop over a period of hours, days or weeks following the initial trauma (Graham et al., 2000). Secondary perturbations include ischemia, excitotoxicity, spreading neurotoxicity due to disruption of ionic homeostasis and tissue destruction, energy failure, cerebral swelling, initiation of necrotic and apoptotic neuronal death cascades, neuronal degeneration due to downstream deafferentiation/denervation and inflammation, which may in turn compound other responses and add to the spread of neurotoxic effects (Maxwell et al., 1997; Finnie and Blumbergs, 2002; Bayir et al., 2003; Chen et al., 2003a; Liou et al., 2003; Povlishock and Katz, 2005). Secondary alterations may be diffuse and/or focal depending on the form of the initial injury.

1.3.3 Models of brain injury

Several *in vivo* models of brain injury have been developed with the aim of providing insight into improving the treatment of head trauma and therefore reducing the mortality, morbidity and costs associated with this condition. A variety of *in vitro* models have also been developed to complement the *in vivo* models and may provide a more complete representation of the neuronal response to brain trauma. Surprisingly, the evolution of degenerative and regenerative changes following a traumatic incident are highly reproducible across both *in vivo* and *in vitro* models, despite the simplified nature of the latter, and follow a predictable sequence over time (for recent reviews see Maxwell et al., 1997; King et al., 2000a; Vickers et al., 2000; Finnie and Blumbergs, 2002). However, it should be noted that there is a certain level of failure in the translation of experimental investigations to the clinical scenario of TBI (Statler et al., 2001).

1.3.3.1 In vivo models of TBI

In vivo experimental models of head injury primarily aim to simulate the types of brain damage that occur in human head trauma (Adams, 1992; Graham et al., 2000; Finnie and Blumbergs, 2002). In this regard, a variety of lesion models have been developed. Animal models generally induce a controlled mechanical injury that is reproducible, quantifiable and clinically relevant (Finnie and Blumbergs, 2002). However, any single model is not capable of replicating the full repertoire of forces involved in human head injury (Statler et al., 2001). *In vivo* brain injury models can be broadly divided into impact acceleration, non-impact (inertial) acceleration and direct brain deformation. Thus, the mode of injury for each of these models involves

direct impact on the skull, acceleration of the head without impact and direct deformation of the brain through the exposed dura/cortex, respectively (reviewed by Graham et al., 2000; Finnie and Blumbergs, 2002). Acceleration/inertial models mimic forces inflicted on the brain in situations such as vehicle accidents. Despite their similarity to human brain injury, many injury models in involving primates (primarily contact and non-contact acceleration models) involve specific economic and ethical implications and therefore have largely been replaced by rodent or non-primate animal studies. In this regard, acceleration injuries are usually performed in species such as sheep and pigs, which have gyrencephalic brains similar to humans (Finnie and Blumbergs, 2002).

The most widely used *in vivo* brain injury models are fluid-percussion and controlled cortical impact, or cortical contusion, models – both of which involve direct brain deformation (Finnie and Blumbergs, 2002). Fluid-percussion models produce injury by creating pressure transients in the cranium using a device that transmits pressure waves hydraulically (Steward, 1999). The resulting elastic deformation of the brain resembles a direct impact injury (Adams, 1992). Pneumatically driven mechanical impactors can be used to produce a controlled compression of the exposed brain (Adams, 1992; Steward, 1999), or can directly impact on the skull, resulting in skull fractures in addition to brain damage (Steward, 1999). Both fluid-percussion and control cortical impact techniques are capable of producing DAI (Adams, 1992). Another model involves stretching the optic nerve in the adult guinea pig (Jafari et al., 1997, 1998; Maxwell et al., 1997), which can produce axonal damage that is morphologically identical to DAI in humans (Adams, 1992). Defined surgical lesion

(eg King et al., 2001; Chung et al., 2002, 2003) and excitotoxic models have also been developed, which produce relatively comparable consequences (reviewed by Steward, 1999).

Although *in vivo* models aim to replicate various aspects of human head trauma they are often inherently simplified and do not incorporate certain key, clinically relevant factors such as multiple trauma, secondary insults (for example hypotension and hypoxemia, intracranial hypertension and cerebral oedema), gender, age, alcohol consumption, genetic predisposition, frontal lobe injury, intracranial haemorrhage and surgical intervention (for review see Statler et al., 2001). Furthermore, Statler et al. (2001) highlight several additional factors that are implicated in clinical cases of TBI. For instance, fever, seizures, electrolyte disturbances and blood transfusion thresholds. Additionally, coma and administration of neurointensive care monitoring and management (for example choice of anaesthetics, use of sedatives, inspired oxygen concentration, hemodynamic support and surgical decompression) are often not addressed in animal models (Statler et al., 2001). Despite their obvious set backs, the importance of animal models cannot, however, be underestimated as they are proving extraordinarily useful in elucidating many of the molecular, biochemical and cellular factors implicated in the sequela of TBI. In this respect, certain models and animal species are more suited to investigating the various aspects of human brain trauma, such as rodent versus large animal and models, which produce focal as opposed to diffuse lesions.

1.3.3.2 In vitro models of TBI

The establishment of neuronal culture models has allowed extensive study into neuronal development and the neuronal response to injury. Cultured neurons are easily accessible and economical. Additionally, cultured cells can be chemically, genetically or physically manipulated and monitored with relative ease, particularly through live cell imaging technologies (Bekkers, 1997). Cultured neurons can be exposed to a variety of physical insults that may be implicated in TBI. Such insults include acceleration, compression, shear, stretch and transection (see review by Morrison et al., 1998). Cultures of dissociated neural tissue involve removing explants of tissue and plating them as dissociated cell suspensions. It is important to note that many studies are performed using neuron-like cell lines, for example neuroblastoma lines, or neurons from the PNS, rather than primary CNS derived cultures, which are more difficult to culture and manipulate. Additionally, very few neuronal injury models are able to generate specific axonal injury (Dickson et al., 2000; Chung et al., 2002; Haas et al., 2004; Taylor et al., 2005) and instead tend to be models of global neuronal trauma (Morrison et al., 1998).

The major disadvantage of neuronal cultures is that much of the three-dimensional architecture of the intact nervous system is lost. Therefore, anatomical data and the role of brain vasculature cannot be examined. Nevertheless, these models are important for investigating the morphological and physiological response of single cells, are particularly amenable to manipulation and provide a major platform for the initial testing of neuro-therapeutic agents. Moreover, neuronal slices can be harvested from the intact brain. Cultured brain slices retain much of the connectivity

typical of the origin from which they were removed. Importantly, tissue for both slice and dissociated cell culture can be removed from either embryonic or neurologically mature animals. A variety of neuron-like cell lines also exist. Taken together, data derived from the vast array of *in vivo* and *in vitro* experimental brain and neuronal injury models has provided valuable insight into the mechanisms involved in the neuronal response to injury.

1.4 The neuronal response to injury

Data gathered from both experimental models of neuronal injury and cases of human TBI indicate that neurons undergo a stereotypical sequence of reactive and potentially regenerative alterations following injury (Maxwell et al., 1997). Importantly, DAI often occurs in the absence of other parenchymal alterations, particularly in mild to moderate brain injuries (Maxwell et al., 1997; Smith and Meaney, 2003, Povlishock and Katz, 2005). Axonal injury, in turn, may signal characteristic alterations in the soma (Vickers et al., 2000), as discussed below.

1.4.1 Axonal injury

Axonal injury is present in human head trauma of varying severity as well as in numerous experimental models of brain injury (Gennarelli, 1996). Due to their relatively long-distance projection and fragility, axons are particularly vulnerable to injuries involving rapid acceleration/deceleration and rotational forces (Maxwell et al., 1997; Smith and Meaney, 2000). In this regard, widespread axonal damage, or DAI, is a frequent consequence of such trauma (Gennarelli, 1996; Vickers et al., 2000). DAI is the most common cause of vegetative state following brain trauma (Adams, 1992) and, in severe head injuries, axonal damage is associated with significant mortality (Gennarelli, 1996). Extensive axonal disruption results in the loss of multiple synaptic connections throughout the brain parenchyma and, in turn, the disruption of the circuits into which they are integrated.

Whether axonal disconnection, or axotomy, occurs during an injury or sometime later, the eventual consequence is the formation of two axonal segments. The

severed axonal ends seal over almost immediately following disconnection, separating the axon into a proximal and distal portion, which retract away from one another and begin to swell due to the continued anterograde and retrograde transport of organelles and other material, including mitochondria, vesicles, multivesicular bodies and neurofilaments (Kelly, 1981). The distal fragment becomes completely separated from its neuron of origin, whereas the proximal segment remains attached to the soma. Around the injury site rapid myelin degeneration occurs.

Axonal disconnection results in metabolic deprivation within the distal axonal segment. The distal axonal fragment, therefore, undergoes a degenerative response, referred to as Wallerian degeneration. As degeneration in the distal segment proceeds, the myelin sheath draws back from the axon and degrades, the axon beads and degenerates and the resultant debris is phagocytosed and recycled by glial cells (Kelly, 1981). Interestingly, utilising *in vivo* live imaging techniques, Kerschensteiner at al. (2005) have shown that the severed terminals of both the proximal and distal axonal segments of primary sensory neurons initially undergo a rapid phase of die-back, termed acute axonal degeneration, which is distinct from the slower Wallerian degeneration observed within the distal axonal segment. Once the distal segment degenerates, its postsynaptic targets are also often disrupted and may die. Furthermore, pre-synaptic cells may also be affected due to target loss. Thus, a lesion at one site in the CNS can cause widespread disruption involving distant cells, according to the connections interrupted by the lesion.

1.4.1.1 Primary versus secondary axotomy

TBI may cause axons to be torn immediately at the time of injury. However, studies in both animals and humans, involving mild, moderate and severe injuries, indicate that axotomy usually occurs via a delayed or secondary process (reviewed by Maxwell et al., 1997; Graham et al., 2000). In delayed axotomy, subtle disruptions at the time of injury are believed to result in several detrimental changes, including damage to the axolemma or axoplasm, and alterations in ionic homeostasis (particularly calcium ions) that ultimately culminate in axonal disconnection. Axonal disconnection typically occurs at least several hours following injury (Graham et al., 2000). Altered ionic concentrations, particularly in influx of calcium ions, are postulated to cause abnormal activation of various calcium-regulated enzymes, such as calpains, resulting in disruption of axonal cytoarchitecture (Maxwell et a., 1997; Graham et al., 2000). Interestingly, a variety of cytoskeletal proteins, including the microtubule-associated proteins, MAP-2 and tau, as well as neurofilaments can act as substrates for the calcium-dependant calpains (Zhang et al., 2000). Disruption of calcium regulation and abnormal enzyme-activation may therefore initiate a cascade of events resulting in axonal collapse, impeded axoplasmic transport, axonal swelling (due to organelle accumulation) and ultimately axotomy (Adams, 1992; Gennarelli, 1996; Maxwell et al., 1997; Graham et al., 2000). Alternatively, or additionally, direct disruption to the neuronal cytoskeleton, may result in impeded axonal transport, swelling and disconnection. Whether direct or enzyme-activated, disruption to the axonal cytoskeleton and axonal transport are viewed as fundamental factors contributing to axonal discontinuity (Maxwell et al., 1997; Graham et al., 2000).

1.4.1.2 Cytoskeletal alterations

Studies in animals and humans demonstrate that TBI often results in DAI via a process of delayed axotomy, the final manifestation of which is determined primarily by the severity of the initial injury, as discussed above. Importantly, disruption of axolemmal permeability at the time of injury may be a key factor in the post-injury response (Maxwell et al., 1997; Graham et al., 2000; Smith and Meaney, 2000). In this regard, brain trauma, without disruption of the axolemma tends to cause cytoskeletal misalignment,* whereas more severe injury, in which axolemmal permeability is compromised, typically results in loss, accumulation or compaction of certain cytoskeletal components (Maxwell et al., 1997; Graham et al., 2000) (Figure 1.8). This suggests that alterations in the neuronal cytoskeleton may be central to the neuronal response to injury. Indeed, numerous studies (eg Hoffman et al., 1987, 1993; Goldstein et al., 1988; Hoffman and Cleveland, 1988; Muma et al, 1990; Nixon and Shea, 1992) have shown that following axonal injury and during regenerative attempts neurofilament expression is down-regulated, whereas expression of other cytoskeletal elements is elevated, suggesting that the sequence of cytoskeletal gene expression occurring in development is recapitulated during regenerative attempts (Hoffman and Cleveland, 1988; Lee and Cleveland, 1996).

Interestingly, ß-amyloid has been shown to accumulate within the brain following injury (Smith et al., 1999). ß-amyloid is an integral transmembrane glycoprotein synthesized in the neuronal perikarya and transported into axons. It is proteolytically cleaved from the larger precursor molecule, ß-amyloid precursor protein (APP). Immunohistochemical labelling for APP is now widely used to differentiate injured

*Cytoskeletal misalignment occurs when cytoskeletal elements, particularly neurofilaments, become redistributed and/or compacted following mild neuronal injury, in the absence of altered axolemmal permeability (Maxwell et al., 1997; Vickers et al., 2000).

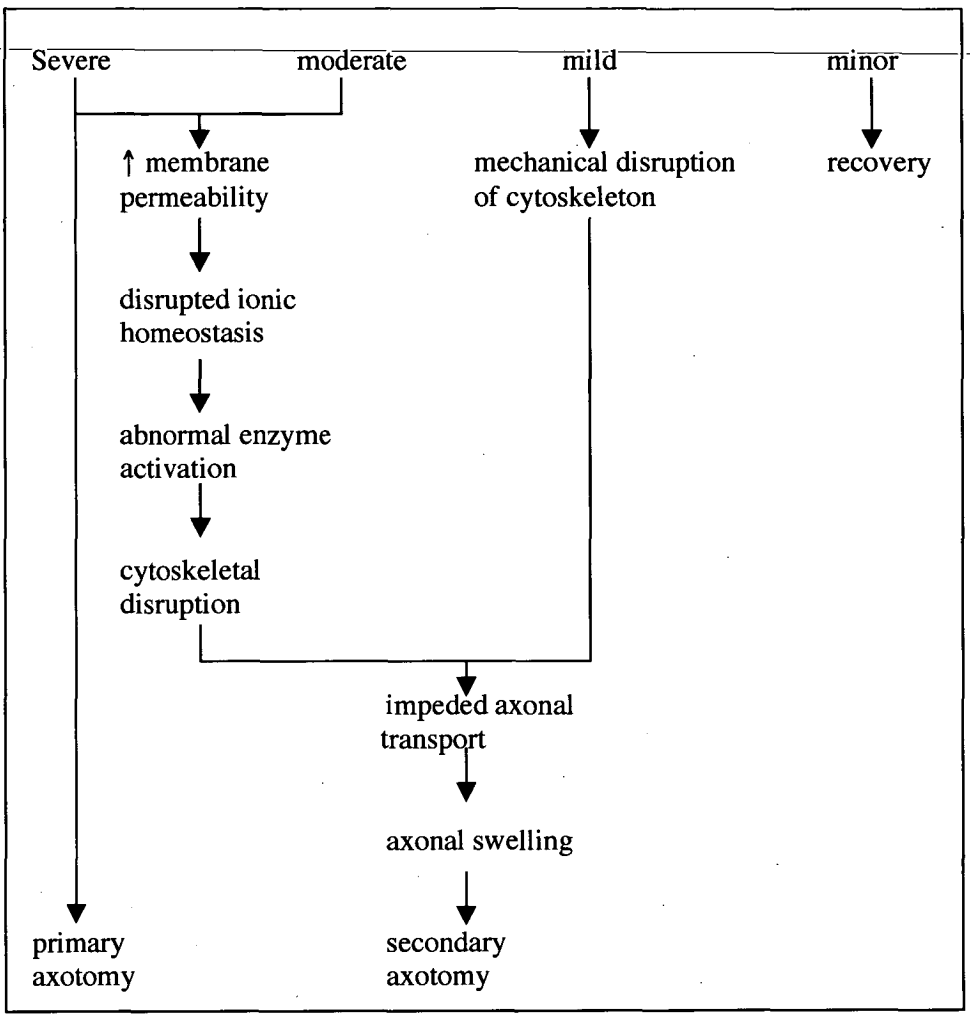


Figure 1.8 Schematic overview of pathological events that are postulated to result from diffuse axonal injury of varying severities

Essentially, mild and moderate head injuries may cause cytoskeletal disruption either directly or through aberrant enzyme activation. Cytoskeletal disruption impedes axonal transport and may ultimately result in axonal disconnection through a delayed, or secondary, process. Axons may undergo secondary axotomy in following severe head trauma, or may be axotomised at the time of injury.

from non-injured axons (Graham et al., 2000). Both APP and ß-amyloid accumulate within neurons following injury and are implicated in the pathology of Alzheimer's disease (Smith and Meaney, 2000; Vickers et al., 2000). In this respect, brain injury has been proposed as a risk factor for the development of Alzheimer's disease later in life (Vickers et al., 2000; Jellinger et al., 2001).

1.4.1.2.1 Alterations in microtubules following axonal injury

Microtubules and their associated motor proteins are fundamental to the process of fast axonal transport, forming an assemblage along which membrane-bound vesicles are continually conveyed to distal axonal regions. Although axons can completely recover from mild compression, more severe levels of compression may irreversibly block fast axonal transport due to mechanical breakage of the axoplasm (Gallant, 1992). Several studies have shown a reduction in the levels of microtubules in areas of axonal disruption (Hoffman et al., 1984, 1985; Jafari et al., 1997, 1998; Maxwell and Graham, 1997; Adlard et al., 2000). This loss is most notable at the nodes of Ranvier (Maxwell and Graham, 1997) and may be accompanied by a loss of tau and dendritic MAP-2 in the damaged neurons (Saatman et al., 1998; Zhang et al., 2000). Alternatively Smith et al. (1999) observed accumulations of tau in axonal stumps following acceleration injury. It has been postulated that a post-traumatic calcium influx (particularly in severe axonal injury) contributes to the generation of an intra-axonal environment favouring microtubule dissolution, which subsequently results in a focal disruption of fast axonal transport (Graham et al., 2000). In this respect, Maxwell et al. (1997) suggest that fast axonal transport continues in areas where microtubules remain intact, distant to the focal area of their loss. This may in turn

result in the accumulation of cytoskeletal and other cellular organelles at several sites along the damaged axons, thus contributing to multi-focal axonal swelling.

1.4.1.2.2 Alterations in neurofilaments following axonal injury

The neurofilament intermediate filament cytoskeleton is an important structural component of the neuron. Neurofilaments provide tensile strength as well as stabilising neuronal structure (Hamberger et al., 2003). Neurofilament disruption, therefore, has profound effects on neuronal structure and function (Posmantur et al., 2000). Neurofilaments provide neurons with mechanical strength by resisting the compressive forces of the local environment (Hoffman et al, 1984; Cleveland et al., 1991). Additionally, neurofilament density is relatively constant over a wide range of axonal calibres (Julien and Grosveld, 1991); thus larger axons will tend to contain a greater number of neurofilaments than smaller axons. It is therefore plausible that different sized axons respond in different ways when damaged, particularly when inflicted with tensile strain (Maxwell et al., 1997). Indeed, Reeves et al. (2005) have recently demonstrated that axon calibre, and associated myelination, influence axonal recovery following injury.

Neurofilaments are present in all major compartments of the neuron except the axon terminals, where calcium-activated proteases, such as calpains are thought to be responsible for their breakdown (Schlaepfer, 1987). Neurofilament degradation by calpains possibly provides feedback information to the perikarya, signalling normal synaptic function (King et al., 2000a). In the perikarya and dendrites, serine/threonine phosphatases may maintain neurofilaments in a predominantly

dephosphorylated form, while in the axon protein kinases extensively phosphorylate these filamentous structures (Maxwell et al., 1997). Following injury neurofilament phosphorylation and distribution is altered. Abnormally phosphorylated neurofilaments tend to accumulate in the perikarya, while neurofilaments within the axons may become dephosphorylated. Neurofilament phosphorylation in the soma is believed to impede neurofilament transport into the axon, subsequently contributing to decreased axon calibre following axonal damage (Schlaepfer, 1987). Using a rotational acceleration injury in the rabbit, which generated a force equivalent to that occurring in human head trauma, Hamberger et al. (2003) demonstrated alterations in the distribution of the high molecular weight subunit of the neurofilament triplet protein, NF-H, where phosphorylated NF-H accumulated in the neuronal perikarya and was lost from axons in several brain regions.

In the post-traumatic period, neurofilaments can be observed in distended axonal regions as well as infiltrating axon terminals, possibly indicating a disruption in calpain mediated neurofilament breakdown (King et al., 2000a). Additionally, *in vitro* (Dickson et al, 2000) and *in vivo* (King et al., 2000b, 2001) studies have demonstrated extensive accumulations of neurofilaments into ring- and bulb-like structures in severed axonal stumps. Both structures lacked microtubules and appeared to contain a dense core of organelles (including mitochondria), surrounded by either a whorl (in ring-like structures) or dense ball (in bulb-like structures) of neurofilaments. Interestingly, these damaged axons exhibit similar morphological changes to a subtype of dystrophic neurites in Alzheimer's disease (King et al., 2000b). Nixon (1998), noted that despite access to an expended axoplasmic space in

the acute swellings that develop at the transected axonal tips, neurofilaments remain bundled. Additionally, neurofilaments can persist in both proximal and distal axonal stumps for long periods. Hall and Lee (1995) suggest this may be due to a steric protection offered by the remaining filamentous structure.

Numerous investigations have demonstrated neurofilament compaction within the axon shaft following axonal injury. Studies by Jafari et al. (1997, 1998) and Povlishock et al. (1997) suggest that neurofilament compaction is associated with disruption of the axolemma, which may or may not manifest, depending of the severity of injury. In this regard, in injuries where axolemma permeability is not disrupted, focal neurofilament disarray and misalignment occurs, but at more severe levels of axonal injury, neurofilament compaction occurs (Maxwell et al., 1997). However, Chung et al. (2005) have demonstrated that neurofilament compaction may also occur following relatively mild axonal stretch injury. Neurofilament compaction occurs within minutes of injury and may persist for hours (Maxwell et al., 1997). Although the mechanisms leading to neurofilament compaction remain to be elucidated, compaction is thought to be the result of loss and/or collapse of neurofilament side-arms, with concomitant preservation of filamentous structure. Accumulating literature provides evidence for both scenarios (reviewed by Maxwell et al., 1997).

Altered interaction between protein kinases and phosphatases may occur following axonal injury, causing neurofilament sidearm collapse. Alternatively, post-traumatic disruption of calcium homeostasis may abnormally activate proteolytic calpains,

which cleave the neurofilament side-arms from the core filamentous structure. Both processes could theoretically lead to a reduction in intra-filament spacing and therefore neurofilament compaction. Some studies have shown a reduction in the levels of neurofilaments occurring in areas of axonal disruption (Hoffman et al., 1984, 1985; Maxwell and Graham, 1997) and Zhang et al. (2000) demonstrated the loss of phosphorylated neurofilaments is delayed when compared to the reactive changes occurring in other cytoskeletal components.

1.4.2 Cell body reaction

Because axonal transport occurs in both the anterograde and retrograde direction following axonal disconnection, signals from the disconnected distal axonal segment are no longer sent to the soma. This lack of input may in turn evoke alterations in the soma. Changes in the soma may culminate in cell atrophy, death or survival (Kelly, 1981; Tuszynski and Gage, 1995; Singleton et al., 2002; Selzer, 2003). Often the damaged neuron survives. In this instance, the soma and remaining portion of the injured axon undergo reactive changes involving the specific regulation of proteins, particularly cytoskeletal components, in preparation for an attempt at sprouting or regeneration (King et al., 2000a). This process is referred to as the "cell body" or "retrograde" reaction and is characterised by perikaryal hypertrophy, displacement of the nucleus to the cell periphery and dispersion of the Nissl substance (Torvik, 1976).

1.5 Regeneration and repair following brain injury

Accumulating evidence indicates that brain injury evokes an adaptive sequence of alterations directed at healing and remodelling, much like the wound healing reaction observed in other tissues such as the skin (Maxwell et al., 1990; Hunt et al., 2000; King et al., 2001; Yamaguchi and Yoshikawa, 2001; Oehmichen, 2004). In this regard, many of the cell populations disrupted by the lesion or affected by secondary events and signalling cascades are the same cellular populations that are involved in brain healing. Thus, astrocytes, microglia/macrophages, oligodendrocytes, neurons, neural progenitor cells and brain vasculature have all been shown to undergo adaptive alterations in the aftermath of a physical injury (Maxwell et al., 1990; Christman et al., 1997; Deller and Frotscher, 1997; Fujita et al., 1998; Pastor et al., 2000; Vickers et al., 2000; King et al., 2001; Kozorovitskiy and Gould, 2003; Finnie and Blumbergs, 2002; Tonchev et al., 2003; Chen et al., 2003a; Lee et al., 2003; Brazel and Rao, 2004; Lie et al., 2004; Davalos et al., 2005; Emsley et al., 2005).

Astrocytes undergo a process of reactive astrogliosis involving proliferation, hypertrophy and the secretion of various factors that culminate in the formation of a glial scar (Maxwell et al., 1990; Ridet et al., 1997; Raivich et al., 1999; Kernie et al., 2001; McGraw et al., 2001; Chirumamilla et al., 2002; Chen et al., 2003a, b). This structure effectively segregates the injured from non-injured tissue to prevent the spread of cytotoxicity. Brain-resident microglia are also activated to transform into phagocytosing macrophages and along with macrophages derived from the circulatory system act to clear cell debris from the lesion site (Thomas, 1992; Lotan and Schwartz, 1994; Bruce-Keller, 1999; Stence et al., 2001; Carbonell et al., 2005;

Davalos et al., 2005). More long-term alterations include tissue re-modelling, which may involve maturation of the glial scar, angiogenesis (or vascular growth) and attempted neuronal regeneration (Maxwell et al., 1990; King et al., 2001). Damaged axons in most regions of the mature CNS do not spontaneously regenerate and instead tend to undergo an abortive regenerative response restricted to the local neuropil (Cotman et al., 1994; Kapfhammer, 1997; Rhodes and Fawcett, 2004).

1.5.1 Inhibitors of regeneration in the adult CNS

The incapacity for axonal regeneration in the adult CNS has been ascribed to a range of factors including an intrinsic incapacity of CNS neurons to re-initiate growth, myelin and myelin-associated factors, the development of glial scars, cavitation of the injured tissue, a lack of growth factors or support cells and the presence of growth inhibitors (Fawcett, 1992, 1997; Berry et al., 1994; Caroni, 1998; Fawcett and Asher, 1999; Bandtlow and Schwab, 2000; Goldberg and Barres, 2000; Qiu et al., 2000). Thus, axonal regeneration is limited by the ability of neurons to extend new processes in a non-permissive environment (Compston, 1995). Berry et al. (1994) postulate that during normal development some of these mechanisms may act to confine growing axons to their appropriate pathways, thus limiting overgrowth and preventing the mixing of functionally different fibre systems. For example, CNS myelination is associated with cessation of axon growth (Nagashima et al., 1999) and loss of neuronal plasticity (Goldberg and Barres, 2000). Myelination may, therefore, provide growth inhibitory signals as the nervous system nears maturity.

Myelination alone does not appear sufficient to prevent regeneration, as studies

conducted by Nagashima et al. (1999) have demonstrated that although central white matter substantially inhibits neuronal regeneration, both myelin-rich CNS white matter and relatively myelin-free CNS grey matter have this capability. Additionally, Davies et al. (1997) found that regenerating mature axons were able to extend for considerable distances in un-injured CNS white matter tracts. Thus, it is likely that injured, rather than un-injured, CNS tissue contains components which are inhibitory to axonal re-growth (Rhodes and Fawcett, 2004). In this respect, formation of the glial scar, including incorporation of inhibitory extracellular matrix molecules, is also a major molecular and physical barrier to axons regeneration (Fawcett and Asher, 1999; Rhodes and Fawcett, 2004).

1.5.2 Mechanisms of endogenous neuronal regeneration

Neurons respond to injury with a complex sequence of morphological, biochemical and gene expression alterations, some of which may be directed towards sprouting, regeneration, synaptogenesis and even the generation of entirely new neurons (Deller and Frotscher, 1997; Christman et al., 1997; King et al., 2000a; Magavi et al., 2000; Pastor et al., 2000; Kernie et al., 2001; Rice et al., 2003). In this regard, axonal sprouting and neurogenesis are the two major mechanisms by which lost neuronal connections and degenerated neurons can be replaced.

1.5.2.1 Axonal sprouting

Axons in the CNS often respond actively, rather than passively, to injury and may undergo a distinctive sprouting response or regenerative attempt. Importantly, axonal regeneration and axonal sprouting are two distinct responses to axotomy,

which may occur at the proximal stump of surviving injured axons. Regeneration implies successful restoration of the specific contacts between an axotomised neuron and its target, whereas sprouting describes the abortive response of the proximal stump to regenerate, leading to multiple local axonal collaterals without the restoration of normal connectivity (Berry et al., 1994; Deller and Frotscher, 1997). Collateral sprouting from uninjured axons may also occur following axonal injury (Deller and Frotscher, 1997), possibly due to availability of vacant synaptic territory (McKinney et al., 1997). Thus, injury in the CNS may induce a dramatic reorganisation of surviving circuitry manifesting as axonal sprouting.

Although the PNS is capable of regeneration over relatively long distances, regeneration in the CNS is comparably very limited, however, can be promoted in a facilitative environment. Differences in the glial cells that perform myelination appear to be pivotal in unravelling the disparity between the regenerative capacities of the CNS and PNS (Watkins and Barres, 2002). Axons of the CNS are firmly enclosed in myelin membranes produced by oligodendrocytes, whereas Schwann cells perform myelination in the PNS. Myelin within the PNS is rapidly removed following axonal injury. Conversely, this process takes considerably longer in the CNS, indicating that certain components of CNS myelin are likely to inhibit regeneration (Watkins and Barres, 2002). Additionally, in the PNS, myelin sheaths may not be disrupted, or myelinating cells may form new myelin conduits to guide and nurture regenerating axons (Kelly, 1981).

The level of severity of TBI plays an important role in the manifestation DAI (Figure

1.8). It has been postulated that if TBI occurs in the absence of parenchymal change (as it often does in less severe trauma), axons are well equipped to mount a regenerative attempt (Christman et al., 1997). Following injury, cytoskeletal responses in the axon and perikarya appear to be directed towards axonal regeneration and synaptogenesis. A key event in axonal survival and the potential for regeneration is a substantial reorganisation of the cytoskeleton (Christman et al., 1997). Neurofilaments may play a crucial role in determining whether and how central neurons regenerate following axon transection (Hall and Yao, 2000) as indicated by the disrupted regeneration exhibited by neurofilament-deficient mice (Zhu et al., 1997) and quail (Jiang et al., 1996) following peripheral nerve injury.

It has be demonstrated, both *in vitro* (Dickson et al., 2000) and *in vivo* (Christman et al., 1997; King et al., 2001), that axons can mount a regenerative attempt, which is associated with cytoskeletal reorganisation and accompanied by an up-regulation of GAP-43. GAP-43, a neuron specific growth and membrane-associated phosphoprotein, is one of the most abundant proteins in neuronal growth cones, where it is implicated in filopodial motility, axon guidance, synaptogenesis and synaptic plasticity (Brandt, 1998). It is therefore not surprising that its expression is down-regulated in the mature CNS, but elevated during neuronal development and regeneration (Goslin et al., 1988; Cantallops and Routtenberg, 1999).

1.5.2.2 Neurogenesis

The long held belief that adult neurons are post-mitotic, terminally differentiated and held in a rather static state, lacking the capacity to re-enter the cell cycle or

regenerate following injury is being increasingly challenged. Although mature neurons within the brain are generally regarded as post-mitotic, accumulating data indicate that various forms of brain lesion are capable of evoking a neurogenic response at sites both directly and indirectly associated with the lesion (Magavi et al., 2000; Kernie et al., 2001; Rice et al., 2003). In this regard, new neurons may be generated from parenchymal-resident progenitor cells or from stem or progenitor cells located within germinal regions of the adult brain, such as the SVZ (Emsley et al., 2005). Additionally, some studies have also indicated that migrating neuroblasts, produced throughout life and largely destined for the olfactory bulb, may be recruited to sites of brain injury (Ramaswamy et al., 2005).

During brain development and exit from the cell cycle extracellular signals may initiate a genetic program that results in the commitment of a progenitor cell to a particular differentiated state (Brewer, 1999). One of the triggers for neurogenesis in the adult brain may be injury-induced neuronal death. Indeed, Magavi et al. (2000) demonstrated that targeted apoptosis in the neocortical grey matter resulted in the neuronal differentiation of parenchymal-resident neural progenitor cells. Additionally, several studies have demonstrated the existence of neuronal stem cell populations in the adult CNS that can give rise to new neurons when the brain is not compromised (Kuhn et al., 1996; Eriksson et al., 1998; Gould et al., 1999; Johansson et al., 1999; Cameron and McKay, 2001; Bernier et al., 2002, Bedard and Parent, 2004) as well as in response to various lesion paradigms (Magavi et al., 2000; Kernie et al., 2001; Braun et al., 2002; Chen et al., 2003b; Rice et al., 2003; Tonchev et al., 2003).

1.5.3 Therapeutic interventions following TBI

There are currently no treatments available to effectively counteract the sequence of neurodegenerative changes following TBI. Brain trauma and subsequent axonal injury may lead to degeneration and death of affected neurons or an attempt at regeneration by the damaged neurons. Regenerative attempts manifest as extensive axonal sprouting (Christman et al., 1997; Dickson et al., 2000; King et al., 2001) or the generation of new neurons (Picard-Riera et al., 2004). Additionally, although early studies suggested that DAI resulted from axonal tearing at the time of injury, later investigations have emphasised that this form of direct axonal injury is usually only present following severe brain trauma (reviewed by Maxwell et al., 1997). It is now recognised that axonal damage frequently results from delayed axotomy, that is axonal disruption occurring several hours following an injury, which is triggered by subtle disruptions at the time of injury (for reviews see Povlishock and Christman, 1995; Povlishock and Jenkins, 1995; Maxwell et al., 1997; King et al., 2000a; Vickers et al., 2000).

Delayed neurodegenerative events indicate that a therapeutic window exists in which brain damage and its progression can be slowed, halted or even reversed (Adams, 1992; Smith and Meaney, 2000; Finnie and Blumbergs, 2002), improving long-term prognosis. Several therapies have been show to decrease neuronal damaged and degeneration following brain injury. For example hypothermia (Büki et al., 1999; Povlishock et al., 1999, Liou et al., 2003; Strauch et al., 2005) and Cyclosporin A (CsA) infusion (Sullivan et al., 1999, 2000; Okonkwo et al., 1999a, b, 2003; Strauch et al., 2005) demonstrate neuroprotective effects when administered in the post-

injury period. Hypothermia is a global neuroprotective approach, which might provide antiexcitotoxic, and antioxidant effects, reduce metabolic demands, prevent intracranial hypertension and reduce the activity of destructive enzymes (Liou, 2003). CsA, an immunosuppressive drug, limits the inflammatory response, which may have detrimental effects in exacerbating neural injury and is thought to inhibit opening of the mitochondrial permeability transition pore and related abnormalities, such as disrupted calcium homeostasis and release of apoptosis-related components (Büki et al., 1999; Sullivan et al., 1999, 2000, 2005; Orrenius et al., 2003). Disrupted calcium homeostasis has been implicated in many aspects of the axonal response to injury. Povlishock et al. (1999) have shown that calcium chelators may have a protective effect following brain trauma in axons where axolemmal permeability is disrupted. Additionally, neuroexcitotoxicity has been postulated to result from excessive glutamate release following neuronal trauma. In this respect, competitive and non-competitive antagonists of the glutamate receptor, N-methyl-D-aspartate (NMDA), have been shown to have therapeutic effects (Graham et al., 2000).

1.5.4 Promoting neuronal regeneration following TBI

Numerous experimental approaches are currently being tested for their ability to promote CNS axonal regeneration (Selzer et al., 2003). For example, implantation of growth-promoting cells, such as olfactory ensheathing cells or peripheral nerve Schwann cells (Stichel et al., 1999) may act as a bridge and facilitate invasion of the lesion area by newly generated axonal sprouts. Additionally, immature neurons can be used to replace damaged cells and microglial cells used to remove cellular debris

(Frisén et al., 1998; Pincus et al., 1998; Kruger and Morrison, 2002; Lie et al., 2004; Mitchell et al., 2004; Schouten et al., 2004; Kulbatski et al., 2005; Taupin, 2005). Blocking myelin and other associated regeneration inhibitors, for example using antibodies or competitive antagonist peptides for certain receptors, as well as administering neurotrophic substances or counteracting the glial scar may all be mechanisms by which CNS regeneration can be facilitated (Bregman et al., 1995; Keirstead et al., 1998; Vanek et al., 1998; Watkins and Barres, 2002). A rapidly developing area of neuroregeneration research also involves the transplantation of various stem or progenitor cells, which may be induced to differentiate into neurons and glial cells to replace those degenerated by injury. Moreover, stem cells may be genetically engineered to produce factors that facilitate neuronal regeneration (for recent reviews see Calzà et al., 2004; Mitchell et al., 2004; Picard-Riera et al., 2004; Schouten et al., 2004; Pluchino et al., 2005).

Using *in vitro* and *in vivo* models, Fournier et al. (2003) demonstrated that inhibiting various steps in the Rho signalling pathway (which is pivotal in regulating the actin dynamics) resulted in enhanced neurite outgrowth on inhibitory substrates reduced glial scar formation following lesion as well as promoting axonal regeneration and functional recovery. Watkins and Barres (2002) review recent work indicating that blockade of NgR, a common receptor for the three different myelin proteins, Nogo, myelin-associated glycoprotein (MAG) and oligodendrocyte-myelin glycoprotein (OMgp) enhances CNS repair and functional recovery following injury. Indeed, GrandPre et al. (2002) have developed a competitive antagonist peptide, NEP1-40, which blocks Nogo-66 (one of the two domains of Nogo-A, an isoform of Nogo) and

has the capacity to enhance axonal regrowth, with limited functional recovery following spinal cord injury in rats.*

The extent of functional recovery following injury to the adult CNS remains controversial. However, it is now becoming apparent that certain populations of mature CNS neurons have a remarkable capacity to regenerate if the appropriate growth environment is provided (reviewed by Fawcett and Geller, 1998; McKerracher, 2001), indicating that mature central neurons do retain an intrinsic capacity for axonal regeneration and the lack of regeneration may be more attributed to inhibitory factors in the CNS environment. Several studies have established that the hippocampus is particularly plastic and able to restore function by adaptive regenerative changes following injury including both axonal sprouting and neurogenesis (for example Deller and Frotscher, 1997; Cameron and McKay, 2001; Jin et al., 2001; Braun et al., 2002). However, functional regeneration in the neocortex is a more disputed topic and few studies have specifically addressed the role of neurogenesis and sprouting in contributing to brain healing following focal structural cortical injury.

1.5.5 Implications of aberrant neuronal regeneration

Although research efforts are now directed at replacing lost neurons and promoting axonal growth in the inhibitory environment of the CNS following injury, experimental endeavours should be treated with a certain degree of caution if they are to be translated to the clinical setting. For emerging along side the considerable advances that have led to the discovery and production of potential therapeutic

* Nogo and the Nogo receptor, NgR, are postulated as primary myelin-associated inhibitors. Accordingly, a significant increase in regenerative axonal sprouting occurs following treatment with agents that neutralise these components. Interestingly, however, gene knockouts of both Nogo and NgR confer relatively limited protection to myelin-associate axonal growth inhibition. These findings indicate that the effects of Nogo-NgR neutralising agents may go beyond the inhibition of these myelin components and/or that Nogo-NgR are not as pivotal in axonal regeneration as previously thought (reviewed by Hsuan and Tang, J. Neurochemistry, 2005, 94, 865-847).

agents for CNS trauma have been hints that promoting neurogenesis, neuritogenesis or synaptogenesis in an unregulated manner may generate adverse effects. Indeed, McKinney et al. (1997) have demonstrated that many of the new connections that form when damaged axons sprout and re-synapse with targets are hyperexcitable. Additionally, post-lesion sprouting and neurogenesis has also been implicated in the generation of abnormal circuitry in other brain lesion paradigms (Jacobs et al., 2000; Parent, 2002; Parent and Lowenstein, 2002). These findings indicate that aberrant sprouting and/or abnormal integration of newly born neurons in existing neural circuits may be implicated in the delayed development of post-traumatic epilepsy.

Post-traumatic epilepsy is a common consequence of traumatic head injury (Willmore, 1995; Jacobs et al., 2000; Santhakumar et al., 2001). The onset of epileptic seizures typically occurs 1 to 3 years after head injury and the likelihood of seizure evolution is correlated with the severity of the initial injury (Willmore, 1995). McKinney et al. (1997) postulate that the delay occurring between brain injury and the onset of post-traumatic epilepsy may be attributed to the time required for the elaboration of new axonal processes and formation of new synaptic connections. Importantly, the gradual, albeit partial, recovery of function often observed following human brain injury may also be attributable to processes involving injury-induced neurogenesis, axonal sprouting, synaptogenesis and general brain plasticity. Clearly any intervention that prevents post-traumatic neurodegeneration, inappropriate axonal sprouting or maladaptive neurogenesis and enhances adaptive neural responses has the potential to substantially improve the prognosis of TBI. A great deal of emphasis is therefore being placed upon developing strategies to promote

neural repair following injury. However, many of the basic mechanisms underlying endogenous repair mechanism are not fully understood and need to be discovered to prevent aberrant outcomes.

Aims

Despite an accumulating literature regarding the capacity for neural remodelling and regeneration following structural brain injury many aspects of this response remain elusive. This thesis will, therefore, seek to explore the reactive and regenerative alterations associated with the neural response to physical injury in the adult mammalian brain in an acute model of structural injury to the rodent neocortex. Additionally, the intrinsic regenerative potential of individual mature brain neurons, free of their normal glial matrix, will be assessed in an *in vitro* model of axonal injury. To meet these goals a variety of procedures will be utilised, including immunohistochemical, molecular and imaging techniques, to examine the morphological, cellular and molecular events that characterise the neural response to injury. Specific emphasis will be placed upon investigating the potential for induction of frank neuronal replacement following injury, alterations within the damaged neurons themselves which may be indicative of an intrinsic capacity for regeneration, and the role of the neuronal cytoskeleton in neuronal regenerative events, as the mechanisms underlying these processes are currently poorly understood.

Specific aim 1

To determine the contribution proliferating and progenitor cells make towards brain healing following acute focal brain injury and whether these cells undergo neuronal differentiation which contributes to neuronal replacement within injured brain tissue

Recent data suggest that injury-induced proliferating and progenitor cells may participate in brain healing and cell replacement in a wide range of experimental brain lesion models. However, the potential for neurogenesis in typically non-neurogenic brain regions, such as the neocortex, and in response to acute structural brain injuries has not yet been fully established. Therefore, to address this aim, a rodent model of structural injury specific to the neocortical grey matter of the somatosensory cortex was utilised. The cellular response to acute neocortical injury was investigated utilising a battery of antibodies for proliferating, progenitor, immature and mature neural cell types and alterations in nestin, a marker of putative neural progenitor cells, was extensively investigated using immunohistochemical and quantitative real time RT-PCR. These studies aimed to determine the ultimate fate of injury-induced proliferating and progenitor cells and the potential for these cells to contribute towards neuronal replacement in the injured adult neocortex.

Specific aim 2

To investigate the reactive and regenerative changes that characterise the neural response to physical injury and explore the co-ordinated alterations, in both neuronal and glial cell populations, in contributing to this response

The extent to which the adult brain undergoes recovery following injury remains controversial. Indications of brain healing have been demonstrated following various brain lesions, however, the extent of neuro-glial regeneration remains questionable. Utilising the *in vivo* model of structural brain injury from Aim 1, the response of various populations of neural cells, including neurons, astrocytes, microglia and macrophages as well as alterations in brain vasculature and CNS

myelin, were analysed with immunohistochemical techniques. Additionally, quantitative one-step real-time RT-PCR was utilised to examine alterations in gene expression of specific neuronal cytoskeletal- and growth-associated proteins, to determine whether neuronal alterations evoked by injury at the cellular level are reflected by complementary changes in gene expression. One specific sub-aim of this study was to assess injury-induced alterations in α-internexin, which, along with the neurofilament triplet proteins, is a member of the neuronal intermediate filament family of proteins. Although the neurofilament triplet proteins have been widely cited as being disrupted and undergoing abnormal accumulation in response to brain injury in the adult, α-internexin is largely viewed as a developmentally important protein and few data exist demonstrating the role of α-internexin in injury and neuronal regeneration.

Specific aim 3

To determine the cytoskeletal mechanisms utilised by axotomised CNS neurons during regenerative events and how these changes compare to initial neurite development

The axonal cytoskeleton plays a crucial role in initial neurite outgrowth and elongation and has been proposed to be important in axonal sprouting and/or regeneration. This aim examines the cytoskeletal alterations, specifically the reactive and regenerative changes that characterise the axonal response to physical injury. To meet this aim a novel *in vitro* model of axonal injury was utilised, whereby neocortical neurons from rat embryos were grown in culture to relative maturity.

The growth conditions of this system promoted neuronal aggregation and axonal fasciculation. Axonal bundles were then transected, under microscope guidance. The response of normally reacting and cytoskeletally perturbed axons was analysed using multi-labelling immunohistochemistry, live cell imaging and transmission and scanning electron microscopy. Specifically, all major components of the axonal cytoskeleton were examined including the neuronal intermediate filament proteins α-internexin, NF-L, NF-M and NF-H, microtubules (and their associated proteins) and microfilaments. Cytoskeletal perturbations were performed by the addition of cytoskeletal stabilising and destabilising agents, including latrunculin A, taxol and nocodazole.

In summary this study explores, characterises and clarifies the reactive and regenerative events that constitute the consequences of physical injury to mature CNS neurons. These studies are imperative in understanding the regenerative potential of nerve cells in the adult mammalian brain and provide insight into aspects of the post-injury neural response that may be targeted through therapeutic intervention to facilitate adaptive alterations in the neural response to physical injury. Results from this investigation will enhance current knowledge regarding the basic mechanisms underlying neuronal and axonal regeneration and have clinical implications regarding appropriate targets for therapeutic intervention during neuronal degenerative and regenerative events.

Experimental procedures

All experimental procedures were based on investigating the specific cellular and molecular alterations evoked following structural neuronal or brain injury using either an *in vivo* or *in vitro* model of physical injury, as detailed below.

2.1 Experimental procedures relating to *in vivo* structural brain injury

2.1.1 General animal conditions and care

All experimental procedures involving animals were approved by the Animal Ethics Committee for Animal Experimentation of the University of Tasmania and are in accordance with the Australian code of practice for the care and use of animals for scientific purposes. Animals were obtained from the Central Animal House (University of Tasmania). For all procedures, adult male Hooded Wistar rats (250-270g corresponding to approximately 7.5 weeks old) were utilised. Animals were housed in standard conditions (20°C, 12/12 hours light/dark cycle), with access to food and water *ad libitum*. Animals were monitored daily for signs of illness and stress. Water, food and bedding were replenished twice weekly.

2.1.2 *In vivo* structural brain injury

Focal injures to the rodent neocortex were performed as previously described (King et al., 2001). Briefly, following intraperitoneal administration of anaesthetic (pentobarbitone sodium, 72mg/kg, Abbot Laboratories, Paramatta, NSW, Australia)

and analgesic (Carprogen, 4mg/kg, Pfizer), animals were immobilised in a Stoelting stereotaxic frame. After retracting the scalp, a burr hole was drilled into the skull, 5mm anterior and 4.5mm lateral to lambda and a focal injury was made in the somatosensory cortex (Par1 region) by lowering a 25 gauge Hamilton needle to a depth of 1.5mm into the grey matter (Figure 2.1). The needle was left in place for ten minutes prior to removal and suturing of the wound. Animals were terminally anaesthetised (pentabarbitone sodium, 140mg/kg) at a range of post-injury time intervals up to 84 days following injury (n = 3-4 per time point). Using gravity feed, blood was cleared by transcardial perfusion of 0.01M phosphate buffered saline (PBS) for two minutes. Brains were processed for either immunohistochemistry or quantitative real time RT-PCR. For RNA extraction and real time RT-PCR, the brain was removed from the skull and processed as described in Section 2.1.5. For immunohistochemical analysis, blood was cleared by gravity feed with 0.01M PBS. Additionally, the tissue was fixed by gravity feed perfusion with 4% paraformaldehyde (PFA)/0.01M PBS for five minutes.

2.1.3 BrdU preparation and animal injection

To examine populations of proliferating cells animals were administered with the thymidine analogue, 5-Bromo-2'-deoxyuridine (BrdU, Sigma, St Louis, MO) by intraperitoneal injection. A 12.5mg/ml solution of BrdU was prepared in 7mM NaOH/0.9% NaCl, and filter sterilised. Animals were injected with BrdU twice daily (25mg/kg/day) between 1 and 6 days post-injury and were transcardially perfused (4% PFA/0.01M PBS) at 7, 21 or 42 days post-injury. Prior to immunohistochemistry, antigen retrieval was performed by incubating tissue sections

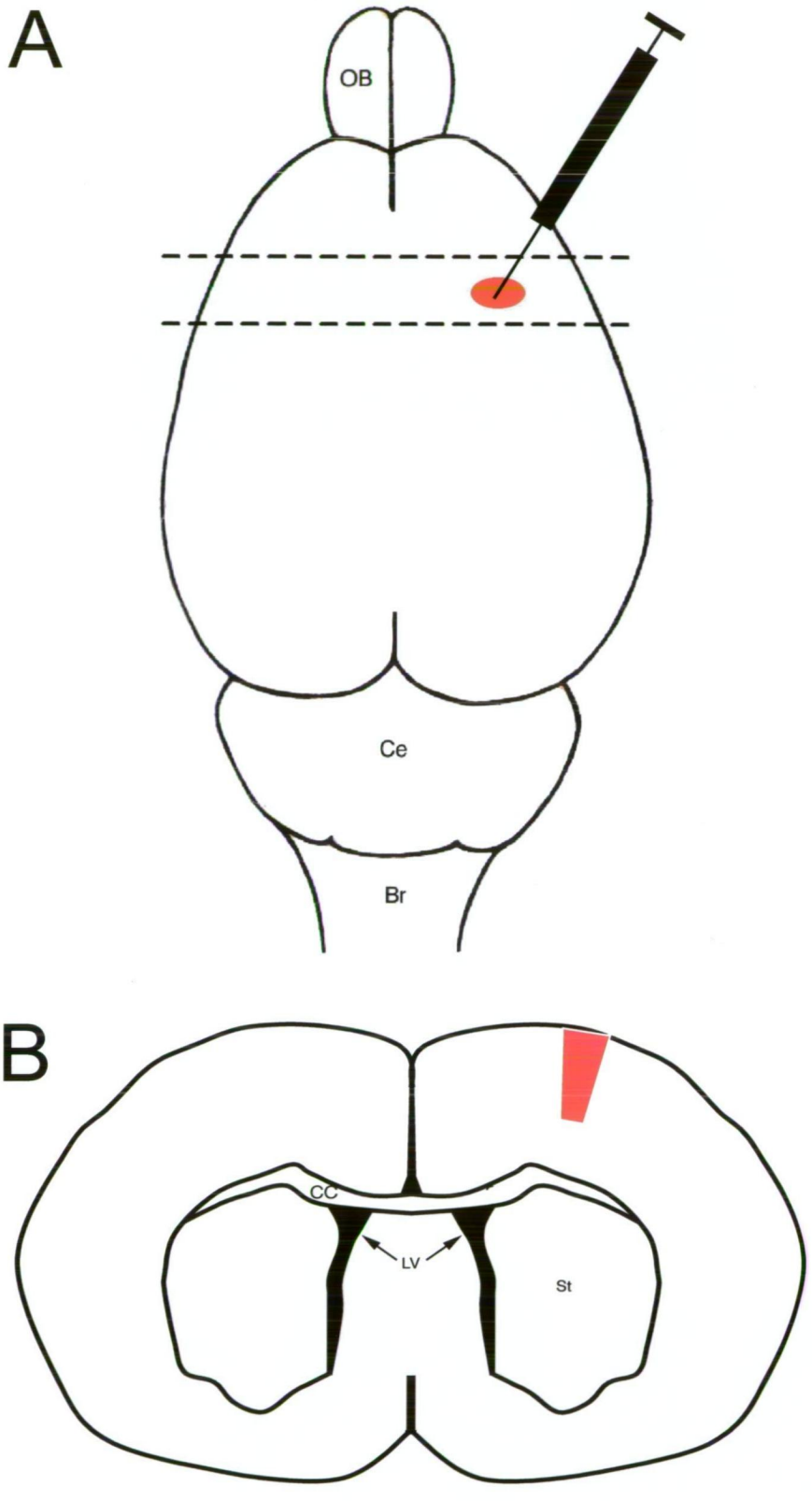


Figure 2.1. Diagrammatic representation of *in vivo* focal neocortical structural injury model. A, top view; B, coronal section

OB, olfactory bulb; Ce, cerebellum; Br, brain stem; CC, corpus callosum; LV, lateral ventricle; St, striatum

in 2M HCl for one hour at 37°C, followed by three neutralisation washes in 0.1M borate buffer (pH 8.5). Immunohistochemistry was performed as described Section 2.1.4, utilising an antibody directed against BrdU (BD Biosciences, Table 2.1).

2.1.4 Immunohistochemistry and staining of rat brain tissue sections

Prior to immunohistochemistry, brains were post-fixed in 4% PFA/0.01M PBS for 24 hours and subsequently cryoprotected in solutions of 18% and 30% sucrose. Coronal brain sections were cut at 40μm on a freezing microtome to include the entire injury site or equivalent region in control brains. Tissue sections were incubated simultaneously in combinations of primary antibodies (Table 2.1) for two hours at room temperature followed by overnight incubation at 4°C. Mouse, rabbit and guinea pig primary antibodies were visualised with Alexa Fluor goat anti-mouse, goat anti-rabbit or goat anti-guinea pig secondary antibodies as appropriate (Molecular Probes, dilution 1:1000), however BrdU and PCNA* were visualised with anti-mouse rat-adsorbed Fluorescein secondary antibody (dilution 1:500, Vector Laboratories, Burlingame, CA, USA). Additionally, to examine microglial and perivascular cells following injury, brain sections were incubated in the presence of Isolectin IB_4, Alexa Fluor 594 conjugate (Molecular Probes, 1:100). All primary and secondary antibodies as well as Isolectin IB_4 were diluted in 0.3% Triton X-100 in 0.01M PBS. Controls, omitting the primary antibody were processed concurrently to ensure they lacked immunoreactivity for the cellular components under investigation.

* Proliferating cell nuclear antigen (PCNA) is an endogenous cell replication marker, essential for DNA synthesis. In fixed tissue, PCNA antibodies will label cells in various phases of the cell cycle, that were proliferating at the time of tissue fixation. Conversely, BrdU is an artificial thymidine analogue that, when administered to an anima/cell will incorporate into DNA during S-phase of the cell cycle and is thus useful for retrospectively tracking cell proliferation. Utilised in combination, PCNA and BrdU allow the dynamics of cell proliferation and cell fate to be determined and provide a more complete picture of cell proliferation that either marker used alone.

Table 2.1. Primary antibodies utilised in immunofluorescent cell labelling

Antibody	Type	Immunoreactivity	Dilution	Supplier
Anti-NF-L	R	68kD neurofilament triplet light/low molecular weight subunit	1:1000	Novus Biologicals
Anti-NF-M	R	150kD neurofilament triplet medium molecular weight subunit	1:2000	Serotec
Anti-NF-H	R	200kD neurofilament triplet heavy/high molecular weight subunit	1:2000	Serotec
Anti-parvalbumin	R	Interneuron calcium-binding protein	1:2000	Swant
Anti-calretinin	R	Interneuron calcium-binding protein	1:2000	Chemicon International
Anti-ferritin	R	Microglial iron-binding protein	1:2000	Dako
Anti-GFAP	R	Glial fibrillary acidic protein	1:2000	Dako
Anti-tau	R	Phosphorylation independent tau	1:5000	Dako
Anti-NSE	R	Neuron-specific enolase	1:500	Dako
Anti-α-internexin	R	Class IV intermediate filament subunit, distinct from neurofilament triplet	1:2000	Novus Biologicals
Anti-α-internexin	M	Class IV intermediate filament subunit, distinct from neurofilament triplet	1:1000	Chemicon
Anti-synaptophysin	M	Presynaptic vesicles	1:100	Boehringer Mannheim
Anti-GAP43	M	Growth-associated protein 43	1:1000	Sigma
Anti-nestin	M	Intermediate filament protein specific for neural progenitor cells	1:1000	BD Biosciences
Anti-NeuN	M	Post-mitotic neuronal nuclei	1:100	Chemicon International
Anti-βIII-tubulin	M	Neuron specific β-tubulin subunit	1:5000	Promega
Anti-MAP2	M	Microtubule associated protein 2	1:1000	Chemicon International
Anti-PCNA	M	Proliferating cell nuclear antigen	1:500	Zymed
Anti-BrdU	M	Bromodeoxyuridine, thymidine analogue	1:500	BD Biosciences
Anti-BrdU	M	Bromodeoxyuridine	1:1000	Sigma
Anti-myelin/ oligodendrocyte specific protein	M	Oligodendrocytes and CNS myelin	1:500	Chemicon
Anti-SMI312	MC	Phosphorylated neurofilaments	1:5000	Sternberger
Anti-Doublecortin	GP	Microtubule associated protein specific for migrating neuroblasts	1:1000	Chemicon

R, Rabbit polyclonal antibody; M, mouse monoclonal antibody; MC, mouse monoclonal cocktail; GP, guinea pig polyclonal antibody

2.1.5 Total RNA extraction

Animals were terminally anaesthetised and transcardially perfused with 0.01M PBS (n = 4 per time point) at 1, 7, 14 and 28 days post-injury. Non-injured, age-matched controls corresponding to the 1 and 28 day post-injury time point (n = 3 per time point) were processed concurrently. Following removal, all brains were snap frozen in liquid nitrogen and stored for later dissection. Prior to dissection brains were thawed slightly and a 2mm coronal section of tissue was harvested from each brain, to include the injury site, or equivalent region in control brains. RNA extraction was performed using the TRIzol® method according the manufacture's instructions (Invitrogen, Carlsbad, CA, USA), under RNase-free conditions. Tissue samples were homogenized in 500µl TRIzol® reagent using a 1ml syringe with a 26G, followed by 29G, needle attached.

2.1.6 Quantitative one-step real-time RT-PCR

Quantitative analysis of mRNA levels was performed using the QuantiTect one-step SYBR Green RT-PCR Kit (Qiagen, Doncaster, VIC, Australia), following the manufacturers instructions, with minor modifications. Briefly, all amplification reactions were carried out in duplicate with 20ng of total RNA per reaction. Each reaction contained 1 x QuantiTect SYBR Green (containing HotStarTaq DNA Polymerase, buffer, dNTP mix, SYBR Green I, reference dye and 5mM $MgCl_2$), 0.5µM of forward and reverse primers and 0.1µl of QuantiTect RT Mix (containing Omniscript and Sensiscript Reverse Transcriptases) in a total of 10µl. Quantitative real-time RT-PCR was performed using the Rotorgene 3000 (Corbett Research,

Sydney, NSW, Australia). Amplification conditions included 30 minutes at 55°C (reverse transcription), 15 minutes at 95°C (inactivation of reverse transcriptase and activation of HotstartTaq Polymerase), and then 45 cycles at 94°C for 15 seconds (denaturation), 55°C for 30 seconds (annealing), 72°C for 30 seconds (extension and data acquisition). Additionally, a melt curve analysis was performed at the end of each run at 60-95°C. For each run, no-template-controls as well as cDNA and RNA standards were included for each primer set to identify potential genomic DNA contamination and to check reaction efficiency, respectively. Standard curves for each primer set were performed using total rat brain RNA and samples were normalised with the housekeeping gene, GAPDH. Primers sequences were all approximately 20 base pairs in length and produced amplicons ranging in size from 150-450 base pairs (Table 2.2). Primer sequences for βIII-tubulin and NF-L were obtained from Pernas-Alonso et al. (1999). Sequences for all other primer pairs, including NF-H, GAP-43, α-internexin, nestin and GAPDH, were designed using the Primer Express Software (Applied Biosystems, Foster City, CA, USA).

2.1.6.1 Statistical analysis

All data are reported as a mean fold change relative to non-injured control brains ± standard error of the mean (SE). Microsoft Excel was used to prepare data/graphs and determine statistical significance. A p value of < 0.05 was considered statistically significant. Preliminary statistical analysis was performed to determine whether the young (age matched to the 1 day post-injury) and old (age-matched to the 28 day post injury) control groups differed significantly from one another in the expression of each gene examined ($n = 3$ in each group). No statistically significant

Table 2.2. Primer pairs for one-step quantitative real-time RT-PCR

Gene/Primer	Sequence 5'-3'	Nucleotide size
GAPDH		
Forward	TTCATTGACCTCAACTACAT	20
Reverse	GTGGCAGTGATGGCATGGAC	20
Nestin		
Forward	AGCTCTTGGAACCCTGTTCA	20
Reverse	CTGCCTCGCTTTCTTCTCTG	20
GAP-43		
Forward	TGTCAAACCGGAGGATAAGG	20
Reverse	TGCATCGGTAGTAGCAGAGC	20
α-internexin		
Forward	GGTACAAGTVVAAGTTCGCCA	21
Reverse	TGCCCAATGCTATCCTGGTAG	21
βIII-tubulin		
Forward	TTGCAGCTGGAGAGAATCAA	20
Reverse	CTCGGCACCCTCTGTGTAGT	20
NF-L		
Forward	ATGAGCAGGAGATCCGTGATCT	22
Reverse	CAGGAAGGCTATCTCGTCCATC	22
NF-H		
Forward	AGCTGCTCGGTCAGATTCAG	20
Reverse	GCATCCGTGTTCACTTTGG	19

differences in mRNA levels between these control groups were indicated for any of the genes examined, therefore, these control groups were pooled (n = 6).

2.2 Experimental procedures relating to *in vitro* neuronal injury

2.2.1 Primary dissociated neocortical neuron culture preparation

Primary dissociated cortical neuron cultures were prepared from rat embryos at 18 days of gestation using standard techniques (Banker and Goslin, 1998) and grown in Neurobasal™ media, to selectively promote the growth of neuronal cells (Brewer et al., 1993; Brewer, 1995, 1997). Pregnant Hooded Wistar rats were killed by CO_2 exposure. Embryos were rapidly removed using sterile techniques, placed on ice and, following removal of the skull and meninges, the neocortical hemispheres were dissected out and placed in 10mM HEPES buffer (in 0.01M PBS), pre-warmed to 37°C. Cortical tissue was dissociated by enzymatic digestion (0.025% trypsin) and gentle agitation at 37°C for 20 minutes. Enzymatic digestion was halted by rinsing the tissue in fresh 10mM HEPES buffer and dissociation was completed by gentle trituration using a 1ml pipette.

Prior to plating, cell viability was assessed using trypan blue vital dye exclusion. Cells were plated, at a density of 4.5 x 10^5 cells/coverslip, onto circular 19mm diameter glass coverslips (Marienfeld, Lauda-Koenigshoten, Germany), immersed in an initial plating media, consisting of Neurobasal™ media, 10% foetal bovine serum, 2% B-27 supplement (all from Invitrogen BRL, Life Technologies, Grand Island, NY, USA), 0.5mM L-glutamine, 25μM glutamate and 1ml/L gentamicin. Coverslips

had previously been nitric acid treated and pre-incubated in 1mg/ml poly-L-lysine (Sigma, St. Louis, MO, USA) in borate buffer (pH 7.4) overnight, before being placed into individual wells of 12-well microplates (Iwaki, Tokyo, Nihonbashi, Japan), each containing 2ml of initial plating media. Cultures were maintained in a 37°C humidified atmosphere of 5% CO_2 for up to 28 days. After 1 day *in vitro* (DIV), the initial plating media was removed and replaced with a "subsequent", serum-free growth media containing Neurobasal™ media, 2% B-27 supplement, 0.5 mM L-glutamine and 3ml/L gentamicin. Half the volume of culture media was replenished every 3 to 4 days with fresh subsequent growth media.

2.2.2 *In vitro* axonal injury

Axonal injury was performed as described by Dickson et al. (2000). At 20 DIV, coverslips were transferred to individual 35mm sterile plastic Petri dishes (Iwaki, Nihonbashi, Tokyo, Japan) and allowed to re-acclimatise to the incubator conditions. At 21 DIV, individual axonal bundles, interconnecting discrete neuronal aggregates, were transected under microscope guidance using a 12cm Barkan goniotomy curved blade diamond knife (Altomed, London, England). Thick axonal bundles, adherent to the underlying substrate were selected for transection. The knife blade was carefully pressed upon the appropriate axonal bundles to completely transect the axons and form a cell-free lesion between two previously interconnected neuronal aggregates. Cuts were made equidistant between neuronal aggregates to produce lesions approximately 50-150μm wide. Several (10-15) injuries were made per coverslip. Cultures were removed from the incubator for no longer than 5 minutes (as longer durations compromised neuronal integrity). Cultures were re-incubated

for a range of time points following injury (as described in individual chapters).

2.2.3 Immunohistochemistry and staining of cultured neocortical cells

Cultured neurons were fixed in 4% PFA at room temperature for 30 minutes. Indirect double immunofluorescent labelling was utilised to visualise the distribution of specific cellular components in developing neurites as well as injured and non-injured mature axons. Briefly, cultured monolayers were incubated in combinations of mouse and rabbit primary antibodies for two hours at room temperature, followed by incubation in secondary antibodies for one hour at room temperature. Mouse and rabbit primary antibodies were visualised with goat anti-mouse and goat anti-rabbit Alexa Fluor 488™ and 594™ secondary antibodies (Molecular Probes, Eugene, OR, USA, dilution 1:1000) as appropriate. To label for filamentous actin, cultures were incubated with AlexaFluor 488 phalloidin (Molecular Probes, Eugene, OR, USA, dilution 1:200), at room temperature for 30 minutes subsequent to immunohistochemistry. Following washing, coverslips were mounted onto glass slides using Permafluor mounting media (Immunotech, Marseille, Cedex, France). All antibodies were diluted in 0.3% Triton X-100 in 0.01M PBS. Controls, omitting the primary antibodies, were processed concurrently and lacked immunoreactivity for the cellular components under investigation.

2.3 Microscopy

All fluorescence labelled sections and cell monolayers were viewed with a Leica DMIRB or DMLB2 microscope equipped with a range of objectives. Filter blocks within the microscope enabled both selective and combined visualisation of

fluorescence labelled epitopes within each specimen. Digital images were captured with an Optronics Magnifire cooled CCD camera (Optronics, Chelmsford. MA, USA) attached to a G4 Macintosh computer (Apple Computers, Cupertino, CA, USA), using Magnifire Software.

Acute structural injury to the brain induces substantial alterations in cell proliferation and endogenous progenitors cells directed towards brain healing

3.1 Introduction

The adult mammalian brain is incapable of undergoing full structural and functional restoration following physical injury, however, accumulating evidence indicates that the damaged brain undergoes a specific sequence of adaptive alterations directed at tissue healing, which may involve the recruitment of endogenous proliferating and progenitor cells. Understanding the mechanisms employed by the damaged brain to self-heal is of paramount importance in devising effective therapies to treat brain injury. In this regard, similar to other tissues, structural injury to the brain evokes a distinct sequence of events indicative of a wound healing response. A tissue wound, defined as a morphologic-functional disruption of the continuity of a tissue structure (Oehminchen, 2004), induces a healing response involving distinct phases of coagulation, inflammation, proliferation, migration and remodelling (Maxwell et al., 1990; Hunt et al., 2000; Yamaguchi and Yoshikawa, 2001; Oehminchen, 2004) and may involve processes directed at tissue regeneration or repair. Regeneration essentially infers the complete restoration of injured tissue, whereas repair involves the replacement of lost tissue with scar tissue and is associated with varying degrees of functional recovery.

Injury to the CNS evokes tissue specific processes directed at healing and scar formation, including the activation of a variety of glial cell populations, which

ultimately contribute to formation of a glial scar and may create an environment inhibitory to neuronal regeneration (Fawcett and Asher, 1999). With regard to tissue healing, the discovery of proliferating and progenitor cells with neurogenic potential in the adult mammalian brain has widespread implications for brain recovery following injury (Kozorovitskiy and Gould, 2001; Brazel and Rao, 2004). Indeed, altered cell proliferation and changes in neural progenitor cell populations have been demonstrated in diverse models of experimental brain lesion and are proposed to contribute to post-lesion brain recovery (Clarke et al., 1994; Duggul et al., 1997; Holmin et al., 1997; Li and Chopp, 1999; Sahin Kaya et al., 1999; Kernie et al., 2001; Arvidsson et al., 2002; Chirumamilla et al., 2002; Shi et al., 2002; Chen et al., 2003a; Nakamura et al., 2003; Tonchev et al., 2003; Douen et al., 2004; Salman et al., 2004).

Interestingly, the SVZ of the anterior lateral ventricles is regarded as the primary germinal zone of the adult brain and, in addition to the hippocampal dentate gyrus, retains neurogenic potential into adulthood (for recent reviews Conover and Allen, 2002; Abrous et al., 2005; Doetsch and Hen, 2005). Indeed, populations of progenitor and proliferating cells in the SVZ give rise to more restricted progenitor phenotypes, generating astrocytes, oligodendrocytes and neurons, and contribute to cell turnover throughout life (Lendahl et al., 1990; Lois and Alvarez-Buylla, 1993; Weiss et al., 1996; Doetsch et al., 1999; Alvarez-Buylla and Doetsch, 2002; Conover and Allen, 2002; Limke and Rao, 2002; Okano, 2002; Marshall et al., 2003; Mignone et al., 2004). Moreover, neural progenitors have also been demonstrated in several typically non-neurogenic brain regions (reviewed by Emsley et al., 2005). Thus, the

recruitment of proliferating and progenitor cells from the SVZ, as well as parenchymal-resident progenitor cells, are postulated sources for cell replacement following brain lesion. Ultimately, injury-induced proliferating and progenitor cells may generate new neurons and/or glia (Gu et al., 2000; Magavi et al., 2000; Jin et al., 2001; Chen et al., 2003a, b; Douen et al., 2004; Salman et al. 2004). Collectively, reports of injury-induced neuro- and glio-genesis indicate intrinsic repair mechanisms are initiated in the damaged brain (review by Pluchino et al., 2005). However, whether brain injury evokes a neurogenic response that contributes to functional recovery remains contentious.

In light of accumulating data implicating cell proliferation and progenitor cell differentiation as mechanisms of brain repair following injury, the current study utilised a model of acute focal injury to the rat neocortex to examine the contribution of these cells towards brain healing. This injury model produces a wound-like lesion within the grey matter of the neocortex (King et al., 2001). Immunohistochemical and quantitative real-time RT-PCR techniques were used to examine the spatio-temporal alterations and neurogenic potential of injury induced proliferating and progenitor cells. The cellular response to acute neocortical injury was investigated utilising a battery of antibodies for proliferating, progenitor, immature and mature neural cell types. Injury-induced alterations in neural progenitor cells were identified based on expression of the embryonic cytoskeletal protein, nestin. Findings from this study are discussed with regard to an emerging body of literature indicating that the damaged brain undergoes endogenous repair, involving cell proliferation, glial activation and, potentially, neurogenesis.

3.2 Experimental procedures

3.2.1 Immunohistochemistry of rat brain tissue sections

Following *in vivo* structural brain injuries as described in Section 2.1.2, Figure 2.1, a battery of antibodies, directed at different neural cell populations and differentiation states, was utilised to determine the response and fate of proliferating, progenitor and glial cells as well as immature and mature neurons. Immunohistochemistry was performed as described in Section 2.4.1, using antibodies specific for a variety of different neural cell populations (see Table 3.1 below). Specific antibody details are provided in Table 2.1. BrdU and PCNA were utilised to identify proliferating cells. Neural progenitor cells were examined based on expression/content of the embryonic cytoskeletal protein, nestin, which has previously been associated with this cell population (Lendahl et al., 1990; Dahlstrand et al., 1995; Taupin and Gage, 2002; Michalczyk and Ziman, 2005). Doublecortin (DCX), a microtubule-associated protein specific for migrating and differentiating neuroblasts (Francis et al., 1999; Gleeson et al., 1999) and potentially indicative of neurogenesis (Couillard-Depres et al., 2005) was utilised to determine the potential recruitment of migrating neuroblasts to the injury site. Neuron-specific βIII-tubulin was utilised to identify early neurons, whereas mature pyramidal neurons and non-pyramidal/interneurons were distinguished based on immunolabelling for the neuronal intermediate filament proteins, NF-M and α-internexin, or calcium binding proteins, calretinin and parvalbumin, respectively. Activated microglia/macrophages were visualised based on labelling for the iron binding protein ferritin and astrocytes were identified based on GFAP immunoreactivity.

To determine alterations in different cell populations following cortical injury, rats were terminally anaesthetised (pentabarbitone sodium, 140mg/kg) at 1, 7, 14, 21, 42 and 84 days following injury (n=3 per time point) and transcardially perfused with 4% PFA/0.01M PBS. Non-injured, age-matched controls corresponding to the 1, 42 and 84-day post-injury time points (n=3 per time point) were also perfused. For double labelling of nestin versus BrdU or PCNA, a sequential labelling protocol was performed whereby tissue sections were first incubated with anti-BrdU/PCNA antibody, followed by saturation with anti-mouse rat-adsorbed Fluorescein secondary antibody (dilution 1:50). Nestin immunolabeling was subsequently performed with anti-mouse AlexaFluor 594 secondary antibody. Negative controls, omitting the primary antibodies, were processed concurrently to ensure the absence of non-specific labelling. This protocol was a satisfactory method of labelling as nestin and BrdU/PCNA were localised to different cellular compartments, negating the possibility of ambiguous labelling.

Table 3.1. Antibodies utilised to identify different neural cell populations

Cell type	Marker
Proliferating cells	BrdU
	PCNA
Neural progenitor cells	Nestin
Neuroblasts	Doublecortin
Young neurons	βIII-tubulin
Pyramidal neurons	NF-M
	α-internexin
Interneurons	Calretinin
	Parvalbumin
Astrocytes	GFAP
Activated microglia and macrophages	Ferritin

3.2.2 Immunofluorescence quantitation

To determine alterations in nestin protein levels within perilesion tissue, the intensity of immunolabelling was quantitatively assessed by optical density measurements (Adobe Photoshop 7) from regions immediately bordering the injury site (Figure 3.1) and used as a relative measure of alterations in protein level following needle injury to the rat neocortex. Images were captured with a 40x objective immediately lateral and medial to the injury site, or in equivalent regions of non-injured control brains and mean optical density measurements were quantitated relative to non-injured control tissue. To limit discrepancies in the period each brain section was exposed to both ambient and immunofluorescent light, immunofluorescence images required for analysis were captured from several brain sections at each time point on the same day and at the same camera exposure level.

3.2.3 Quantitative one-step real-time RT-PCR

To determine alterations in nestin mRNA levels following cortical injury, rats were terminally anaesthetised at 1, 7, 14 and 28 days following injury (n = 4 per time point). Non-injured, age-matched controls corresponding to the 1 and 28 day post-injury time points (n = 3 per time-point) were also perfused. Tissue was dissected from three regions within each brain section (Figure 3.1): the lesion site, perilesion tissue immediately lateral and medial to the lesion and the SVZ, including some sub-cortical white matter. Medial and lateral perilesion tissue samples for each brain were pooled. Tissue was processed for mRNA analysis as described in Section 2.1.6, utilising forward and reverse primers for nestin and GAPDH displayed in Table 2.2.

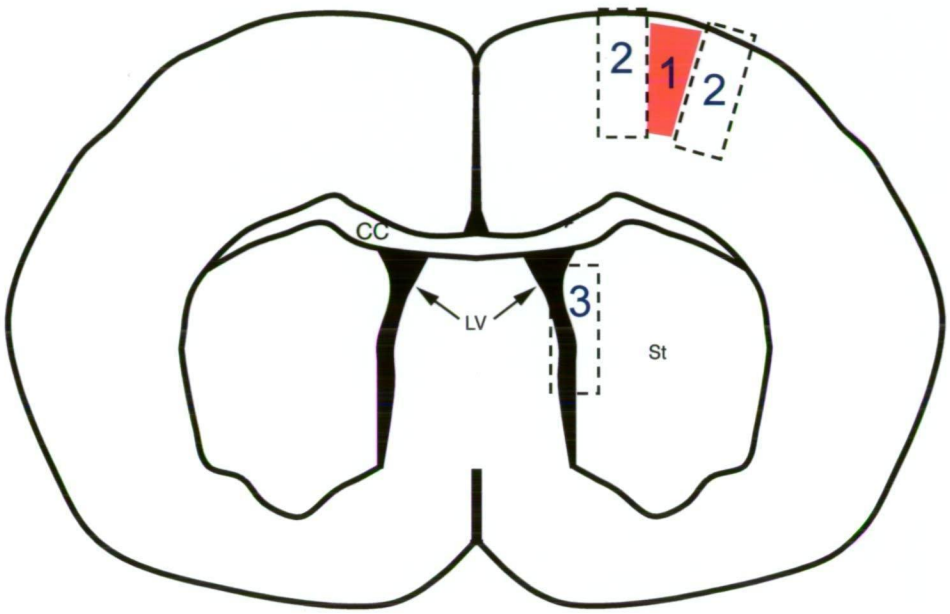


Figure 3.1 Diagrammatic representation of sites from which tissue was harvested for RNA extraction to be assessed by quantitative real time RT-PCR analysis

Tissue was harvested from three brain regions: (1) the lesion site, (2) perilesion tissue and (3) the SVZ. Tissue from the perilesion region was pooled prior to RNA extraction. Tissue harvested from the SVZ included some sub-cortical white matter. CC, corpus callosum; SVZ, subventricular zone; St, striatum.

3.3 Results

3.3.1 Alterations in cell proliferation in response to acute neocortical injury

Proliferating cells were assessed utilising the mitotic markers, BrdU and PCNA. BrdU and PCNA immunopositive nuclei were rarely observed within non-injured tissue sections, where cell proliferation was limited and generally restricted to the SVZ and pial covering, with occasional labelled nuclei distributed elsewhere in the brain parenchyma. However, cell proliferation was a prominent feature of the lesioned neocortex (Figure 3.2). At 1 day post-injury, BrdU immunoreactive nuclei were present within perilesion tissue (Figure 3.2A), as well as the SVZ and corpus callosum (Figure 3.2B). By 7 days following injury, both BrdU and PCNA labelling was extensive. BrdU immunoreactive proliferating cells were distributed in a broad zone surrounding the injury site (Figure 3.2C) and extending into the corpus callosum and SVZ (Figure 3.2D) and PCNA labelled cells were distributed in a similar pattern (Figure 3.2E and F). Over the time course examined, BrdU immunoreactive nuclei decreased in the ipsilateral lesioned neocortex. At 21 days following injury, proliferating cells were generally restricted to sites immediately surrounding the lesion (Figure 3.2G) and, by 42 days following injury, these cells were retained within a narrow band at the lesion border (Figure 3.2H).

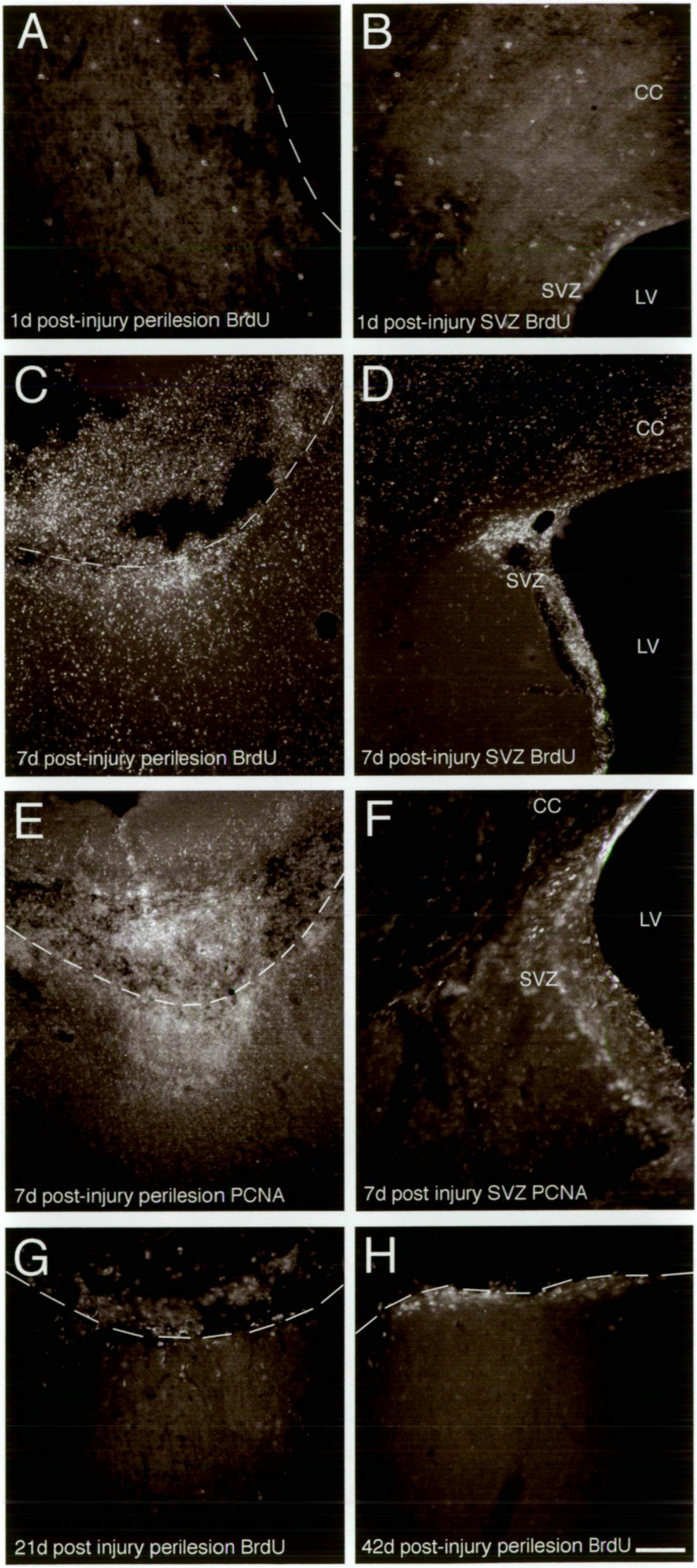
3.3.2 Alterations in nestin expression and distribution in response to acute neocortical injury

The injury-induced response of a population of endogenous neural progenitor cells was assessed based on the cellular expression/content of nestin. Figure 3.3 illustrates

Figure 3.2 Acute structural neocortical injury induced a substantial increase in cell proliferation

Immunofluorescence labelling for proliferation markers, BrdU and PCNA following transient localised injury to the rat somatosensory cortex. At 1 day following injury, BrdU immunoreactive nuclei were scattered sporadically within perilesion tissue (A) as well as in the corpus callosum and SVZ (B). By 7 days following lesions, BrdU immunoreactive nuclei were abundant and distributed within the lesion cavity as well as in a broad zone surrounding the lesion site (C) and extending into the corpus callosum and SVZ (D). Labelling for PCNA at 7 days post-injury demonstrated a similar labelling pattern to that observed for BrdU at the lesion site (E) and within the SVZ (F). By 21 days following injury, BrdU immunoreactive nuclei were more restricted in distribution and were localised predominantly to the perilesion region (G), forming a narrow band at the lesion border by 42 days post-injury (H). Dotted lines indicate the lesion border. SVZ, subventricular zone; CC, corpus callosum; LV, lateral ventricle.

Scale bar: A, B, F = 100μm; C, D, E, G, H = 200μm.


A
1d post-injury perilesion BrdU
B
CC
SVZ
LV
1d post-injury SVZ BrdU
C
7d post-injury perilesion BrdU
D
CC
SVZ
LV
7d post-injury SVZ BrdU
E
7d post-injury perilesion PCNA
F
CC
LV
SVZ
7d post injury SVZ PCNA
G
21d post injury perilesion BrdU
H
42d post-injury perilesion BrdU

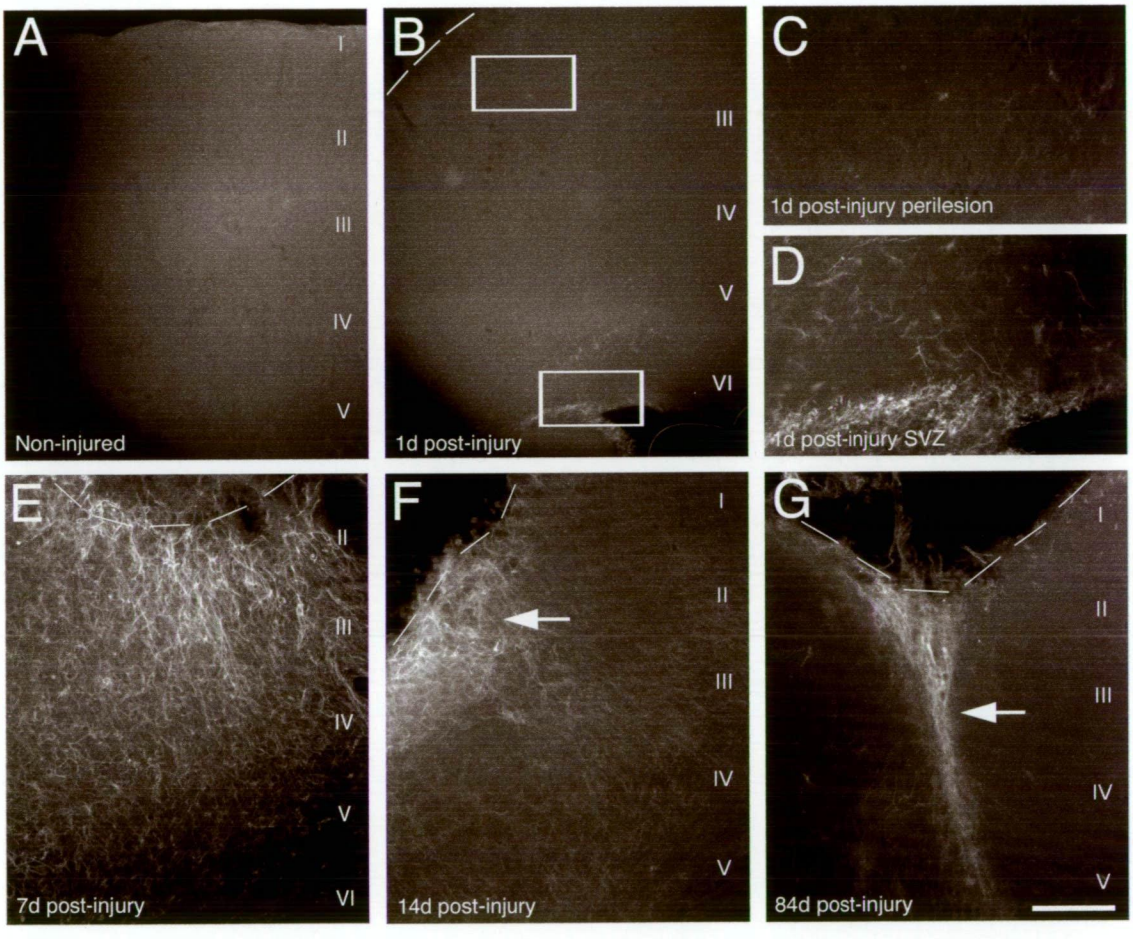
alterations in the distribution of nestin immunoreactivity over a time course ranging from pre-injury to 84 days post-injury. Nestin immunoreactive cells were not evident in the neocortex of control non-injured brains (Figure 3.3A), although some labelling was observed in the periventricular region. In response to injury, nestin labelling became a prominent feature of the ipsilateral cortical tissue. At 1 day post-injury, occasional nestin immunoreactive cells were observed within the deeper cortical laminae and corpus callosum, emanating from the SVZ (Figure 3.3B, C and D). By 7 days post-injury, nestin immunoreactive profiles were localised throughout the neocortical laminae and were especially abundant within the perilesion tissue (Figure 3.3E). Nestin immunolabelling was also evident at later post-injury time intervals, however, from 14 days post-injury, distinct nestin immunoreactive cellular profiles were less frequent (Figure 3.3F) and nestin immunoreactivity became progressively restricted to a narrow zone surrounding the injury site. By 42-84 days post-injury, a remnant of relatively low nestin immunoreactivity was observed lining the lesion cavity (Figure 3.3G), a region in which a dense plexus of GFAP immunoreactive astrocytes has previously been demonstrated to form in this lesion model (King et al., 2001, see also Chapter 4). Alterations in the re-distribution of nestin cells within the neocortex were restricted to the ipsilateral cortex and were not observed in cortical tissue contralateral to the lesion.

Alterations in nestin gene expression, in response to localised neocortical injury, were examined at 1, 7, 14 and 28 days post-injury utilising quantitative real time RT-PCR, based on changes in nestin mRNA levels in tissue from the injury site, the perilesion region and the SVZ, relative to equivalent regions from non-injured

Figure 3.3 Acute structural to the rodent neocortex induced substantial alterations in the distribution of nestin

Nestin labelling in the non-injured cortical parenchyma was negligible (A). At 1 day post-injury, occasional nestin-labelled cells were observed within the corpus callosum, extending from the SVZ, but not perilesion tissue (B, boxes in B are represented in C and D as high magnification images). By 7 days following injury, nestin labelling was abundant, extending in a broad zone surrounding the lesion (E) and although still present at 14 days following injury, nestin labelling was reduced to a narrow zone in the perilesion tissue (arrow in F denotes perilesion nestin labelling). Although nestin labelling was retained within the perilesion tissue up to 42 days following injury it was restricted to a narrow zone in original lesion tract (arrow in G). Dotted lines in B, E, F and G define the lesion border.

Scale bar: A, B, E, F, G = 80μm; C, D = 20μm


A
I
II
III
IV
V
Non-injured
B
III
IV
V
VI
1d post-injury
C
1d post-injury perilesion
D
1d post-injury SVZ
E
II
III
IV
V
7d post-injury
VI
F
I
II
III
IV
V
14d post-injury
G
I
II
III
IV
V
84d post-injury

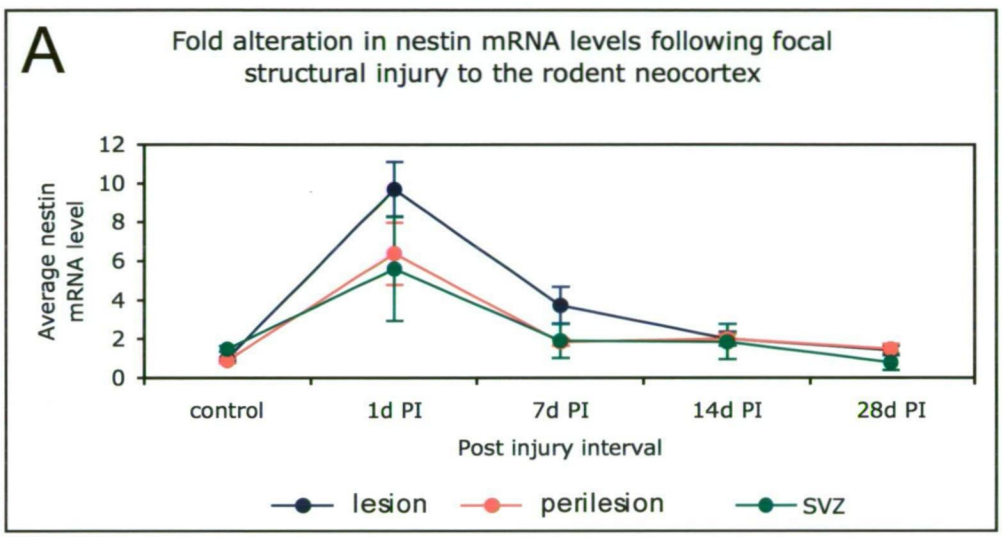
control brains (Figure 3.4A). Nestin mRNA levels were significantly ($p < 0.05$) and maximally increased in tissue from all three brain regions at 1 day following injury, with fold increases of 9.70 ± 1.40 (SE), 6.38 ± 1.61 (SE) and 5.61 ± 2.67 (SE) in the lesion site, perilesion tissue and SVZ tissue, respectively. Significant increases in nestin mRNA expression persisted until 14 days post-injury in both the lesion site and perilesion tissue. To determine whether alterations in nestin mRNA levels were reflected in equivalent changes at the protein level, we quantitated relative nestin immunofluorescence levels within the perilesion tissue, corresponding to regions from which tissue was harvested for quantitative real-time RT-PCR analysis. Quantitative immunofluorescence was performed on tissue sections from 1, 7, 14, 21, 42 and 84 days post-injury. Nestin labelling was negligible in control cortical tissue. Likewise, at 1 day post-injury, nestin immunoreactivity was insignificant, with infrequent nestin cells present within the cortical parenchyma. However, quantitation of nestin immunofluorescence demonstrated a significant elevation ($p < 0.05$) in nestin protein in perilesion tissue from 7 days post-injury, which persisted across all subsequent time points examined (Figure 3.4B). This response was most marked at 7 days post-injury, indicated by a 2.18 ± 0.14 (SE) fold increase in nestin within the perilesion tissue relative to non-injured controls.

As previously mentioned, immunoreactivity for nestin was most abundant at 7 days post-injury, at which time a band of nestin immunopositive cells extended from the SVZ to the lesion site (Figure 3.5A). Although this labelling pattern was prominent within perilesion grey matter (Figure 3.5B), nestin-labelled cells were also abundant within the corpus callosum and SVZ (Figure 3.5C). Moreover, nestin-labelled cells

Figure 3.4 Acute structural neocortical injury evoked statistically significant increases in nestin expression and immunofluorescence levels

Nestin mRNA levels were assessed in 3 brain regions, the lesion site, perilesion tissue and the SVZ at 1, 7, 14 and 28 days following injury. Nestin mRNA levels were significantly and maximally elevated at 1 day post injury in all 3 brain regions investigated and this increase persisted until 14 days post injury in both the injury site and perilesion tissue (A). Nestin immunofluorescence levels were quantitated in perilesion tissue. No alteration was observed in nestin immunofluorescence until 7 days post injury. Although nestin immunofluorescence peaked at 7 days following injury, levels remained significantly increased at all subsequent time points assessed up to 84 days post injury (B).

$p < 0.05$. Error bars are standard error of the mean


A
Fold alteration in nestin mRNA levels following focal structural injury to the rodent neocortex
Average nestin mRNA level
12
10
8
6
4
2
0
control
1d PI
7d PI
14d PI
28d PI
Post injury interval
lesion
perilesion
SVZ

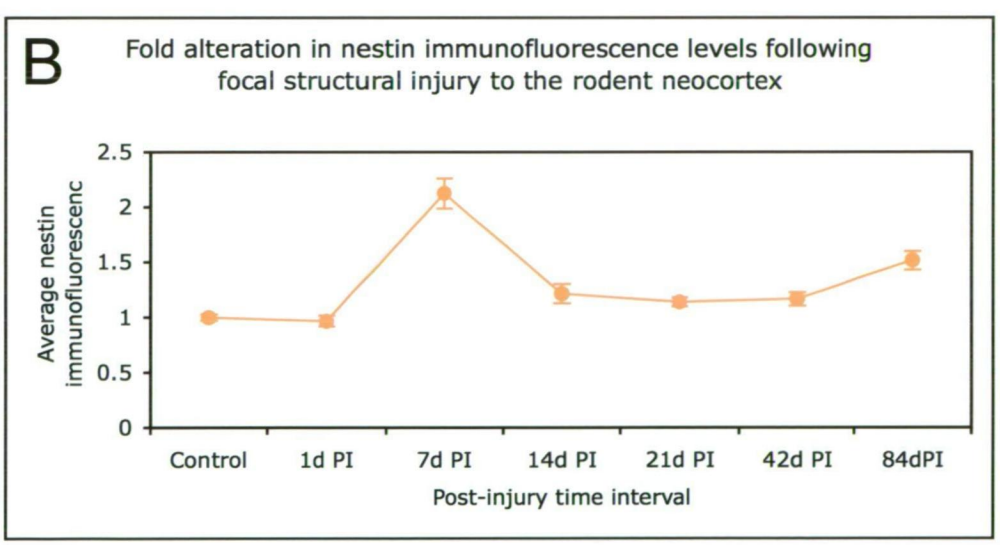
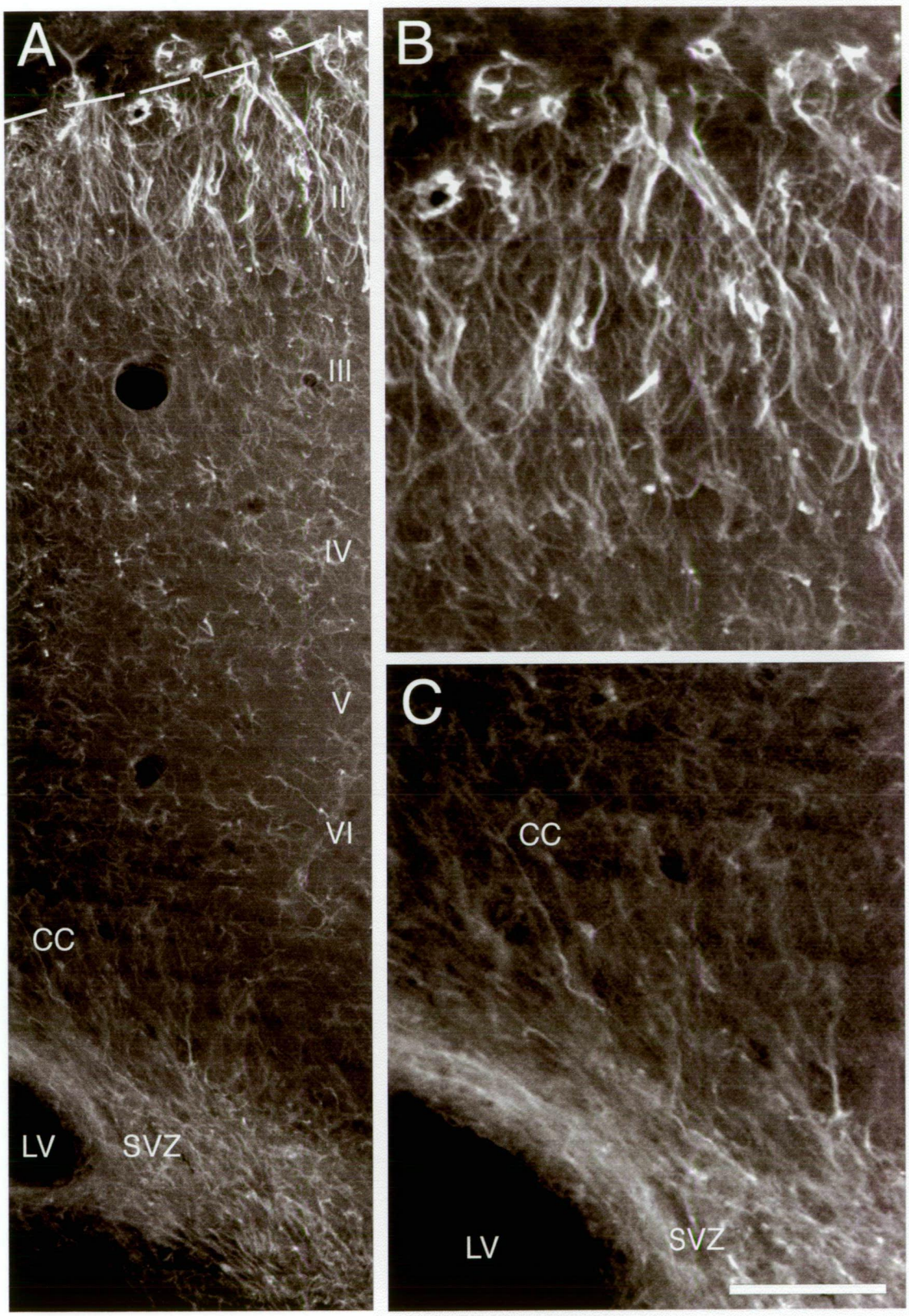

B
Fold alteration in nestin immunofluorescence levels following focal structural injury to the rodent neocortex
Average nestin immunofluorescenc
2.5
2
1.5
1
0.5
0
Control
1d PI
7d PI
14d PI
21d PI
42d PI
84dPI
Post-injury time interval

Figure 3.5 Nestin-labelled cells were distributed between the SVZ and injury site by 7 days following injury

Immunofluorescence labelling for nestin within the ipsilateral hemisphere at 7 days post-injury following transient localised injury of the rat somatosensory cortex. By 7 days post-injury nestin immunoreactive profiles were present in tissue extending from the SVZ and corpus callosum up to the injury site (A). Nestin-labelled cells were particularly abundant within perilesion tissue (B) and within the SVZ (C). Dotted line in A denotes the lesion border. CC, corpus callosum; SVZ, subventricular zone; LV, lateral ventricle.

Scale bar: A = 800μm B, C, = 200μm.


A
I
II
III
IV
V
VI
CC
LV
SVZ
B
C
CC
LV
SVZ

displayed variable morphological features, which correlated with distance from the lesion site and the brain region in which the cells were localised (Figure 3.6). For example, while nestin cells were elongated and displayed parallel arrangement, perpendicular in orientation to the lesion border, within the immediate perilesion tissue (Figure 3.6A), nestin cells in the cortical parenchyma demonstrated a dendritic morphology (Figure 3.6B). Furthermore, nestin cells were narrow and elongated within the sub-cortical white matter (Figure 3.6C), but displayed more spindle-like morphology with distinct rounded cell bodies within the SVZ (Figure 3.6D).

Sequential labelling for nestin and BrdU or PCNA demonstrated that a substantial proportion of the endogenous nestin cell population was mitotically active in the cortical parenchyma and SVZ (Figure 3.7A and B respectively). However, although BrdU/PCNA immunoreactive profiles were present in a broad zone between the lesion site and ipsilateral SVZ, concomitant with the presence of nestin cells within this region, numerous BrdU/PCNA nuclei were not localised to nestin positive cells.

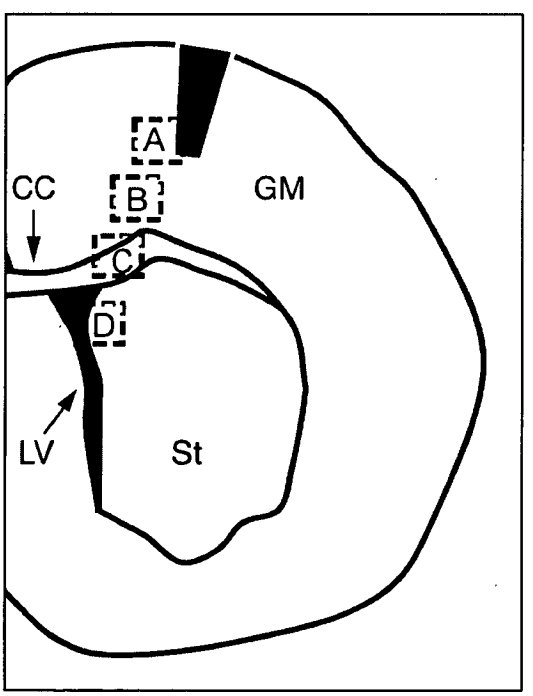
3.3.3 Cells expressing nestin following acute neocortical injury

Previous reports have indicated that nestin may be re-expressed in different populations of mature neural cells types in a variety of experimental brain lesion paradigms (see discussion). Double labelling immunofluorescence studies were performed to determine whether this was a possibility in our brain lesion model. Tissue sections were double labelled for nestin relative to a selection of markers (Figure 3.8) for microglia/macrophages (ferritin), astrocytes (GFAP) and neurons (NF-M, α-internexin, calretinin and parvalbumin). Neuronal markers were selected

Figure 3.6 Acute structural neocortical injury induced alterations in the morphology of nestin-labelled cells, which were associated with the brain region to which these cells were localised

The diagrammatic representation of the ipsilateral injured brain displayed below indicates the brain region from which each image was taken. Nestin cells were elongated and arranged parallel to one another within perilesion tissue (A), but demonstrated stellate morphology at within the cortical parenchyma at site more distant to the lesion (B). In the corpus callosum nestin cells were elongated (C), whereas they demonstrated rounded somas, emanating spiky processes within periventricular tissue (D). Arrows denote examples of nestin-labelled cells in each image. LV, lateral ventricle; St, striatum; CC, corpus callosum; GM, grey matter

Scale bar: A, B, C and D = 100μm



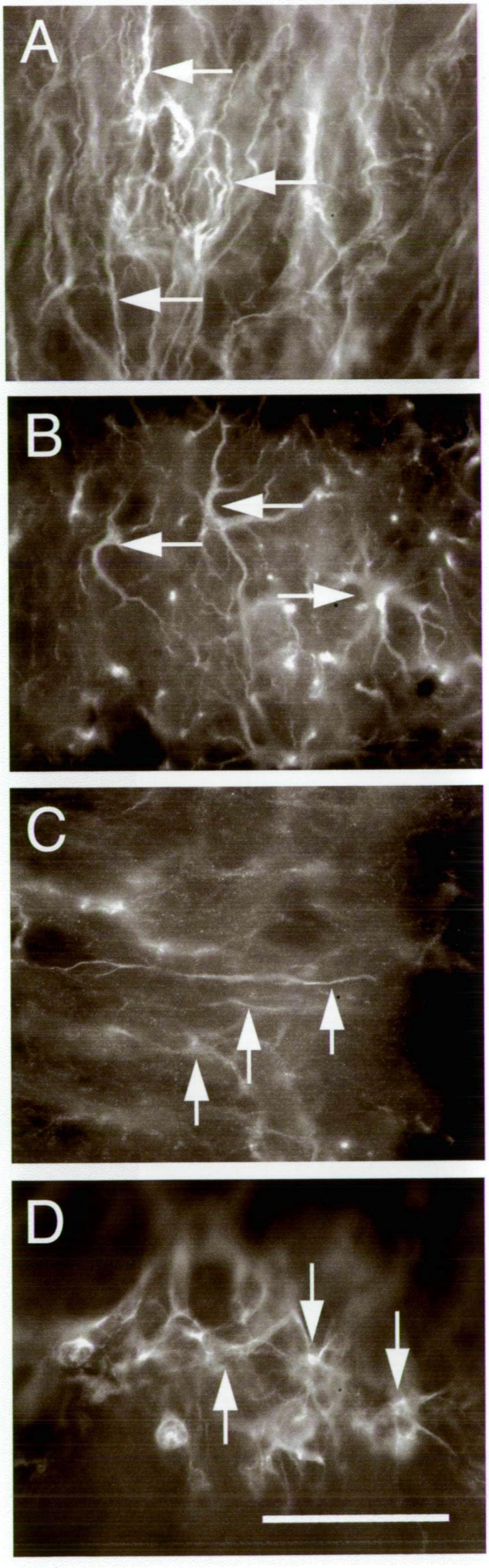
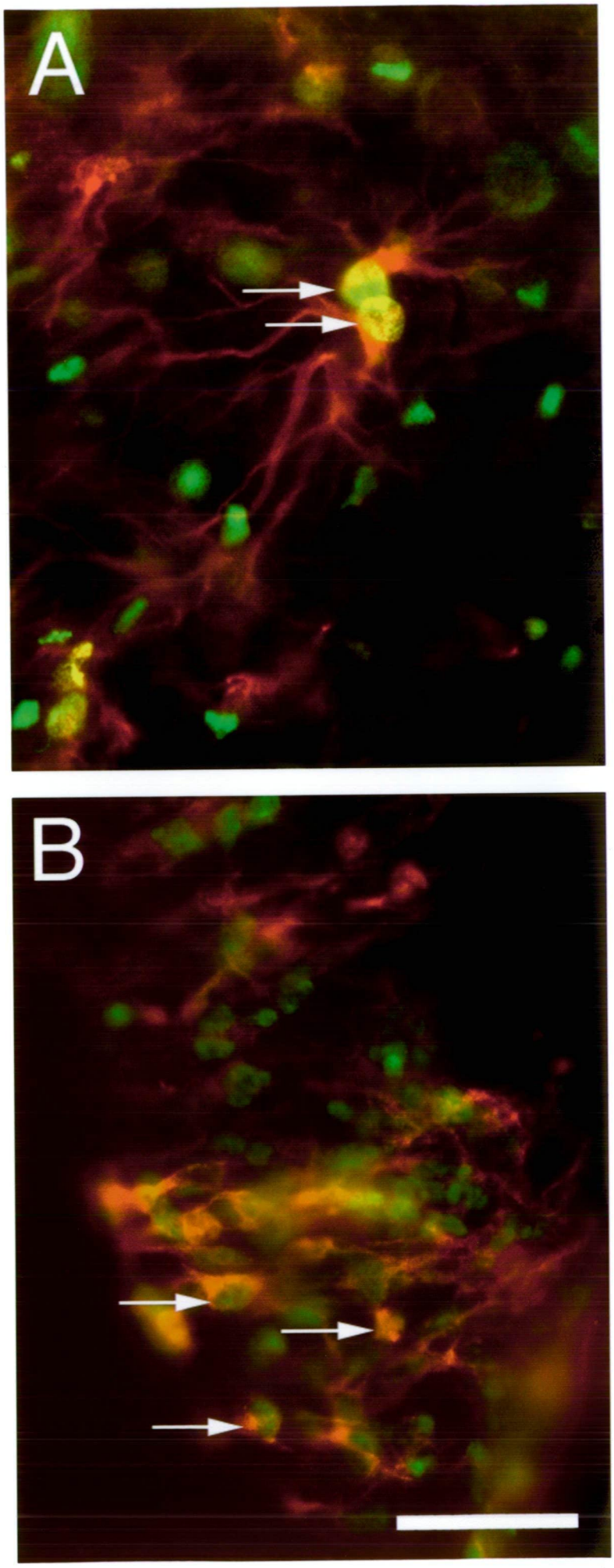

A
B
C
D

Figure 3.7 A proportion of the nestin-labelled cell population was mitotically active, as indicated by co-labelling for BrdU or PCNA, in the perilesion tissue and SVZ

Double immunofluorescence labelling for BrdU relative to nestin demonstrated proliferating nestin cells in cortical parenchyma (A). Likewise, nestin cells were also mitotically active in the SVZ as demonstrated by PCNA-nestin double labelling (B). Arrows denote examples of mitotically active nestin-labelled cells.

Scale bar: A = 50μm; B = 100μm


A
B

to represent cortical interneuron (calretinin and parvalbumin) and pyramidal neuron (NF-M and α-internexin) populations.

Examination of injured tissue from all post-injury time points investigated showed that nestin labelling exclusively co-localised with the astrocytic intermediate filament protein, GFAP (Figure 3.8A). Cells labelled solely for nestin or GFAP, as well as cells demonstrating nestin-GFAP co-localisation, shared similar morphological features within perilesion tissue, characterised by an activated elongated morphology and parallel cell arrangement, perpendicular to the lesion border. Contrary to the widespread co-localisation between nestin and GFAP, nestin was not localised within ferritin-positive activated microglia/macrophages (Figure 3.8B). Furthermore, nestin immunoreactivity was absent from all neuronal populations examined within perilesion tissue, including those positive for NF-M, α-internexin, calretinin and parvalbumin (Figure 3.8C, D, E and F respectively).

3.3.4 Distribution of DCX-labelled neuroblasts following acute neocortical injury

To determine whether new neurons were generated in the SVZ and perilesion tissue, tissue sections were double-labelled for DCX relative to BrdU, nestin and neuron-specific βIII-tubulin (Figure 3.9). DCX immunoreactive cells were present in the SVZ, and occasionally in the subcortical white matter of both non-injured control brains (Figure 3.9A) and injured brains (Figure 3.9B). DCX-labelled cells in both non-injured and injured brains were mitotically active, as indicated by BrdU incorporation (Figure 3.10A), however, these cells rarely, if ever, co-labelled with

Figure 3.8 Nestin was localised to astrocytes, but no microglial/macrophage or neuronal populations

Double immunofluorescence labelling for nestin (green) relative to astrocyte, microglial/macrophage and neuronal markers (red) in perilesion tissue at 7 days following cortical injury demonstrated nestin labelling exclusively co-localised with GFAP (A) and was not present in ferritin positive microglia/macrophages (B), or NF-M- (C), α-internexin- (D), calretinin- (E) or parvalbumin- (F) immunoreactive neurons. Arrows in A denote examples of co-labelled cells, arrows in B-F denote examples of microglia/neurons, arrowheads in B-F denote examples of nestin-labelled cells.

Scale bar: 50μm

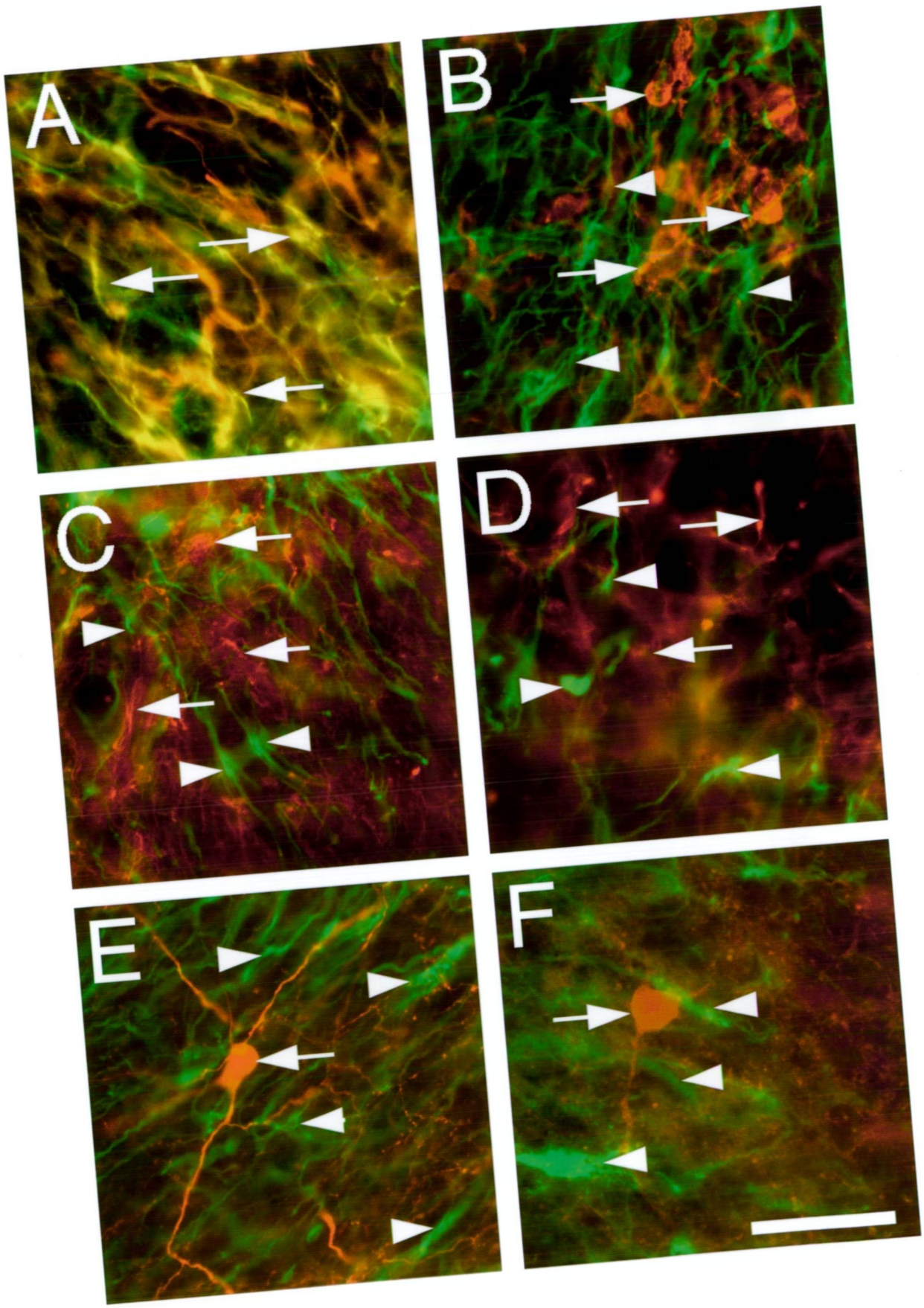
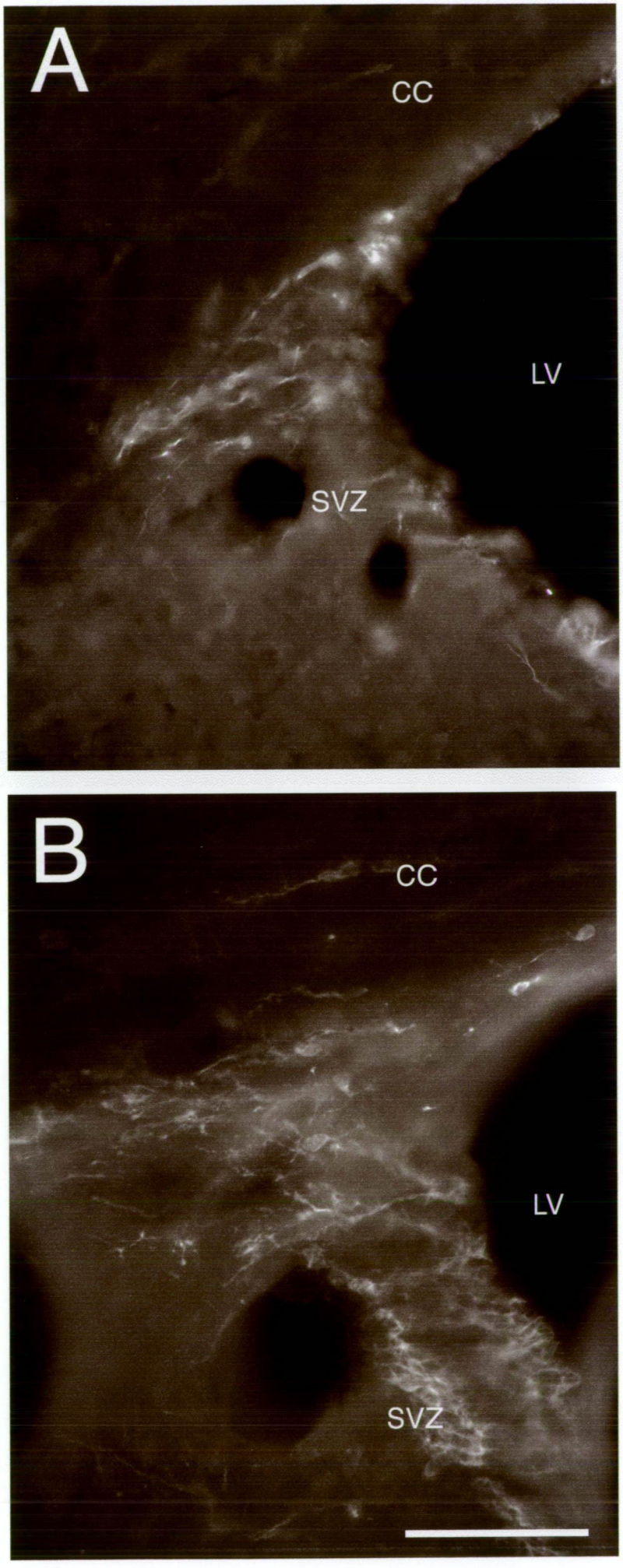

A
B
C
D
E
F

Figure 3.9 Doublecortin-labelled cells were present in the SVZ of both non-injured and injured brains, but were absent from both the injury sited of injured brains and equivalent region of non-injured brains

Doublecortin labelling of the SVZ of a non-injured control (A) and injured brain, 7 days following injury (B). SVZ, subventricular zone; LV, lateral ventricle; CC, corpus callosum.

Scale bar A and B = 100μm


A
CC
LV
SVZ
B
CC
LV
SVZ

nestin (Figure 3.10B), indicating that they were likely to have surpassed the neural progenitor phase of development. Furthermore, a proportion of DCX-labelled cells residing in the SVZ co-expressed the early neuronal marker βIII-tubulin (3.10C). Observations of DCX labelling were restricted exclusively to the SVZ and DCX immunoreactive cells were not observed within perilesion tissue, suggesting that this cell population was not recruited in response to injury. These finding were confirmed up to 21 days following injury.

3.3.5 Fate of injury-induced proliferating cells

As previously demonstrated, cortical injury evoked characteristic alterations in cell proliferation, including the generation of nestin-labelled cells. Differentiation of newly generated cells into neurons following brain injury has been implicated in brain healing. To determine the fate of cells that had incorporated BrdU within the first week following injury and whether these cells went on to develop neuronal or glial phenotypes that contributed towards brain healing, brain sections were derived from animals perfused at 7, 21 and 42 days following lesion and double-immunofluorescence labelled for BrdU relative to the same battery of antibodies utilised for nestin double labelling studies, including GFAP, ferritin, NF-M, α-internexin, calretinin and parvalbumin (Figure 3.11).

Double-labelling immunohistochemistry demonstrated numerous GFAP immunopositive cells were mitotically active (Figure 3.11A). Likewise ferritin positive microglia and macrophages also underwent substantial proliferation in response to cortical injury (Figure 3.11B). Strikingly, however, over the course of

Figure 3.10 Doublecortin-labelled cells located in the SVZ of non-injured and injured brains were mitotically active and expressed the neuronal marker, β-III tubulin, but not the early neural marker, nestin

Double labelling analysis demonstrated that, in both non-injured control and injured brains, doublecortin cells were mitotically active (A), but rarely co-labelled with nestin (B). Doublecortin cells in the SVZ frequently co-expressed βIII-tubulin. Images were taken at 7 days post-injury, but were representative of non-injured control brains. Arrows denote examples of co-labelling, arrowheads denote the localisation of nestin positive cells.

Scale bar A, B and C = 50μm

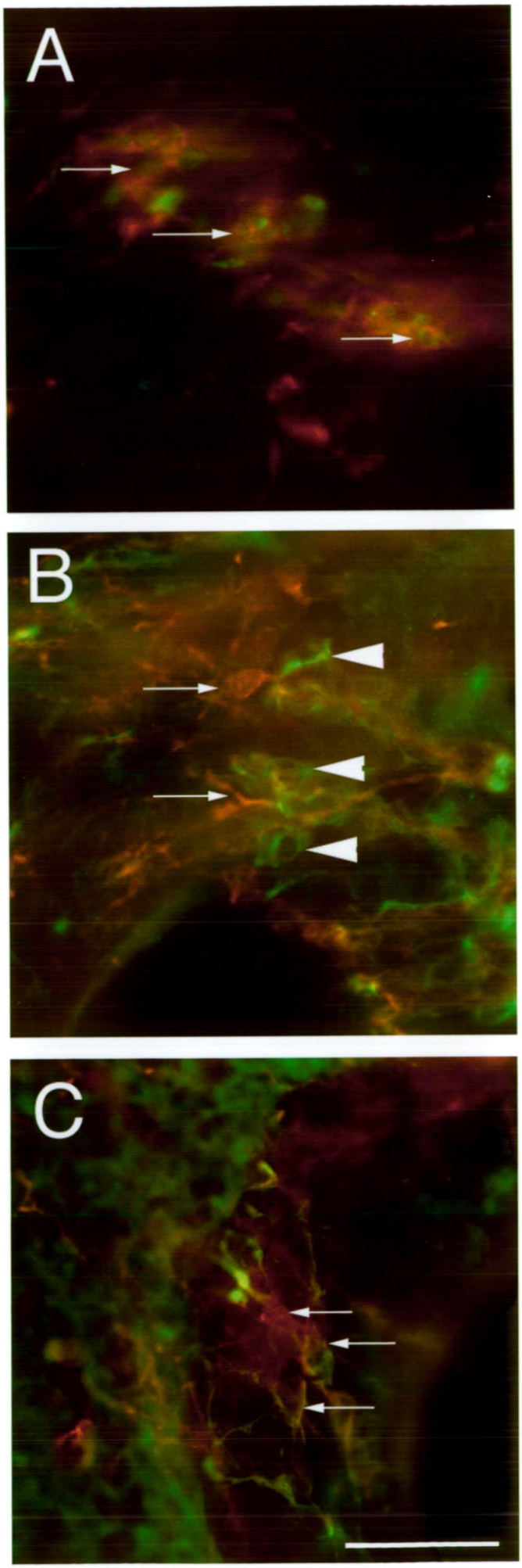
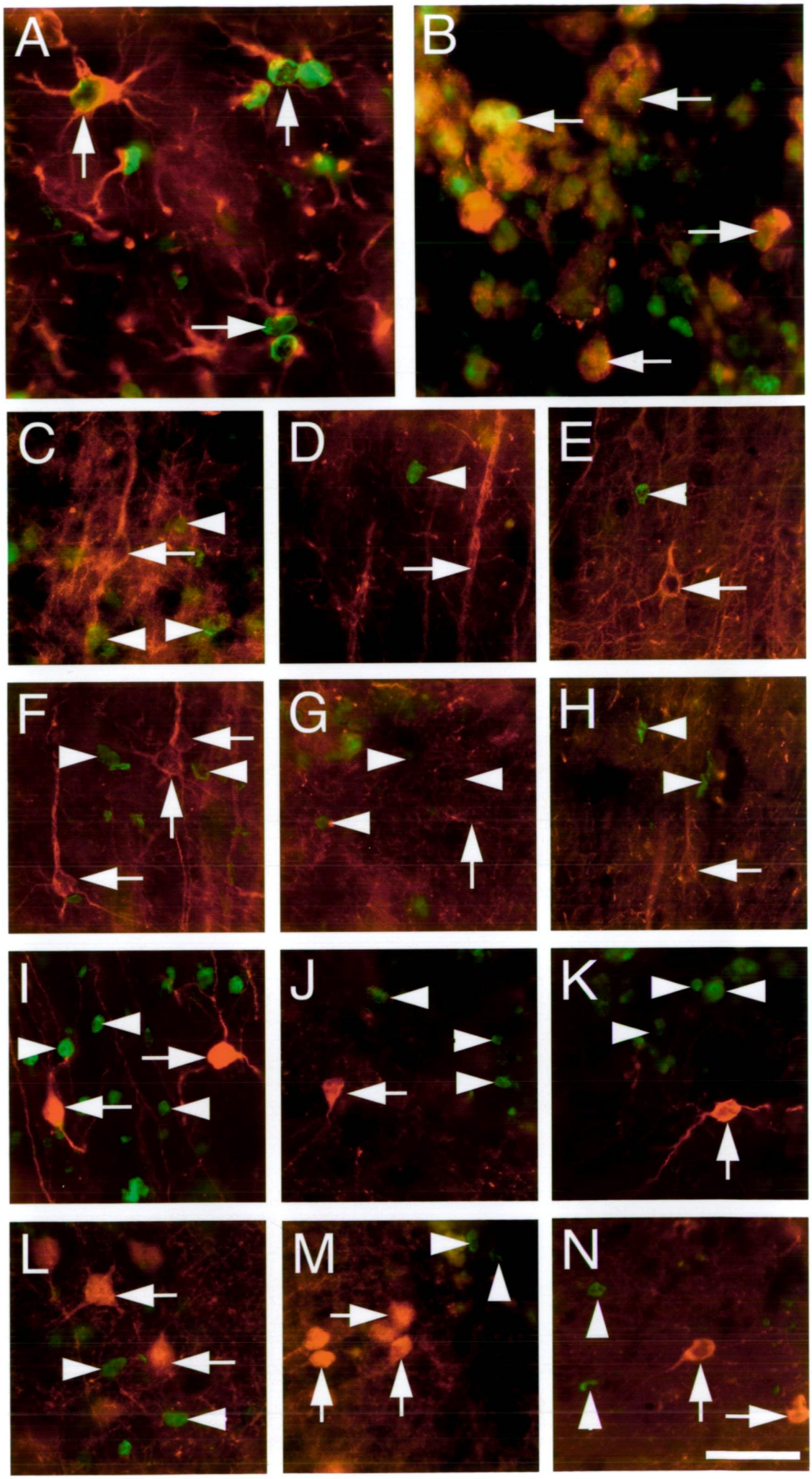

A
B
C

Figure 3.11 Injury-induced proliferating cells were identified to be astrocytes and microglia/macrophages, but not neurons

Double immunofluorescence labelling for BrdU (green) relative to astrocytic, microglial/macrophage and neuronal markers (red) in cortical perilesion tissue at 7 days following transient localised injury to the rat cortex. Numerous GFAP labelled cells also contained BrdU immunoreactive nuclei (A), Additionally, a proportion of ferritin positive microglia/macrophages were also mitotically active following injury (B). However, injury-induced proliferating cells had not differentiated into mature neuronal phenotypes by 1 (left panels), 3 (middle panels) or 6 (right panels) weeks following injury. Labelling is for NF-M- (C-E), α-internexin- (F-H), calretinin- (I-K) and parvalbumin- (L-N) immunopositive neuronal populations.

Scale bar: A and B = 35μm; C-N = 50μm.


A
B
C
D
E
F
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I
J
K
L
M
N

42 days following injury, BrdU immunoreactive nuclei were absent from all neuronal populations examined, including NF-M- (Figure 3.11C-E) and α-internexin- (Figure 3.11F-H) immunoreactive pyramidal cells, as well as calretinin- (Figure 3.11I-K) and parvalbumin- (Figure 3.11L-N) immunoreactive interneurons.

3.4 Discussion

This chapter investigated the spatio-temporal alterations occurring in proliferating and nestin-immunoreactive neural progenitor cells in response to a wound-like structural lesion to the rat somatosensory cortex. Compared to other models of structural brain injury, including weight-drop, controlled cortical impact and lateral fluid percussion (Dixon and Hayes, 1996; Hicks et al., 1997; Hallam et al., 2004; Thompson et al., 2005), the injury model utilised in this study is relatively subtle, in terms of the extent of injury and specifically creates a lesion in the neocortical grey matter. Thus, this lesion paradigm is important for examining degenerative and regenerative alterations within the grey matter of the neocortex as well as alterations occurring at sites not directly affected by the lesion, which may be implicated in brain healing. In this respect, this study demonstrated the recruitment of SVZ-derived cells in contributing towards brain healing, despite the SVZ not being directly damaged by the lesion.

Overall, acute focal neocortical injury induced early reactive alterations, including profuse cell proliferation, as indicated by extensive BrdU incorporation and PCNA labelling, as well as microglial/macrophage and astrocyte activation. Proliferating cells were identified to be nestin-labelled neural progenitors, DCX-immunoreactive neuroblasts, activated microglia/macrophages and astrocytes, but not neurons. Nestin-labelled cells were observed within the SVZ and corpus callosum at 1 day post-injury and by 7 days following injury nestin immunoreactivity extended up to the to injury site. Additionally, double labelling studies indicated co-localisation between nestin and the astrocytic marker, GFAP, however nestin was not re-

expressed in other neural cell types. Moreover, although DCX cells were abundant in the SVZ, these cells did not contribute to re-population of the injured tissue. Collectively, these results demonstrate a sequence of changes directed at brain healing, involving the recruitment of proliferating and progenitor cells. However, this response ultimately culminated in astrogliosis at the wound edge, with an absence of neuronal replacement within the injured tissue.

3.4.1 Acute focal brain lesion induces substantial cell proliferation and alterations in endogenous neural progenitor cells

Similar to previous reports investigating the cellular response to more disruptive forms of brain injury, results from this chapter demonstrated that cell proliferation was a prominent feature of the post-injury response. In this regard, cell proliferation has been demonstrated to occur in a variety of brain lesion paradigms (for example Dash et al., 2001; Jin et al., 2001; Kernie et al., 2001; Chirumamilla et al., 2002; Parent et al., 2002a, b; Gotts and Chesselet, 2005; Ramaswamy et al., 2005; Tatsumi et al., 2005). Importantly, injury-induced proliferating cells may include populations of endogenous neural progenitor cells. In this chapter, neural progenitor cells were assessed based on immunoreactivity and expression of nestin.

Both this and other investigations have reported that the basal population of nestin-labelled cells is relatively small within normal non-compromised mature brain tissue, particularly in regions such as the neocortex (Douen et al., 2004), and is generally restricted to small populations of endothelial and SVZ cells (Sahin Kaya et al., 1999). However, various models of brain insult, including physical/mechanical

trauma (Holmin et al., 1997; Sahin Kaya et al., 1999; Kernie et al., 2001; Chirumamilla et al., 2002; Chen et al., 2003a; Douen et al., 2004), ischemia/stroke (Duggal et al., 1997; Li and Chopp, 1999; Arvidsson et al., 2002; Tonchev et al., 2003), intracerebral haemorrhage (Nakamura et al., 2003), chemical lesions/seizure (Clarke et al., 1994) and ionising radiation (Shi et al., 2002), have demonstrated prominent spatio-temporal alterations in the neural progenitor cell population, including proliferation and lesion-directed migration as well as changes in nestin expression, localisation and distribution.

Results from this chapter demonstrated that nestin-labelled cells, which had proliferated within the first post-injury week, were localised to the SVZ and perilesion cortical parenchyma. Additionally, nestin immunoreactivity was increased within the SVZ and corpus callosum at 1-day post injury, which extended to the lesion site by 7 days following injury and thereafter gradually declined, to be retained as a remnant within the original lesion tract. This spatio-temporal pattern of nestin re-distribution was suggestive of the mobilisation of nestin-labelled cells towards the lesion site. Moreover, utilising quantitative real time RT-PCR, alterations in nestin immunoreactivity were demonstrated to reflect changes in nestin expression, which was significantly elevated within the SVZ, lesion and perilesion tissue at 1 day following post-injury and returned to control levels by 28 days post-lesion. Collectively, these results support those of previous studies implicating progenitor cell proliferation and migration in the neural response to injury. The onset of alterations in nestin expression/induction has been reported to vary from a few days to several weeks post-injury (for example Duggal et al., 1997; Holmin et

al., 1997; Li and Chopp, 1999; Sahin Kaya et al., 1999; Chen et al., 2003a; Douen et al., 2004).

3.4.2 Nestin induction and cell proliferation are important makers of neural injury and are implicated in brain healing

Importantly, injury-induced nestin cell production is not the only source of nestin expression following injury and previous reports have indicated nestin re-induction following brain injury may arise from two potential sources; the proliferation of resident nestin progenitors and the embryonic reversion of the mature cytoskeleton of reactive astrocytes, oligodendrocytes, microglia, monocytes/macrophages and neurons (Clarke et al., 1994; Lin et al., 1995; Duggal et al., 1997; Holmin et al., 1997; Brewer, 1999; Brook et al., 1999; Li and Chopp, 1999; Sahin Kaya et al., 1999; Kernie et al., 2001; Kuroda et al., 2002; Nakamura et al., 2003). To determine the localisation of nestin to various neural cell types, double-labelling immunofluorescence studies were performed. Nestin was not localised within microglial/macrophage or neuronal populations. However, nestin and GFAP were co-localised in glial-like cells surrounding the lesion site.

Observations of nestin cell proliferation, distinct spatio-temporal alterations in nestin expression and re-distribution between the SVZ and injury site, as well as astrocytic proliferation and co-localisation of nestin and GFAP, support roles for both nestin re-expression in reactive astrocytes and the elaboration of astrocytes from resident progenitor and proliferating cells as mechanisms of brain healing following injury. Indeed, these mechanisms have been implicated in formation of the glial scar, brain

remodelling and healing (Sahin Kaya et al., 1999; Kernie et al., 2001; Chen et al., 2003a, b; Douen et al., 2004; Moon et al., 2004; Salman et al., 2004, Tatsumi et al., 2005).

3.4.3 Neurogenesis is a potential mechanism of brain healing following injury

Results from this study suggest that injury-induced proliferating and progenitor cells predominantly adopt a glial (astrocytic) phenotype. However, profound alterations elicited in endogenous neural progenitor cells following brain lesion are particularly remarkable as these cells have the capacity to generate astrocytes, oligodendrocytes and, importantly, neurons (Von Visger et al., 1994; Taupin and Gage, 2002). Indeed, neural progenitor cells residing in germinal regions of the adult brain have been shown to generate neurons throughout life (Johansson et al., 1999) in a range of mammalian species including rodents (Kuhn et al., 1996; Cameron and McKay, 2001), primates (Gould et al., 1999; Bernier et al., 2002) and humans (Eriksson et al., 1998; Bedard and Parent, 2004). Endogenous progenitors may differentiate into neurons from pools of cells within the SVZ and the cortical parenchyma (Dayer et al., 2005; Emsley et al., 2005).

Importantly, recruitment, differentiation and integration of various stem, precursor, progenitor, proliferating and/or migrating cells, into existing neural circuits has provoked speculation that these cells may be responsible for contributing to neuronal replacement and functional recovery following various forms of experimental brain insult (Gu, 2000; Magavi et al., 2000; Kernie et al., 2001; Braun et al., 2002; Rice et al., 2003; Chen et al., 2003b). In this regard, several studies have demonstrated

evidence of neuronal production in response to experimental brain injury (Kernie et al., 2001; Chen et al., 2003b; Rice et al., 2003; Tonchev et al., 2003), providing support for neurogenesis as a mechanism of brain repair following TBI (Kernie et al., 2001; Rice et al., 2003). However, results from this chapter and other investigations (Holmin et al., 1997; Kuroda et al., 2002; Yoshiya et al., 2003; Douen et al., 2004; Salman et al., 2004) have not reported neurogenesis as a mechanism of cell replacement following brain lesion.

3.4.4 Why does the potential for neurogenesis differ with injury paradigm?

The absence of neurogenesis in the present investigation may be due to the location and severity of the injury. Many models of cortical lesion generate widespread neural damage (Dixon and Hayes, 1996; Hicks et al., 1997; Hallam et al., 2004; Thompson et al., 2005), which often directly affects regions associated with high plasticity, such as the SVZ or hippocampus, or at least damages cortex more extensively. The injury model utilised in this chapter, however, specifically targets the grey matter of the neocortex and may not evoke the same signalling mechanisms initiated in more disruptive forms of trauma. The absence of constitutive cortical neurogenesis has been postulated to result from a lack of appropriate micro-environmental cues, rather than a limit of the endogenous precursors themselves (Emsley et al., 2005), and this deficiency may also account for limited or absent neurogenesis following cortical injury in the present study.

This proposition is supported by findings regarding DCX labelling of migrating neuroblasts. Normally, neuroblasts are produced in the SVZ, migrate in the rostral

migratory stream to the olfactory bulb and differentiate into olfactory granule interneurons (Lois and Alvarez-Buylla, 1994; Lois et al., 1996; Francis et al., 1999; Gleeson et al., 1999; Bedard and Parent, 2004; Yang et al., 2004). It has been suggested that these cells may be re-directed to sites of injury, where they contribute to neuronal and/or glial replacement (Jin et al., 2003; Goings et al., 2004; Romanko et al., 2004; Gotts and Chesselet, 2005; Ramaswamy et al., 2005). However, results from this chapter indicate that DCX labelling was absent from perilesion tissue and therefore not involved in neuronal replacement in this injury model. This was potentially due to a lack of appropriate 'pro-neurogenesis' cues. Conversely, using a non-physical brain lesion model that specifically induces apoptotic death in the neocortical grey matter Magavi et al. (2000) have demonstrated the long-term neuronal replacement capability of lesion-induced proliferating and progenitor cells.

Thus, although diverse brain lesion paradigms appear capable of inducing the generation of new cells, the ultimate neurogenic potential of these cells is likely to be dictated by the combined influences of the lesion paradigm, extent of damaged created by the lesion and the brain regions directly and indirectly affected the lesion (Lie et al., 2004). Importantly, reports of injury-induced cells exhibiting an early, robust lesion-directed migratory response highlight a window of opportunity in which neuronal fate may be encouraged prior to establishment of scar tissue. Although results from this chapter did not report the occurrence of neurogenesis in response to cortical injury, they do support the view that the damaged brain instigates an adaptive reparative response and emphasise the role of neural progenitor and proliferating cells in contributing to this response.

3.5 Conclusion

In summary, this study has demonstrated that cell proliferation and neural progenitor cell induction are major reparative mechanisms in the healing response following acute focal brain injury. Importantly, this study provides evidence that a lesion specific to the grey matter of the neocortex is capable of evoking changes within associated parenchymal regions as well as at sites distant to the lesion, that were spared from direct injury. In this regard, the results from this chapter suggest that brain injury induces a sequence of alterations, involving cell generation and activation, which contribute to a wound healing-like response, similar to that observed in tissues such as the skin. The differentiation of proliferating and progenitor cells, as well as nestin re-expression in reactive astrocytes are important mechanisms underlying this healing response and the ultimate formation of a glial scar at the wound border.

Additionally, previous studies have also implicated neurogenesis in this healing process. Collectively, reports of injury induced neuro- and glio-genesis and altered patterns of cell migration indicate intrinsic repair mechanisms are initiated in the damaged brain directed at self-repair (Pluchino et al., 2005). These studies emphasise the generally unappreciated level of plasticity and highly adaptive nature of the adult mammalian brain. Finally, exogenous manipulation of injury-induced proliferating and endogenous neural progenitor cells provides a viable therapeutic target for enhancing the brain's response to injury and disease (for recent reviews see Calzà et al., 2004; Mitchell et al., 2004; Picard-Riera et al., 2004; Schouten et al., 2004; Pluchino et al., 2005).

However, whether brain injury evokes an endogenous neurogenic response that contributes to functional recovery remains contentious. Indeed, along with increased acceptance of adult neurogenesis, scepticism has arisen regarding the existence of neurogenesis in non-neurogenic regions, as well as the techniques used to confirm such assertions (Kornack and Rakic, 2001, Koketsu et al., 2003). This study has highlighted important aspects of the endogenous neural response to injury and provided important avenues for future research into brain regeneration. Full investigation of these mechanisms will provide important clues as to how endogenous brain repair mechanisms can be facilitated.

Acute focal brain injury induces a stereotypical sequence of reactive and regenerative changes in both neuronal and glial cell populations directed at brain healing

4.1 Introduction

Significant pessimism has traditionally surrounded the capacity of the adult mammalian brain to recover both structurally and, more importantly, functionally following TBI. Trauma to the brain causes tissue deformation at the time of injury, which may be associated with axonal shearing, contusions, haemorrhages and oedema (Finnie and Blumbergs, 2002). Collectively, these perturbations culminate in substantial tissue damage, widespread neural disruption and degeneration, including both necrotic and apoptotic neuronal death (Newcomb et al., 1999; Liou et al., 2003). In addition to degenerative cascades, brain injury also initiates complex reactive, cellular, inflammatory, neuro-chemical and metabolic alterations (Maxwell et al., 1997; Finnie and Blumbergs, 2002), which affect astrocyte, microglial, oligodendrocyte and neuronal cell populations, as well as brain vasculature (Chen et al., 2003a).

Despite the limited recovery generally observed following head trauma, several lines of research have demonstrated striking examples of brain plasticity (for example Darian-Smith and Gilbert, 1995; Carlen et al., 2002; Englund et al., 2002; Knott et al., 2002). Moreover, this phenomenon has been supported by observations of spontaneous, though frequently partial, recovery of function following stroke, spinal cord injury and human acquired brain injury (Goldberger and Murray, 1988; Wilson,

1998; Medana and Esiri, 2003; Constantinidou et al., 2005; Ding et al., 2005). Indeed, several studies in animal models of brain trauma suggest that neurons from the adult CNS respond to injury with a complex sequence of morphological, biochemical and gene expression alterations, directed towards sprouting, regeneration, synaptogenesis and even neurogenesis (Christman et al., 1997; Deller and Frotscher, 1997; Pastor et al., 2000; Vickers et al., 2000; King et al., 2001; Lie et al., 2004). Furthermore, the neuronal response to injury involves potentially adaptive alterations, including the re-expression of developmentally important proteins and substantial cytoskeletal reorganisation (Christman et al., 1997; Dickson et al., 2000; King et al., 2001).

Additionally, astrocyte, oligodendrocyte, microglial and macrophage populations have all been shown to undergo substantial reactive alterations following various forms of brain insult (Maxwell et al., 1990; Fujita et al., 1998; King et al., 2001; Finnie and Blumbergs, 2002; Tonchev et al., 2003; Chen et al., 2003a; Lee et al., 2003; Davalos et al., 2005). However, whether these non-neuronal cell populations have a primarily facilitative or detrimental contribution to neuronal regeneration remains to be elucidated (Ridet et al., 1997; Finnie and Blumbergs, 2002; Batchelor et al., 2002). Furthermore, it has been suggested that the ultimate effect of injury-induced glial activation may depend on the combined influences of injury severity, neural disruption, micro-environmental cues and the brain region affected (Ridet et al., 1997). Nevertheless, these studies indicate that CNS injury initiates a sequence of adaptive alterations, involving all major neural cell populations, directed at tissue healing and scar formation (Fawcett and Asher, 1999). In this regard, this response

reflects the stereotypical phases of healing, such as coagulation, inflammation, proliferation, migration and remodelling, observed in other tissues (Hunt et al., 2000). However, the culmination of these changes may ultimately impede neuronal regeneration (Fawcett, 1997, Fawcett and Asher, 1999).

The previous chapter demonstrated the rapid induction of cell proliferation and neural progenitor cell activation following brain injury and discussed the potential role of these cell populations in brain healing. However, brain injury typically affects several neural populations, particularly those at the immediate site of damage. Therefore, this study investigated the alterations occurring in key neuronal and glial cell populations in response to injury and sought to determine which of these alterations may be indicative of endogenous brain repair. Immunohistochemical techniques and quantitative gene expression analysis were utilised to characterise the spatio-temporal co-ordinated neuro-glial response of various cell populations in response to focal brain injury. Specifically, alterations in populations of astrocytes, microglia, macrophages, oligodendrocytes, perivascular cells and, importantly, neurons were examined over a time course of 84 days following localised injury of the rat somatosensory cortex. Real-time RT-PCR was utilised to determine whether the cellular neuronal response to injury was reflected by changes at the gene expression level. Results from this study are discussed in relation to an accumulating body of evidence indicating that neuro-glial interactions are fundamental to brain healing following trauma.

4.2 Experimental procedures

4.2.1 Immunohistochemistry

Following transient neocortical injury (as described in Section 2.1.2, Figure 2.1), animals were terminally anaesthetised at 1, 7, 14, 21, 42 or 84 days post-injury (n = 3 per time point). Non-injured, age-matched controls corresponding to the 1, 42 and 84 days post-injury time points (n=3 per time point) were also perfused. Tissue was processed and sectioned for immunohistochemistry as described in Section 2.1.4. Tissue sections were labelled with a battery of antibodies directed at various cell populations or neuronal compartments as indicated in Table 4.1 (specific antibody details are provided in Table 2.1). Neuronal populations and cellular compartments were identified based on immunoreactivity for NF-M, parvalbumin, calretinin, SMI312, GAP-43 and MAP-2. Astrocytes were identified based on GFAP labelling, microglia/macrophages on ferritin labelling and oligodendrocytes were labelled with myelin/oligodendrocyte specific protein. Cell proliferation was assessed based on BrdU incorporation as described in Section 2.1.3. Specific antibody details are provided in Table 2.1.

Table 4.1. Antibodies/cell stains utilised to identify different neural cell populations

Cell type/compartment	Marker
Pyramidal neurons	NF-M
Interneurons	Calretinin
	Parvalbumin
Axons	SMI312
	GAP-43
Dendrites	MAP-2
Astrocytes	GFAP
Microglia/macrophages	Ferritin
Oligodendrocytes	Oligodendrocyte specific protein
Proliferating cells	BrdU
Microglia and perivascular cells	Isolectin IB4

4.2.2 Total RNA extraction and quantitative one-step real-time RT-PCR

Following transient neocortical injury animals were terminally anaesthetised at 1, 7, 14 and 28 days post-injury (n = 4 per time point). Non-injured, age-matched controls corresponding to the 1 and 28 day post-injury time points (n=3 per time point) were also perfused. Tissue was dissected from the perilesion region immediately lateral and medial to the lesion site, as displayed in Figure 4.1, and these two tissue blocks were pooled. Quantitative one-step real-time RT-PCR was performed as described in Section 2.1.6, to determine alterations in NF-L, NF-H, βIII-tubulin and GAP-43 mRNA following localised neocortical injury, using the primer pairs displayed in Table 2.2. Statistical analysis indicated that the two groups of control animals were not statistically different and the data for these animals were therefore pooled to form one control group (n = 6).

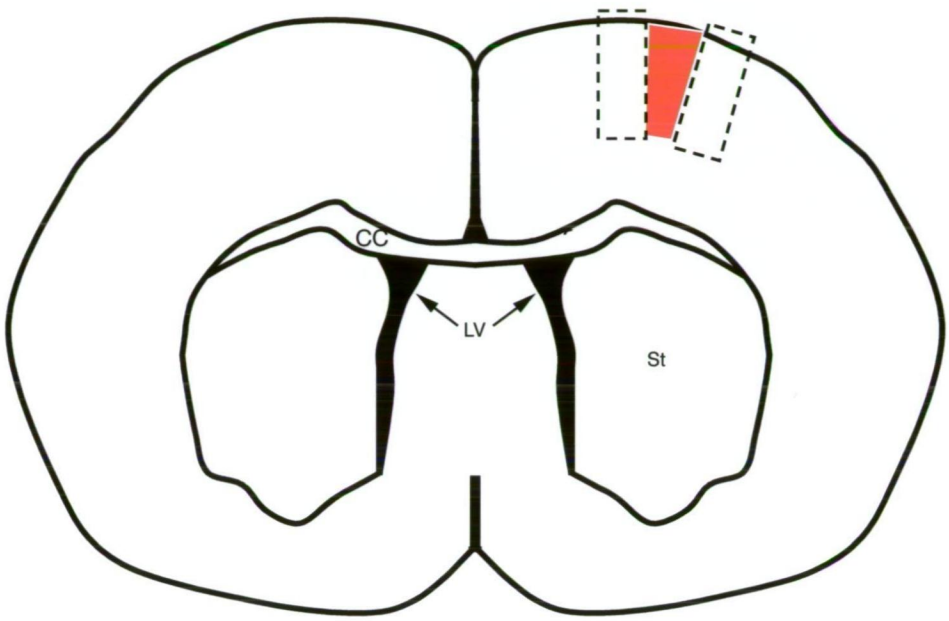


Figure 4.1 Diagrammatic representation of sites from which tissue was harvested for RNA extraction to be assessed by quantitative real time RT-PCR analysis

Tissue was harvested from regions region immediately medial and lateral to the injury site (dotted boxes). These 2 tissue samples were pooled prior to RNA extraction. CC, corpus callosum; SVZ, subventricular zone; St, striatum.

4.3 Results

4.3.1 General lesion morphology and histopathology

Macroscopically, the lesion was initially characterised by tissue cavitation and destruction, vascular damage and bleeding. Over the time course examined, these alterations resolved and were associated with skull and meningeal repair as well as reduced lesion size. Overall, microscopic analysis demonstrated no detectable histopathological changes in control brains or in brain regions contralateral to the lesion site. However, focal neocortical injury resulted in substantial ipsilateral tissue destruction at the site of the lesion as well as in the surrounding perilesion zone, including a disruption of glial and neuronal cell populations as well as blood vessels. Occasionally a tissue 'plug' was retained within the lesion cavity, consisting of degenerating cells and other cell populations that had invaded the injury site.

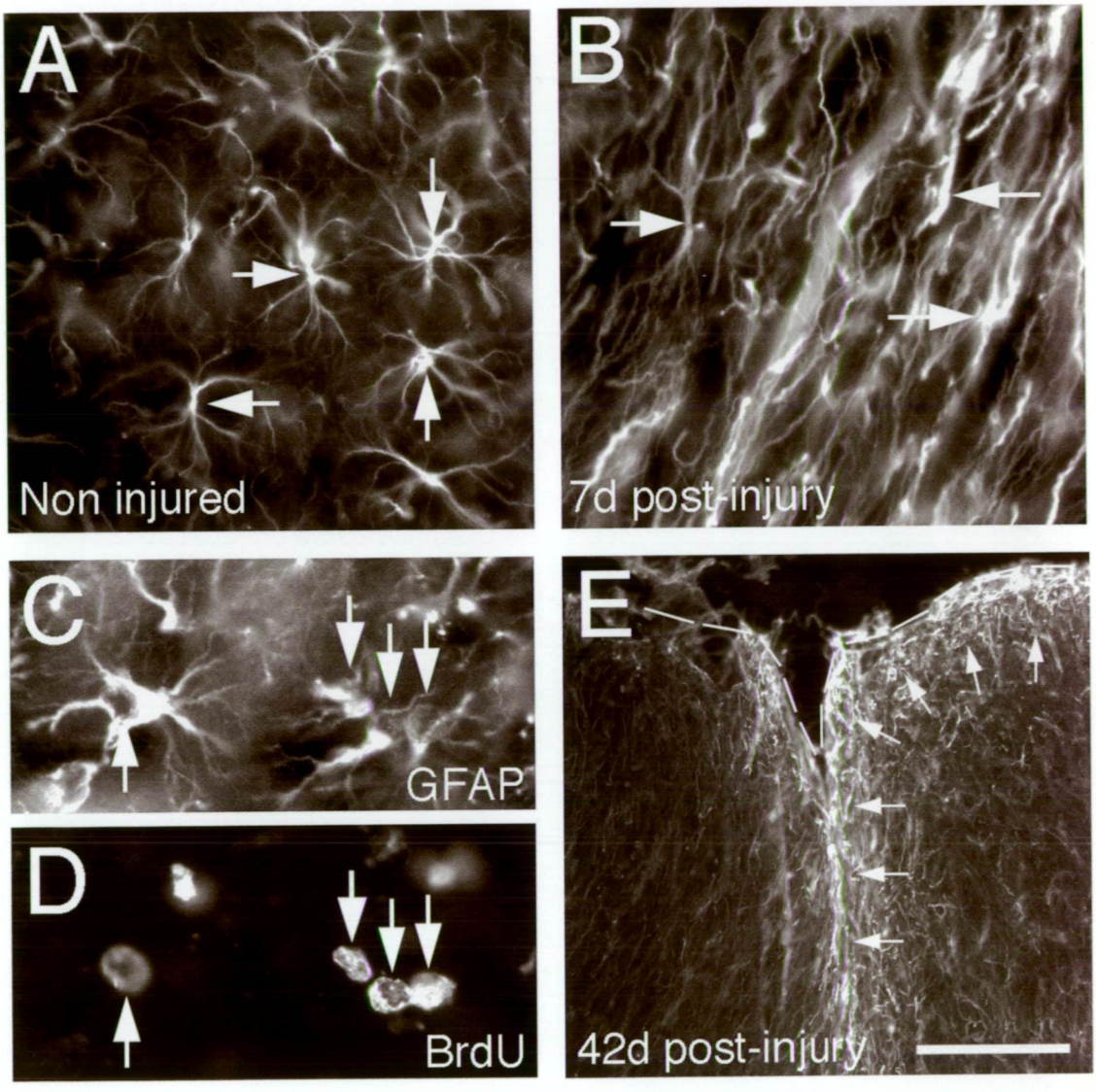
4.3.2 The astrocytic response to acute structural brain injury

The response of the astrocyte population to acute brain injury was determined based on immunoreactivity for the cytoskeletal protein GFAP. Examination of astrocytes within perilesion tissue demonstrated alterations indicative of rapid activation (Figure 4.2). Prior to injury, GFAP immunoreactive astrocytes demonstrated stellate morphology and formed a dense network throughout the brain parenchyma, particularly in the neocortical grey matter. These cells displayed typically small cell bodies and elaborated several slender, tapered processes (Figure 4.2A). GFAP-labelled cells underwent substantial morphological alterations in response to injury, including transformation from a stellate to elongated morphology (Figure 4.2B).

Figure 4.2 Astrocytes underwent changes indicative of activation in response to acute structural neocortical injury, including proliferation, morphological alteration and re-distribution

Astrocytes were assessed based on immunoreactivity for GFAP. In the non-injured cortex and at sites distant from neocortical lesion, astrocytes displayed a stellate morphology defined by a small soma from which emanated several radially extending processes (A, arrows). In response to injury, astrocytes exhibited an activated morphology, including elongated somal shape, parallel cell arrangement and re-orientation towards the lesion (B, arrows). Additionally, double immunofluorescence labelling for GFAP (C) relative to BrdU (D) demonstrated that astrocytes proliferated extensively in response to injury (arrows denote examples of proliferating astrocytes, see also Figure 3.11A). By 42 days following injury, astrocytes had formed a dense plexus at the lesion border, indicative of glial scar formation (E, dotted line denotes lesion border, arrows denote scar tissue, see also Figure 4.5C).

Scale bar: A and B = 50μm; C and D = 10μm; E = 120μm.


A
Non injured
B
7d post-injury
C
GFAP
D
BrdU
E
42d post-injury

Furthermore, astrocyte cell bodies underwent hypertrophy, lost their defined processes and adopted a streamline morphology and parallel cell arrangement, which was directed towards the lesion boarder. Double-labelling immunohistochemistry for BrdU demonstrated that a proportion of GFAP immunopositive cells were mitotically active following injury (Figure 4.2C and D, see also Figure 3.11A from chapter 3). These typically reactive alterations in the astrocyte cell population were evoked as early as one day following injury and persisted up to several weeks following injury. By 42 days following injury a dense astrocytic plexus, or glial scar, had formed lining the lesion border and astrocytes with morphology and GFAP content equivalent to pre-injured tissue populated the perilesion zone (Figure 4.2E).

Interestingly, although astrocytes were shown to be mitotically active within the perilesion tissue, as time progressed fewer GFAP-labelled cells contained BrdU immunoreactive nuclei and BrdU-labelled nuclei had predominantly localised to the injury border. These findings support the view that newly formed astrocytes were contributing to the formation of the glial scar. Additionally, astrocytes may have been dying, accounting for a decrease in GFAP-BrdU labelled cells. Alternatively, astrocytes may have been proliferating excessively, diluting out the concentration of BrdU beyond detectable levels.

4.3.3 The microglial/macrophage response to acute structural brain injury

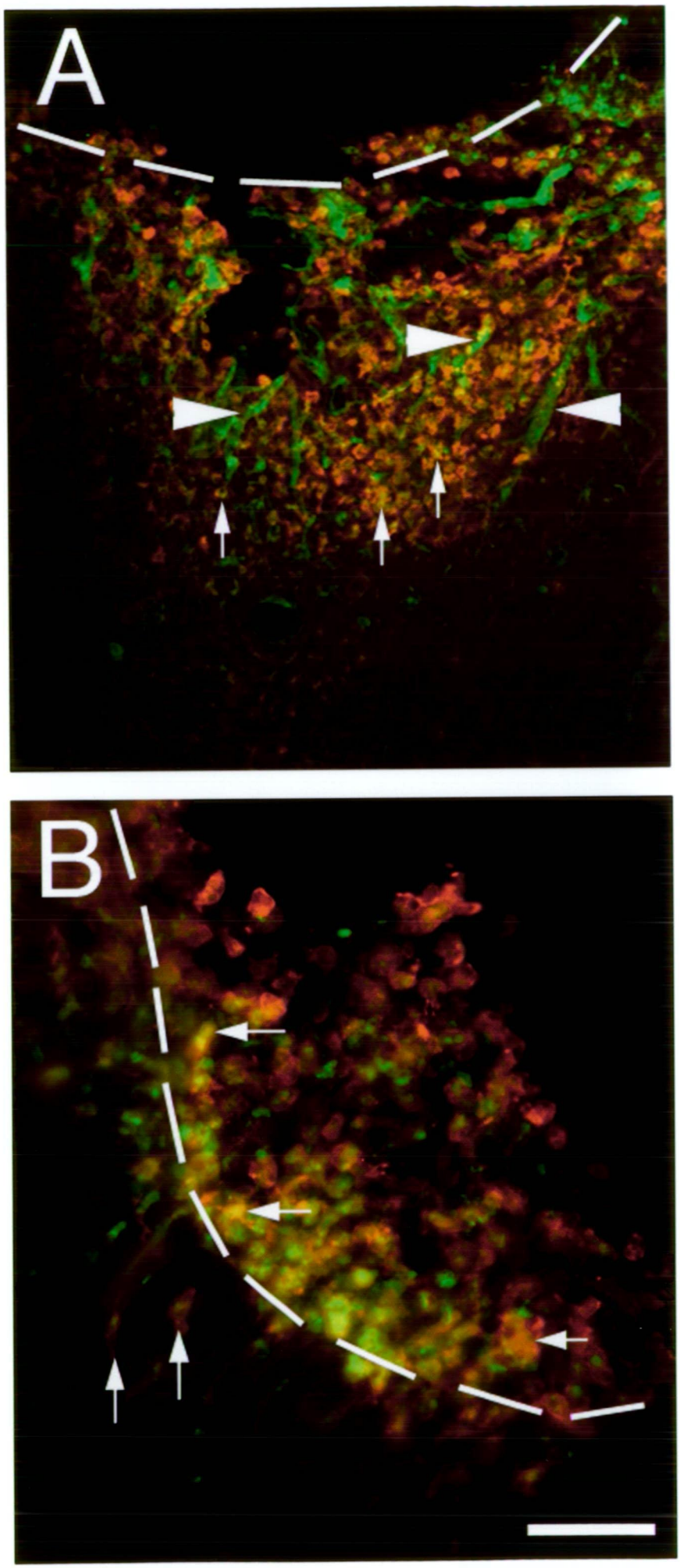
In the present study, alterations in microglial and macrophage populations were examined based on cellular content of ferritin, an iron binding protein expressed in activated microglia and macrophages (Barron, 1995; Zhang and Olsson, 1995;

DiPatre and Gelman, 1997). Additionally, tissue sections were also labelled with fluorescence-tagged Isolectin IB4, which has been demonstrated to label brain microglia (Dailey and Waite, 1999) as well as perivascular cells. Labelling of control, non-injured tissue indicated only a very small population of ferritin-labelled microglia existed within the brain parenchyma.

Following injury, a substantial increase in ferritin and Isolectin IB4 cells was observed associated with the injured cortical tissue (Figure 4.3 and Figure 4.5). As early as one day following injury, numerous ferritin and Isolectin IB4 labelled cells were observed within and immediately surrounding the lesion site. Ferritin-labelled microglia/macrophages were particularly abundant within and surrounding the lesion site by seven days following injury, when labelled cells extended in a large zone surrounding the lesion site in the neocortical grey matter and sometimes extending into the subcortical white matter (Figure 4.3A). Additionally, numerous cells had infiltrated the lesion cavity. Although these cells were a prominent feature of the injured tissue, labelling gradually declined over the time course examined with few labelled cells remaining by 42-84 days following injury. Double-labelling analysis demonstrated that ferritin positive microglia and macrophages underwent substantial proliferation in response to cortical injury, as indicated by BrdU incorporation (Figure 4.3B, see also Figure 3.11B, Chapter 3). Similar to the astrocytic population, the proportion of ferritin-BrdU co-labelled cells (i.e. proliferating microglia/macrophages) decreased over time. This may be explained by cell death, ongoing proliferation, which diluted the BrdU beyond detectable levels and/or the return of some of these cells to circulation.

Figure 4.3 Microglia and macrophages underwent substantial proliferation in response to acute structural neocortical injury and infiltrated the lesion site. Labelling for activated microglia/macrophages (with ferritin, red and Isolectin IB4, green) demonstrated an influx of these cells into injured tissue by 7 days following injury (A). Arrows in A denote examples of ferritin-isolectin IB4 labelled microglia, arrowheads denote examples of isolectin IB4 labelled blood vessels. Double labelling for ferritin (red) relative to BrdU (green) demonstrated that microglia/macrophages were highly proliferative within perilesion tissue (B). Dotted lines denote the lesion border.

Scale bar: A = 20μm; B = 40μm


A
B

Brain microglia underwent marked morphological alterations in response to injury, which correlated with distance from the lesion site (Figure 4.4A). In non-injured control tissue and at sites more distant from the lesion (generally greater that 150-200μm), ferritin labelled-cells demonstrated distinct ramified morphology, whereby numerous branched processes emanated from a rounded cell body (Figure 4.4B). In the perilesion tissue, close to the injury site (generally within 100μm of the lesion border) ferritin-labelled cells demonstrated both rounded and ramified morphology, but were generally rounded, possessing either short stumpy processes or a lack of processes, within closer proximity to the lesion cavity (Figure 4.4C).

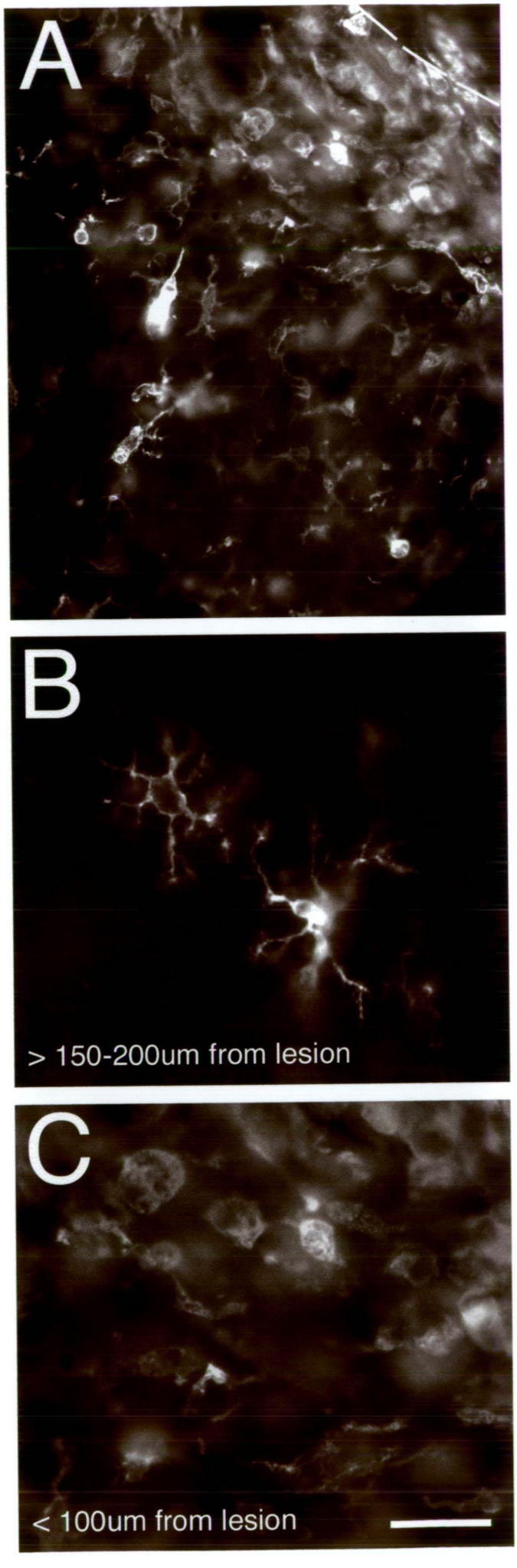
4.3.4 Differential distribution of astrocytes and microglia/macrophages within injured cortical tissue.

To determine the interactions between two of the primary injury-related glial cell populations, namely astrocytes and microglia/macrophages, following acute structural neocortical injury, brain sections were double labelled for ferritin and GFAP (Figure 4.5). These studies showed that astrocytes and microglia/macrophages populated discrete regions of the injured cortical tissue. Although microglia/macrophages were localised throughout the perilesion tissue, they were most abundant within the lesion cavity and by seven days following injury had substantially infiltrated this region, whereas astrocytes were activated immediately around the lesion border (Figure 4.5A). Interestingly, blood vessels were observed within some lesion sites by seven days following injury. Double labelling for ferritin and GFAP demonstrated the different distributions of microglia/macrophages and astrocytes around vascular structures, for example

Figure 4.4 Brain microglia underwent substantial morphological alteration in response to acute structural neocortical injury

Activated microglia/macrophages were generally absent from the non-injured neocortex, as indicted by very few ferritin labelled cells within the cortical parenchyma. In response to injury microglia/macrophages infiltrated the lesion site and underwent marked morphological alterations (A, dotted line in top right denotes lesion border). At sites more distant from the lesion (generally > 150-200μm) microglia demonstrated distinct ramified morphology, indicated by small cell bodies elaborating numerous branched processes (B). With closer proximity to the lesion (generally < 100μm), microglia/macrophages exhibited an activated morphology, characterised by the loss of processes and hypertrophied cell bodies (C).

Scale bar: A = 30μm; B and C= 15μm


A
B
> 150-200um from lesion
C
< 100um from lesion

astrocytes were observed surrounding blood vessels within the perilesion tissue, where they potentially contributed to formation of the blood-brain barrier, whereas microglia/macrophages were localised around blood vessels within the lesion cavity (Figure 4.5A inset). Between 21 and 42 days following injury, activated microglia/macrophages were still located within the perilesion tissue, but had diminished within the lesion cavity, whereas some extended into the lesion cavity (Figure 4.5 B and C). By 42 days following injury, a distinct astrocytic remnant had formed within the tract of the initial needle injury and this site was generally free of microglia and macrophages (Figure 4.5C).

4.3.5 Oligodendrocyte/myelin-associated changes following acute structural brain injury

To determine alterations in oligodendrocytes/myelin following acute structural brain injury, tissue sections were labelled with an antibody specific for CNS myelin/oligodendrocytes (Figure 4.6). As expected, labelling was abundant within the white matter tracts of the brain. Additionally, in the non-injured cortex labelling was distributed throughout the cortical parenchyma, demonstrating punctate distribution which was more abundant in deeper, than superficial cortical laminae (Figure 4.6A). By seven days following cortical injury a loss of labelling was observed within the immediate perilesion zone, this extended deeper into the cortical laminae and myelin degeneration was indicated by granular remnants in the immediate perilesion tissue (Figure 4.6B). At 21 and 42 days following injury, increased labelling for myelin was observed around the lesion border (Figure 4.6C and D). This labelling had a distinct distribution and was localised to filamentous

Figure 4.5 Astrocytes and microglia/macrophages populated different regions of the injury site

Double immunofluorescence labelling for astrocytes (GFAP, green), relative to microglial/macrophages (ferritin, red) demonstrated that microglia/macrophages had infiltrated the lesion site by 1 week following injury, whereas astrocytes were activated at the lesion border (A). Arrowheads and inset in A show a blood vessel that has invaded the lesion site and highlights the different localisations of astrocytes and microglia around this structure. By 3 weeks post-injury some astrocytes had invaded the lesions site and, although still present within the lesion cavity, microglia were diminished in number (B). At 6 weeks following injury few microglia remained in the lesion cavity (C), however, similar to earlier time points, activated microglia were still observed with perilesion tissue. The needle tract had largely closed over by 6 weeks following injury, as indicated by an astrocytic remnant (arrowheads in C). Dotted line in each image denotes the lesion border.

Scale bar: 100μm

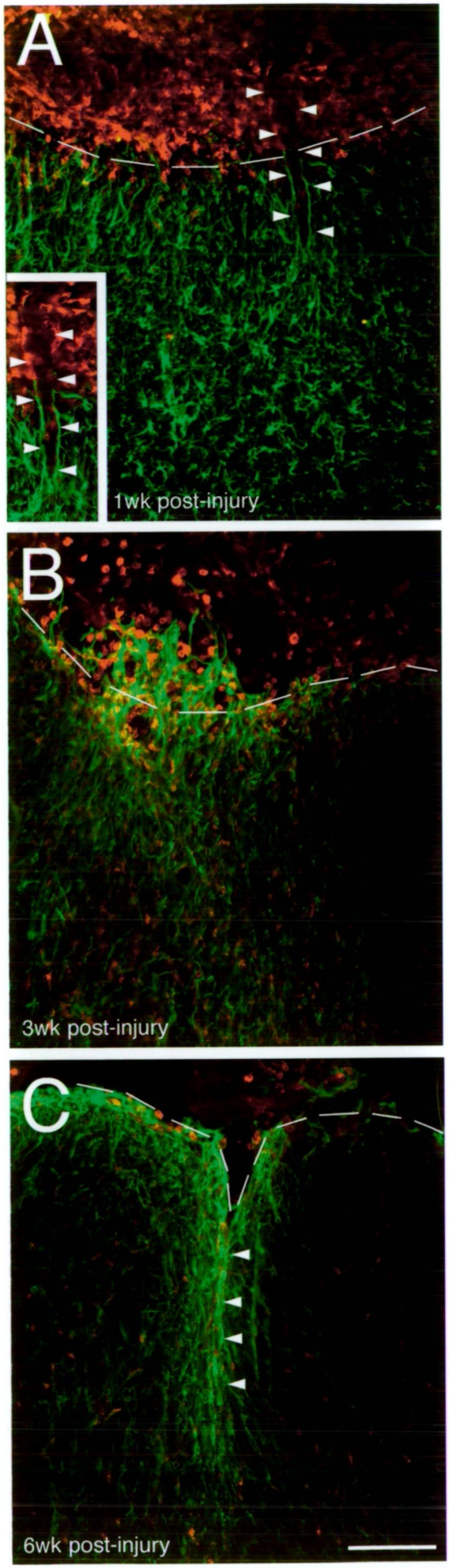
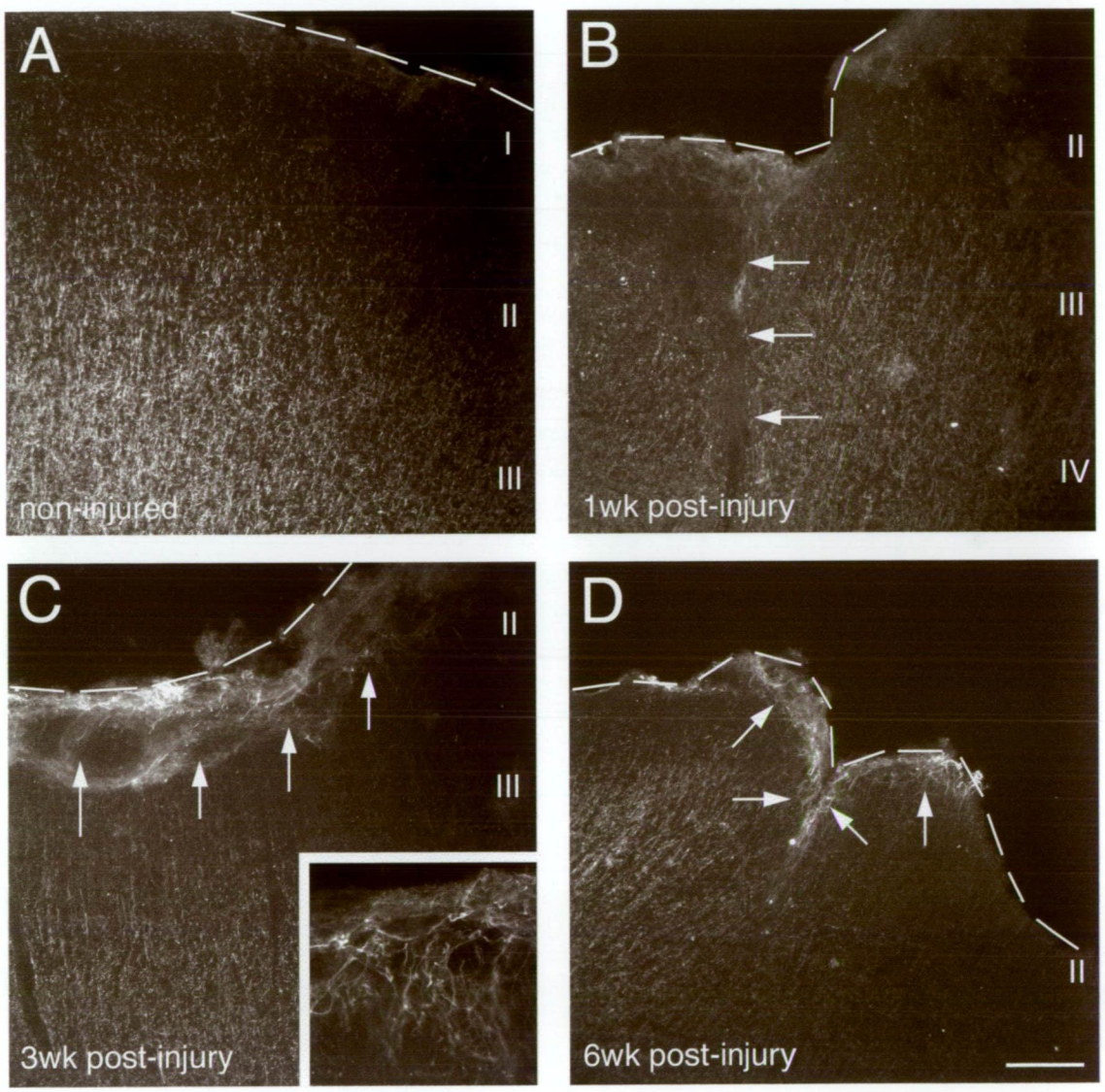

A
1wk post-injury
B
3wk post-injury
C
6wk post-injury

Figure 4.6 Acute structural neocortical injury resulted in a loss of labelling for oligodendrocytes/myelin within perilesion tissue, followed by increased labelling several weeks following injury

Oligodendrocytes/myelin were visualised by labelling tissue sections with an antibody for oligodendrocyte/myelin specific protein, located within the oligodendroglial cell membrane. In non-injured tissue, labelling was relatively punctate throughout the cortical laminae, with relatively little labelling observed within superficial cortical layers (A). By 1 week following injury, labelling within perilesion tissue was diminished relative to controls and sometimes this extended into the initial lesion tract (arrows in B). At both 3 and 6 weeks following injury, increased labelling for oligodendrocyte/myelin specific protein was observed at the lesion border (C and D respectively). Inset in C is a high magnification image of oligodendrocyte/myelin specific protein labelling at the immediate injury border. This labelling was localised as neurite-like structures. Dotted line in each image denotes the lesion border.

Scale bar: A – D = 100μm; inset = 20μm


A
I
II
III
non-injured
B
II
III
IV
1wk post-injury
C
II
III
3wk post-injury
D
II
6wk post-injury

structures within the perilesion zone (Figure 4.6C inset), possibly resembling re-myelination within this region. Double labelling studies for myelin relative to NF-M (Figure 4.7), demonstrated the association of these proteins within the non-injured (Figure 4.7A) and injured (Figure 4.7B) neocortex. Numerous axons were co-labelled throughout the cortical laminae for these proteins. Interestingly, NF-M labelled processes within the injury site were often closely associated with filamentous structures labelled for oligodendrocyte/myelin specific protein (Figure 4.7B, inset).

4.3.6 Changes in brain vasculature in response to brain injury

In an attempt to identify potential alterations in brain vasculature as a result of cortical lesion, tissue sections were labelled with the cellular stain Isolectin IB4 (Figure 4.8). In non-injured tissue sections, numerous blood vessels were labelled with Isolectin IB4. The largest of these vessels were orientated perpendicular to the brain surface and were interconnected by numerous smaller vessels (Figure 4.8A). At 1 day following injury, limited Isolectin IB4-labelled blood vessels remained immediately surrounding the lesion site (Figure 4.8B). However, by seven days post-injury numerous Isolectin IB4-labelled blood vessels were observed around the lesioned region (Figure 4.8C) and by 84 days post-injury vascularisation of the cortical tissue appeared relatively normal (Figure 4.8D), indicating the occurrence of injury-induced vascular re-modelling.

Figure 4.7 Distribution of oligodendrocyte/myelin specific protein relative to NF-M

Double labelling for oligodendrocyte/myelin specific protein (green), relative to NF-M (red) demonstrated co-localisation of these markers throughout the cortical laminae in the non-injured brain (A). By 7 days following injury (B), NF-M was localised to sprout-like protuberances within the lesion cavity and these structures were closely associated similar filamentous structures labelled for oligodendrocyte/myelin specific protein (B, inset).

Scale bar: A and B =100μm; inset = 20μm

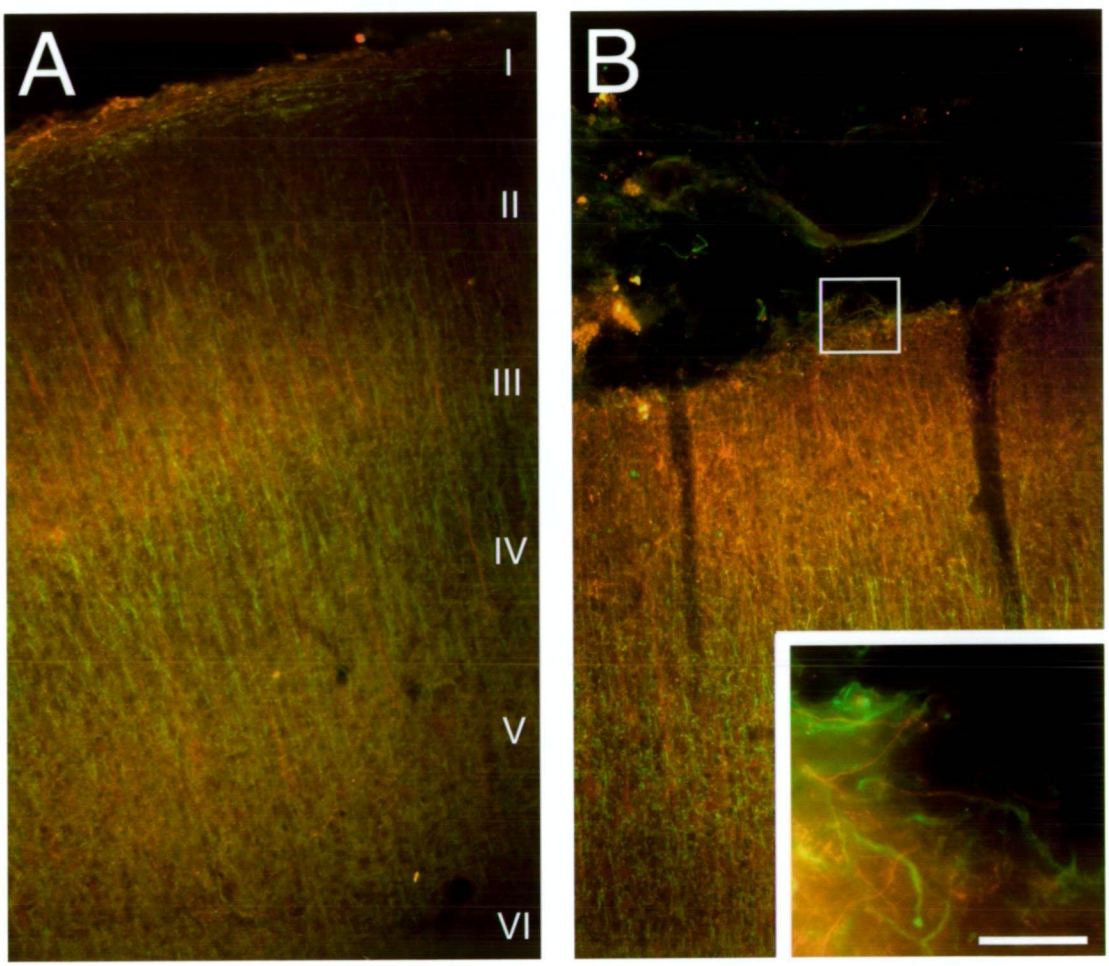
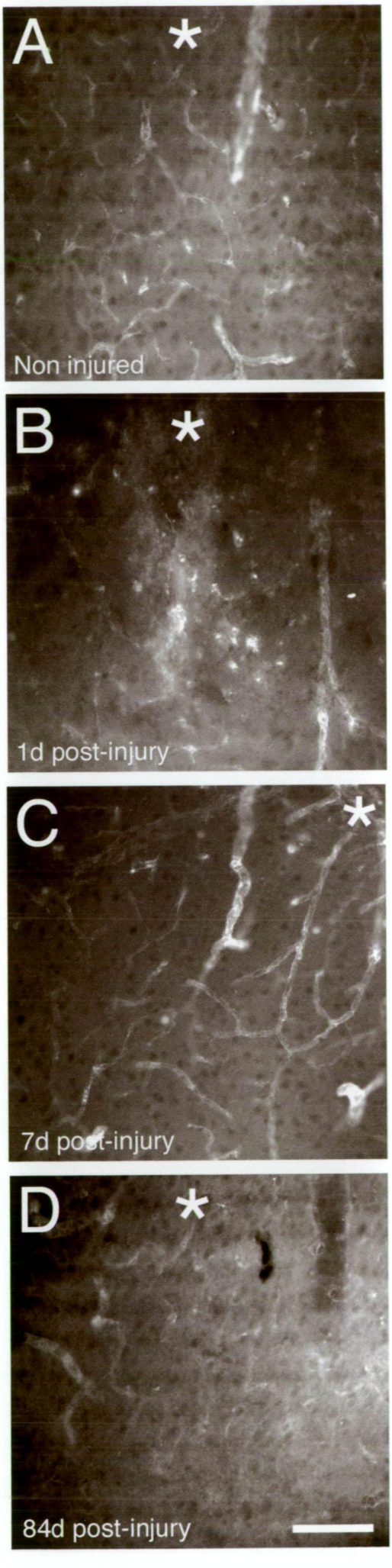

A
I
II
III
IV
V
VI
B

Figure 4.8 Acute structural brain injury induced vascular re-modelling around the lesion site

Alterations in cortical vasculature were analysed based on isolectin IB4 labelling. Blood vessels in the non-injured cortex were characterised by parallel oriented large vessels, interconnected by smaller randomly oriented vessels (A, brain surface lies directly above asterisk). Neocortical injury resulted in a substantial loss of blood vessels within perilesion tissue (B, injury site lies directly above asterisk). By 7 days following injury numerous blood vessels were apparent surrounding the lesion site (C, asterisk lies directly left of lesion border) and by 84 days post-injury relatively normal vasculature had been restored around the lesion site (D, brain surface lies directly above asterisk). Images were taken from equivalent regions of cortical grey matter (layers I-IV) at each time point. Images B-D were taken from perilesion tissue in the immediate vicinity of the lesion site.

Scale bar: 20μm


A
*
Non injured
B
*
1d post-injury
C
*
7d post-injury
D
*
84d post-injury

4.3.7 The neuronal response to acute structural brain injury

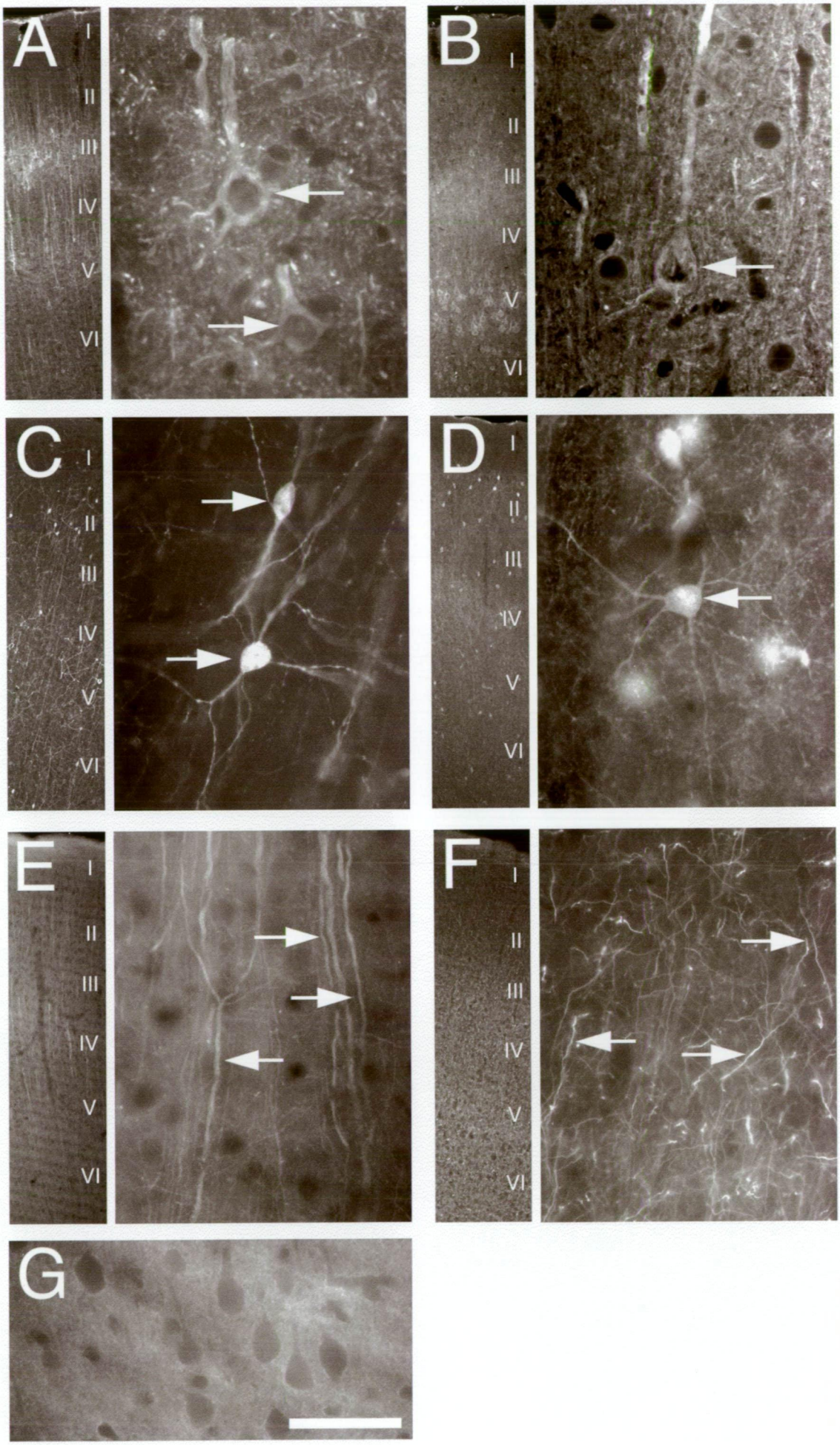
Utilising a battery of antibodies directed at different neuronal cell populations and compartments, as well as techniques to enable identification of specific alterations in neuronal gene expression, the response of neocortical neurons to injury was examined. As anticipated, preliminary immunohistochemical analysis confirmed that the non-compromised neocortex was comprised of specific neuronal populations, including interneurons and pyramidal cells, with distinct columnar and laminar organization (Figure 4.9). Pyramidal cells in layers II/III and V of the neocortex were labelled for the neurofilament triplet proteins, NF-L, NF-M and NF-H (Figure 4.9A). The cell bodies and dendrites of pyramidal cells as well as numerous other neuronal processes demonstrated labelling for βIII-tubulin (Figure 4.9B). Calretinin (Figure 4.9C) and parvalbumin (Figure 4.9D) were localised to distinct sub-classes of cortical interneurons. MAP-2 was typically localised to the apical dendrites of pyramidal cells (Figure 4.9E), whereas phosphorylated neurofilaments (as determined by SMI312 labelling) were localised to axons (Figure 4.9F) and GAP-43 was distributed homogenously throughout the cortical tissue (Figure 4.9G).

Focal neocortical injury evoked a series of reactive and regenerative neuronal alterations (Figure 4.10 and Figure 4.11). Following injury, immunohistochemical labelling demonstrated substantial alterations in a majority of the neuronal markers examined. Initially the lesion boarder was characterised by a range of degenerative and reactive changes. These included the abnormal accumulation of phosphorylated neurofilament proteins, normally localised to axons (Figure 4.10A), into bulb- and

Figure 4.9 A variety of neuronal markers enabled the various neuronal populations and cellular compartments to be visualised with in the rodent neocortex

Low magnification images on left of each panel demonstrate the distribution of each marker throughout the cortical laminae, high magnification images on the right of each panel show the localisation of each marker within different types of cortical neurons. NF-M (A) and βIII-tubulin (B) were largely restricted to cortical pyramidal cells as well as numerous neurites within the cortical parenchyma, whereas calretinin (C) and parvalbumin (D) were localised to cortical interneuron populations. MAP2 (E) was generally restricted to the large apical dendrites of cortical pyramidal neurites, phosphorylated neurofilaments (F, SMI312 labelling) were restricted to axons distributed throughout the cortical laminae and GAP-43 was distributed homogenously throughout the cortical tissue (G). Arrows in each image denote examples of cortical pyramidal/non-pyramidal neurons.

Scale bar: low magnification = 120μm; high magnification = 30μm


A
I
II
III
IV
V
VI
B
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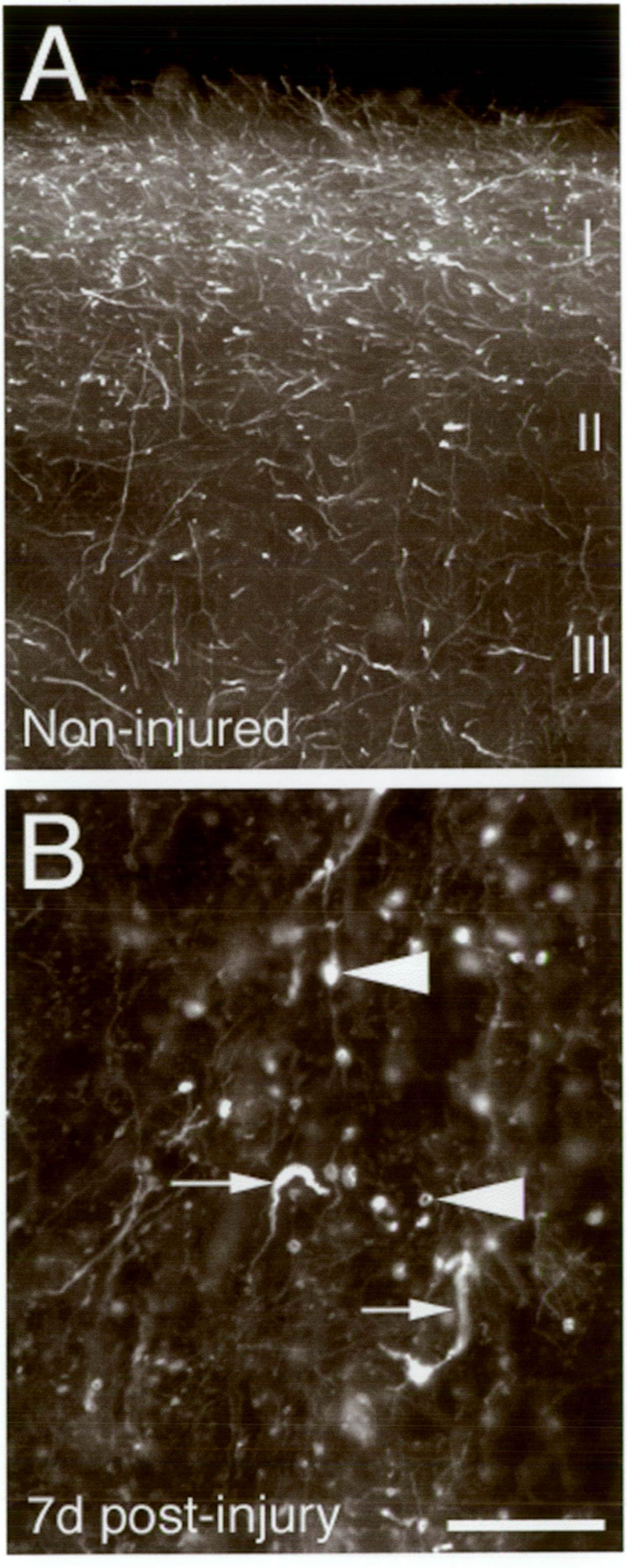
II
III
IV
V
VI
C
I
II
III
IV
V
VI
D
I
II
III
IV
V
VI
E
I
II
III
IV
V
VI
F
I
II
III
IV
V
VI
G

Figure 4.10 Cortical injury evoked substantial neuronal degeneration as well as a series of reactive cytoskeletal associated alterations

SMI312, a cocktail of antibodies directed at phosphorylated neurofilaments typically localised to axons, was utilised to determine to the axonal response to structural injury. SMI312 immunoreactive axons were distributed throughout the non-injured cortex, particularly in layer I (A). By 7 days post-injury phosphorylated neurofilaments had accumulated into abnormal ring- and bulb-like structures (arrowheads in B) and were localised to swollen axons (arrows in B).

Scale bar: A = 40μm; B = 80μm


A
I
II
III
Non-injured
B
7d post-injury

ring-like structures as well as distinctive neurite swelling (Figure 4.10B). Neurite degradation was also a prominent feature of the injury site, manifesting as blebbing and beading of neurite structure. These alterations were most abundant between one and seven days post-injury and after 14 days post-injury were no longer observed.

From seven days post-injury onwards, cellular alterations indicative of attempts by the injured neurons to regenerate were apparent. Concomitant with the subsidence of reactive cytoskeletal alterations was a distinctive neurite sprouting response (Figure 4.11). Neurite sprouts were elaborated into the injury cavity and were most abundant at 7 days post-injury. Although sprouts were also present within the lesion site at post-injury intervals of up to 42 days, they were generally not observed beyond 14 days post-injury. Neurite sprouts were confirmed to be positive for phosphorylated neurofilaments (Figure 4.11A) the neurofilament triplet proteins, NF-L, NF-M, NF-H (Figure 4.11B), βIII-tubulin (Figure 4.11C) and GAP-43 (Figure 4.11D), but not MAP-2, suggesting that they were derived from an axonal, rather than dendritic, origin. Interestingly, injury-induced sprouts were often closely associated with activated microglia/macrophages within the lesion cavity (Figure 4.12). In this regard, sprout trajectories often appeared to bend or weave around microglia/macrophages, which had infiltrated the lesion site.

4.3.7.1 Alterations in neuronal gene expression in response to acute structural neocortical injury

To determine whether the injury-induced sprouting response was reflected by changes at the gene expression level, real-time RT-PCR was performed to analyse

Figure 4.11 Regeneratively sprouting neurites, elaborated into the lesion site within a week following injury, were positive for a range of cytoskeletal and growth-associated markers, excluding the dendritic protein, MAP2

By 7 days post-injury numerous sprout-like protuberances had been elaborated into the lesion cavity. These processes were immunoreactive for phosphorylated neurofilaments (A), NF-M (B), βIII-tubulin (C) and GAP-43 (D), but not MAP2, indicating that they were likely to be of axonal origin.

Scale bar: 80μm

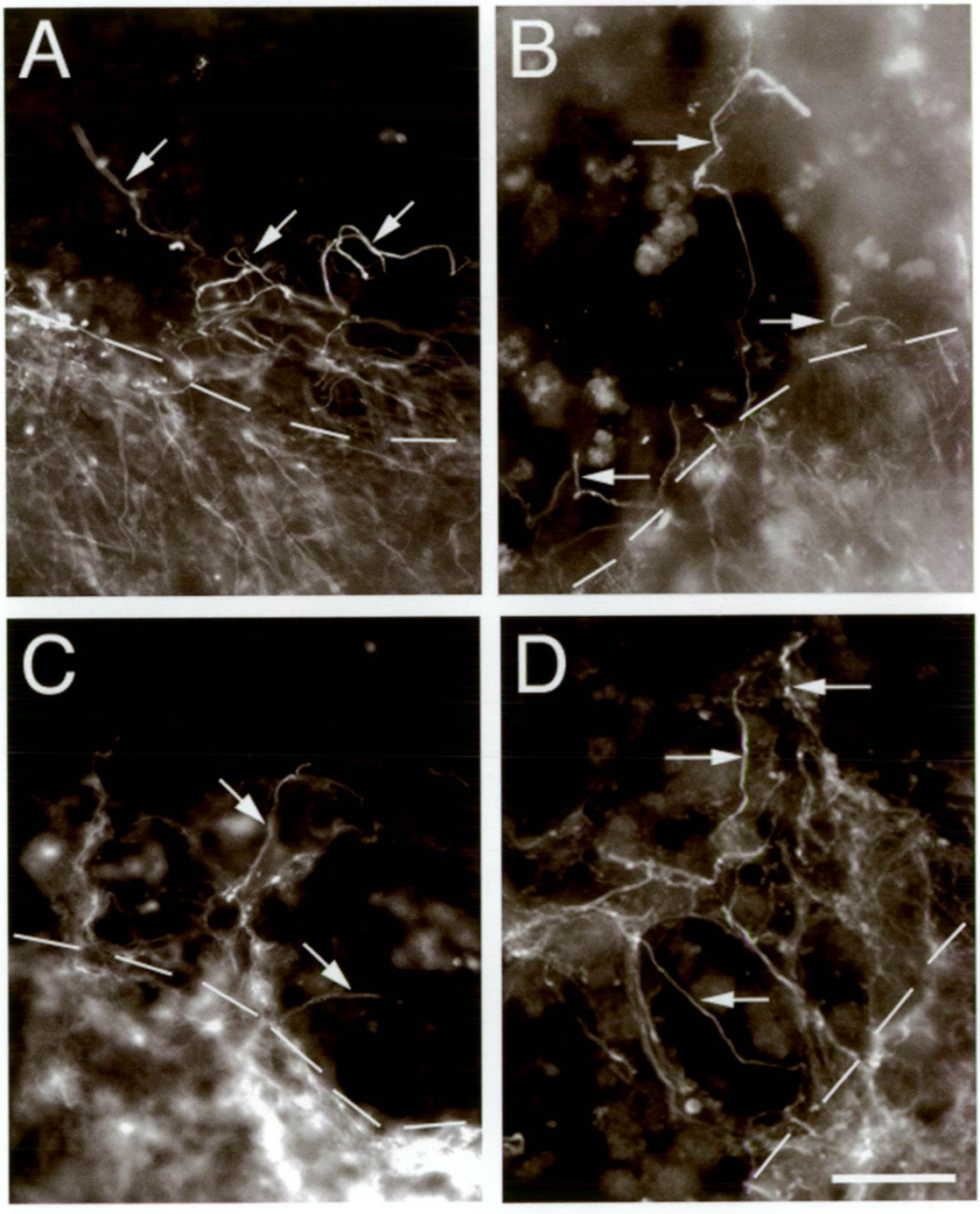
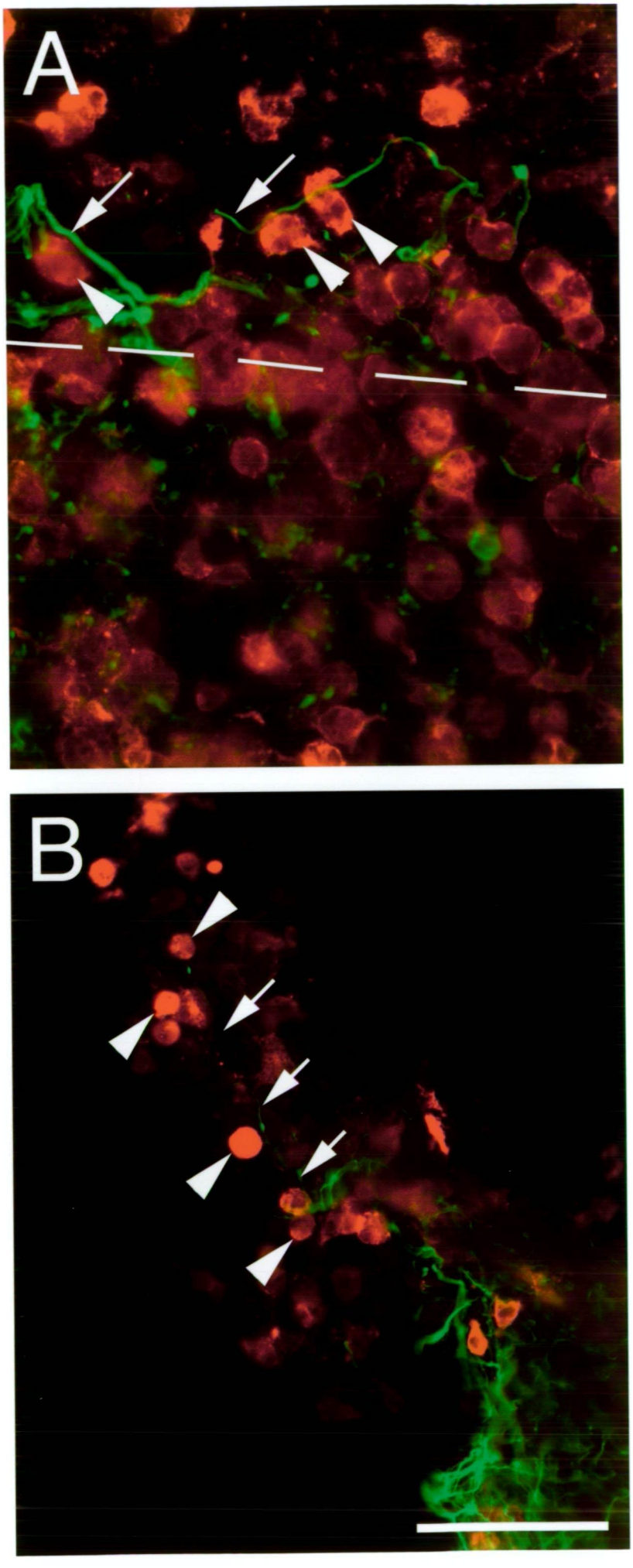

A
B
C
D

Figure 4.12 Injury-induced axonal sprouts were often closely associated with activated microglia/macrophages

Double immunofluorescence labelling for phosphorylated neurofilaments (SMI312) relative to ferritin indicated a close association between sprout-like protuberances (arrows in A and B) and activated macrophages and microglia (arrowheads in A and B) within the injury cavity. Dotted line denotes injury border.

Scale bar: 50μm


A
B

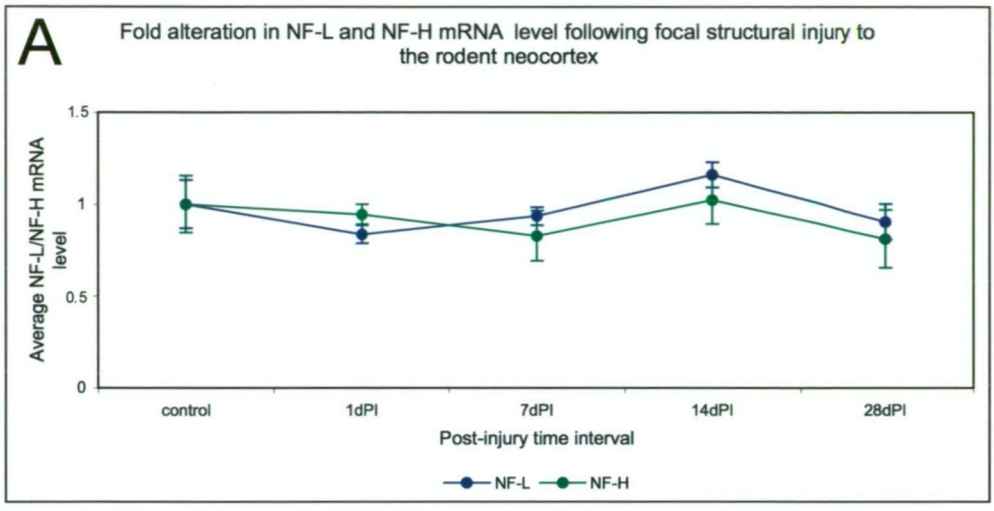
potential alterations in the expression of NF-L, NF-H, βIII-tubulin and GAP-43 between one and 28 days following injury. These investigations demonstrated significant alterations in gene expression (Figure 4.13). Although expression of the neurofilament triplet proteins NF-L and NF-H were unaltered in response to injury (Figure 4.13A), both βIII-tubulin (Figure 4.13B) and GAP-43 mRNA (Figure 4.13C) levels were significantly altered. Specifically, βIII-tubulin mRNA levels were significantly ($p < 0.05$) elevated, by approximately 1.4-1.5 fold, between one and seven days post-injury, but had returned to normal by 28 days following injury. Expression of the growth-associate protein, GAP-43, was significantly ($p < 0.05$) increased at seven and 14 days following injury, by approximately 1.5-1.6 fold, and levels had returned to normal by 28 days post-injury.

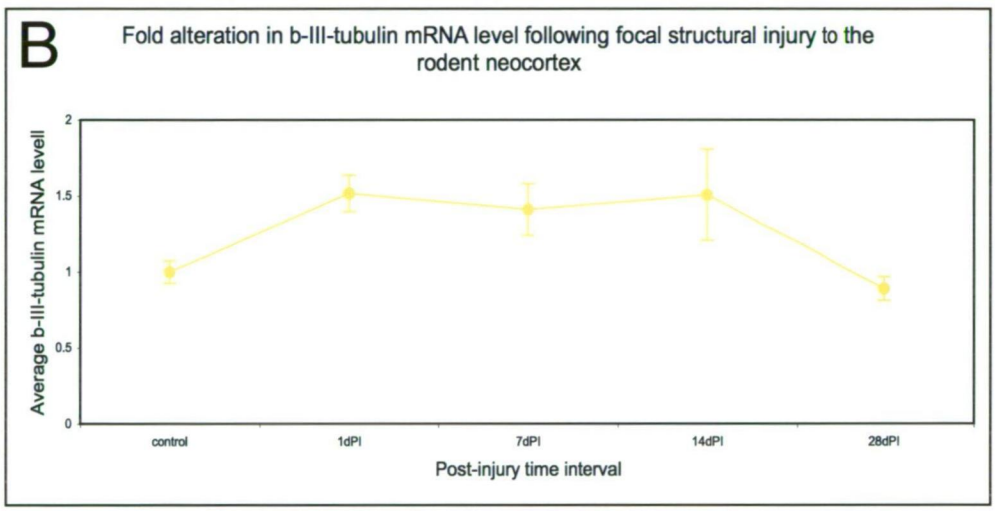
4.3.7.2 Brain extrusion was an occasional consequence of acute structural neocortical injury

Occasionally, by several weeks following injury a small macroscopically visible out-pocketing of brain tissue had formed within or over the injury site. Immunofluorescence labelling demonstrated that these structures were continuous with the underlying cortical tissue and contained both neuronal and glial cell types (Figure 4.14) as well as blood vessels (Figure 4.14B). Indeed, entire cells and/or their processes were located within these structures, including both non-pyramidal (Figure 4.14C) and pyramidal (Figure 4.14D) neurons, astrocytes (Figure 4.14E) and oligodendrocytes/myelin (Figure 4.14F). Features characteristic of injury sites in which these structure had not formed, such as residual microglial infiltration, were often observed around the margins of the extruded tissue, so it appeared that these

Figure 4.13 Acute structural neocortical injury evoked statistically significant alterations in important growth- and cytoskeletal-related, but not neurofilament triplet, proteins

Quantitative real-time RT-PCR was utilised to determine the effect of neocortical injury on the expression of various neuronal genes at 1, 7, 14 and 28 days post-injury. The expression of NF-L and NF-H neurofilament triplet genes were unaltered in response to cortical injury, however, the expression of βIII-tubulin and GAP-43 was significantly elevated. βIII-tubulin mRNA levels were significantly increased between 1 and 14 days post-injury, but had returned to control levels by 28 days post-injury, whereas GAP-43 mRNA levels were significantly increase between 7 and 14 days post-injury and had returned to control levels by 28 days post-injury. $P < 0.05$.


A
Fold alteration in NF-L and NF-H mRNA level following focal structural injury to the rodent neocortex
Average NF-L/NF-H mRNA level
1.5
1
0.5
0
control
1dPI
7dPI
14dPI
28dPI
Post-injury time interval
NF-L
NF-H


B
Fold alteration in b-III-tubulin mRNA level following focal structural injury to the rodent neocortex
Average b-III-tubulin mRNA levell
2
1.5
1
0.5
0
control
1dPI
7dPI
14dPI
28dPI
Post-injury time interval

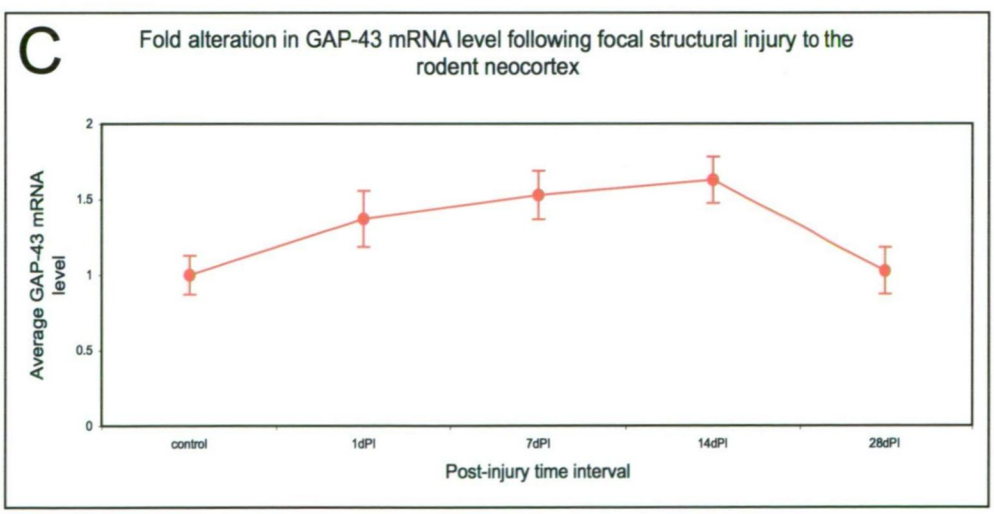
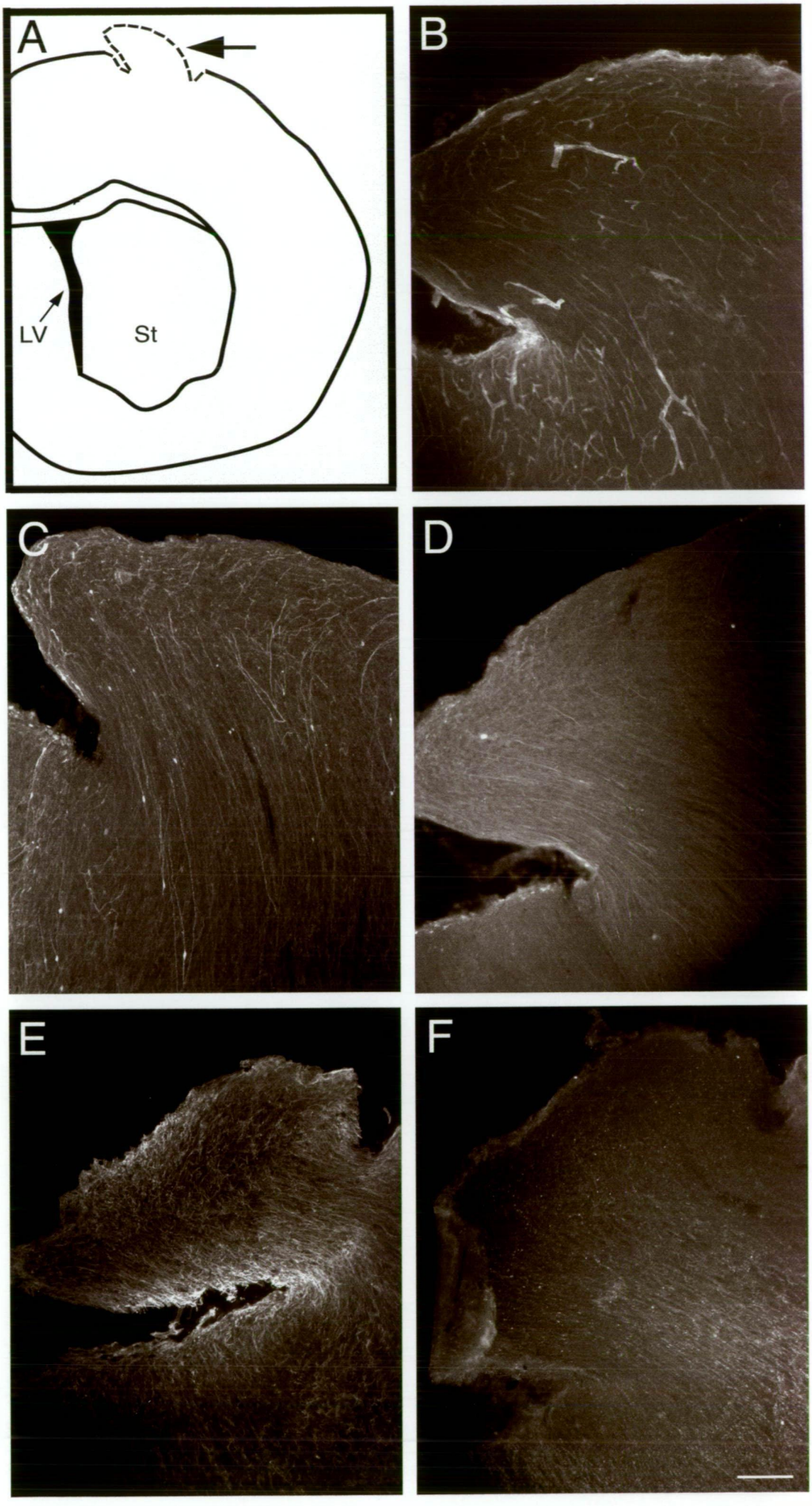

C
Fold alteration in GAP-43 mRNA level following focal structural injury to the rodent neocortex
Average GAP-43 mRNA level
2
1.5
1
0.5
0
control
1dPI
7dPI
14dPI
28dPI
Post-injury time interval

Figure 4.14 Occasionally acute structural neocortical injury resulted in an out-pocketing of cortical tissue to form a lump-like structure in the injury site

The diagrammatic representation in panel A demonstrates the occasional outcome of acute structural injury to the neocortex, i.e. the formation of a lump-like structure within the injury site by several weeks following injury. Immunofluorescence labelling showed that a blood vessels (B) as well as a variety of neural cell types were present in this structure, including interneurons (C, labelling for calretinin), pyramidal neurons (D, labelling for NF-M), astrocytes (E, labelling for GFAP) and oligodendrocytes/myelin (F, labelling for oligodendrocyte/myelin specific protein). All images are from a brain at 6 weeks following injury, in which a lump-like structure had formed within the lesion site as highlighted by the arrow in A.

Scale bar: 100μm


A
LV
St
B
C
D
E
F

structures had been formed by an outgrowth from within the centre of the injury site.

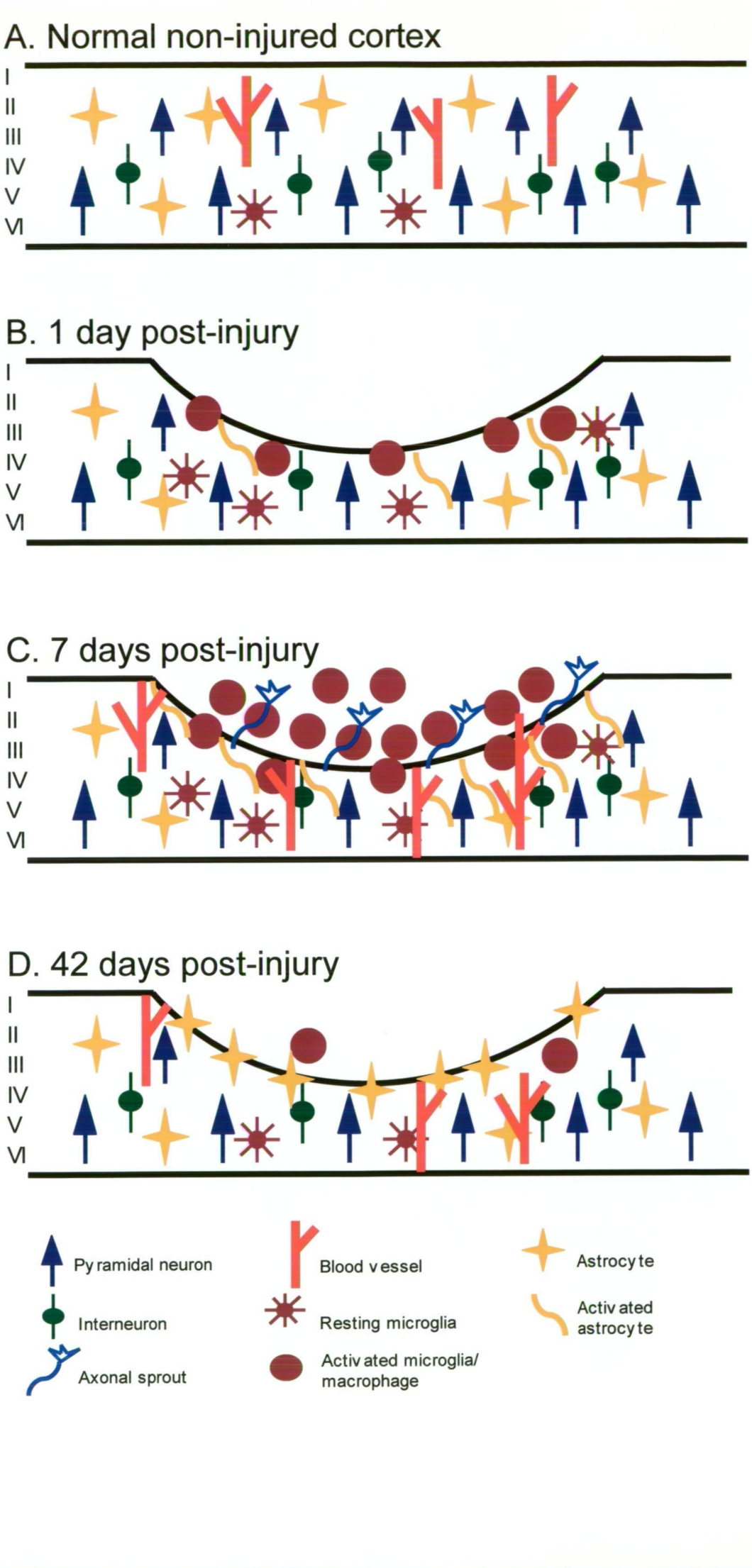
4.4 Discussion

In the present investigation, the long-term neural response to acute neocortical lesion of the rodent somatosensory cortex was determined, focussing specifically on the co-ordinated involvement of astrocyte, microglial, macrophage, and neuronal cell populations as well as potential vascular and oligodendrocyte/myelin alterations. Molecular and cellular alterations were examined at post-injury time points ranging from 1 to 84 days. Specifically, immunohistochemistry was utilised to determine the cellular alterations occurring in different neural cell populations at 1, 7, 14, 21, 42 and 84 days post-injury. Quantitative real-time RT-PCR was then utilised to confirm whether the cellular alterations in damaged neuronal populations were reflected by changes in gene expression of important cytoskeletal and growth-associated genes, including βIII-tubulin, NF-L, NF-H and GAP-43, at 1, 7, 14 and 28 days post-injury.

The injury paradigm utilised in this study was specific to the grey matter of the neocortex and produced a discrete wound-like lesion, which directly damaged the neocortical laminae but not surrounding or underlying structures. Overall, the neural response to acute structural injury was characterised by a progressive sequence of reactive and adaptive changes directed at brain healing and the restoration of neural cytoarchitecture, which proceeded as a typical tissue-healing response. Importantly, astrocyte and microglial activation, as well as reactive and regenerative neuronal alterations, myelin re-distribution and re-vascularisation around the lesion site, were characteristic aspects of this response and culminated in glial scar formation, brain healing and a return to relatively normal cytoarchitecture surrounding the lesion site by 84 days post-injury. Figure 4.15 summarises the reactive and adaptive alterations

Figure 4.15 Diagrammatic representation of the sequence of cellular alterations induced by acute focal injury to the rat neocortex culminating in brain healing

Acute focal injury to the rodent neocortex induced a stereotypical sequence of alterations within perilesion brain tissue, involving all major neural cell populations. The non-injured rat neocortex was characterised by distinct laminar and columnar organisation of both pyramidal and non-pyramidal (interneuron) cells. Large blood vessels were oriented perpendicular to the brain surface and were interconnected by smaller vessels. Astrocytes comprised the prominent glial cell type within neocortical grey matter and displayed distinct stellate morphology, although resting/ramified microglial were occasionally observed. At 1 day following transient needle insertion into the neocortical grey matter, a distinct cavity was formed destroying cells residing within the injury site. Activated microglia/macrophages and astrocytes were observed within the lesion cavity and at the lesion border, respectively. By 7 days following injury, numerous axonal sprouts had been elaborated into the lesion sites, concomitant with an influx of activated microglia/macrophages within this region. Numerous blood vessels were observed within the perilesion tissue and astrocytic activation persisted around the lesion border. By 42 days following injury, both astrocyte and microglial/macrophage activation had generally subsided, blood vessels had undergone a pruning/remodelling process (to resemble pre-injury brain vasculature) and a distinct glial/astrocytic scar had formed at the lesion margin.


A. Normal non-injured cortex
I
II
III
IV
V
VI
B. 1 day post-injury
I
II
III
IV
V
VI
C. 7 days post-injury
I
II
III
IV
V
VI
D. 42 days post-injury
I
II
III
IV
V
VI
Pyramidal neuron
Interneuron
Axonal sprout
Blood vessel
Resting microglia
Activated microglia/ macrophage
Astrocyte
Activated astrocyte

in various neural components, contributing to brain healing.

4.4.1 Damage to the mature brain evokes a wound healing-like response involving the orchestrated response of tissue-specific cell populations

Overall, the molecular and cellular alterations observed in this chapter were indicative of an orchestrated interaction between various key neural cell populations, and resembled a wound healing-like response involving typical phases of inflammation, proliferation/migration and ultimately tissue re-modelling (Maxwell et al., 1990; Hunt et al., 2000; King et al., 2001; Yamaguchi and Yoshikawa, 2001; Oehminchen, 2004). In this regard, damage to the CNS stimulates the co-ordinated activation of glial cell populations, which contribute to the formation of a glial scar in the peri-wound region. However, although this response may be adaptive in some respects, in conjunction with other inhibitory factors within the adult CNS, it often generates a local environment that prevents neuronal regeneration (Fawcett and Asher, 1999). Indeed, the specific interactions between damaged and undamaged neurons and injury-activated glial cells may determine whether glial cells play a predominantly neurotoxic or neurotrophic role during neural repair (reviewed by Aschner et al., 1999).

Utilising a penetrative knife lesion to the cerebrum, Maxwell et al. (1990) demonstrated the stereotypical sequence of changes that ultimately culminate in wound healing, including acute haemorrhage, necrosis and oedema, followed by glial activation, scar formation and wound contraction. In this regard, a study by King et al. (2001) also demonstrated similar reactive alterations leading to the borders of the

lesion being drawn together. These studies, in combination with findings from the present chapter, indicate that the adult brain has a remarkable capacity for repair following injury.

4.4.2 Astrocytes respond rapidly and adaptively to brain injury

In the non-injured brain, protoplasmic astrocytes of the neocortical grey matter provide physical and metabolic support to adjacent neurons (Tacconi, 1998; Anderson and Swanson, 2000; Abbott, 2002; Fields and Stevens-Graham, 2002). Due to this close association it is, therefore, not surprising that they are activated in response to neuronal injury. Astrocytes have been shown to undergo a specific stereotypy of reactive alterations in response to injury, including proliferation, hypertrophy and increased synthesis of GFAP, a process known as reactive astrogliosis (Maxwell et al., 1990; Ridet et al., 1997; Raivich et al., 1999; Kernie et al., 2001; McGraw et al., 2001; Chirumamilla et al., 2002; Chen et al., 2003a). Overall the present study confirmed these alterations in response to acute cortical lesion.

Reactive astrocytes were observed to contribute to the long term re-modelling of the injured tissue through formation of a dense astrocytic plexus, glial scar, and restoration of relatively normal astrocyte distribution within close vicinity of the lesion site. Indeed, within the first few weeks following CNS injury, reactive astrocytes have been shown to create a physical barrier between damaged and non-damaged tissue, effectively segregating non-damaged tissue from the potentially harmful effects of tissue necrosis and blood brain barrier disruption (Fawcett, 1997;

Raivich et al., 1999; Chen et al., 2003a). Although this barrier may protect non-damaged neurons, detrimental consequences of reactive gliosis may ultimately impede axonal re-growth (Ridet et al., 1997; Fawcett and Asher, 1999, Batchelor and Howells, 2003; Hampton et al., 2004). In this regard, certain extracellular matrix molecules are incorporated into the glial scar (McGraw et al., 2001), thus it forms both a physical and molecular barrier to neuronal re-growth. Indeed in the present study, profuse axonal sprouting was observed between seven and 14 days following injury, but was diminished after this time, concomitant with formation of a dense astrocyte remnant at the lesion border.

Interestingly, although astrogliosis and glial scar formation have been viewed as inhibitory to axonal growth a selection of studies indicate that astrocytes may in fact facilitate axonal sprouting and regeneration following injury. For example, Dusart et al. (1999) demonstrated that following a long delay axotomised Purkinje cells were able to elaborate new axonal sprouts that were encased in astrocytic sheaths. The authors postulate that astrocytes may therefore limit contact between sprouting axons and inhibitory molecules and/or express molecules that facilitate axonal re-growth. Moreover, astrocytes may secrete cytokines and growth factors that promote neural repair, either directly or through the activation of macrophages (Lotan and Schwartz, 1994).

4.4.3 Microglia and macrophages invade injured tissue and potentially facilitate axonal sprouting

Microglia and macrophages were assessed regardless of their parenchymal or

systemic origin. As such, ferritin labelling for activated microglia in the non-injured brain was relatively low and labelled cells were rarely observed. The role of microglia in the non-compromised brain has not been fully characterised, but is proposed to involve highly dynamic immune-surveillance functions (Nimmerjahn et al., 2005). Microglia activation (Aihara et al., 1995) and macrophage infiltration (King et al., 2001) are, however, characteristic alterations induced by injury to the brain. Indeed, studies by Davalos et al. (2005) have demonstrated that microglia are activated within minutes following injury. In response to injury, brain-resident microglia proliferate and undergo highly dynamic alterations involving conversion into highly mobile macrophages (Ravivich et al., 1999; Stence et al., 2001; Chirumamilla et al., 2002; Hampton et al., 2004; Carbonell et al., 2005). However, injury-induced mobility is not necessarily lesion-directed (Carbonell et al., 2005).

Furthermore, recent data indicate that microglia respond rapidly to brain injury by extending and coalescing their processes towards sites of injury (Davalos et al., 2005). Utilising cultured hippocampal tissue slices, Stence et al. (2001) demonstrated that that microglial activation proceeds thought a defined sequence of morphological alterations; ramified microglia withdraw their dendritic branches and elaborate a new set of dynamic protuberances, which can be extended and retracted to facilitate motility and migration. As confirmed by the present study, overt morphological alterations include the transformation from a ramified to rounded/amoeboid morphology (Aihara et al., 1995; Stence et al., 2001). The morphological transformation of activated microglia has been associated with their development into phagocytic macrophages, responsible for clearing tissue debris

(Ravivich et al., 1999). Additionally, cells with rounded morphology may also be derived from circulating macrophages, extruded into sites of tissue damage (Barron, 1995; Raivich et al., 1999).

Although microglial/macrophage activation may result in a prolonged inflammatory response and the release of toxic factors resulting in further neuronal destruction (Lotan and Schwartz, 1994; Vilhardt, 2005), these cells have also been suggested to enhance tissue re-modelling and healing through a variety of integrated processes. These processes include inflammation, lipid recycling, secretion of factors that promote neural regeneration and neo-vascularisation, phagocytic removal of tissue debris as well as other immune functions, such as antigen presentation and lymphocyte activation (Thomas, 1992; Lotan and Schwartz, 1994). In this regard, microglia may play important roles in limiting the spread of apoptotic stimuli and promoting neuronal survival and growth following injury (reviewed by Bruce-Keller, 1999).

Microglia and macrophages have also been shown to play important roles in facilitating injury-induced axonal sprouting (Prewitt et al., 1997; Batchelor et al., 1999, 2000, 2002a, b; King et al., 2001), which are supported by observations from the present study. Results from the present study indicated that microglia/macrophages were abundant following injury, declining in number over the time course examined with relatively few, if any, persisting at the longest time interval examined. Interestingly, microglia/macrophages were most abundant at seven days post-injury, consistent with profuse axonal sprouting at this time.

Furthermore, these cells were often observed in close association with sprouting axons and it has been suggested that they may release factors facilitative to axonal regeneration (King et al., 2001; Batchelor et al., 1999, 2002a, b).

Indeed, Batchelor et al. (1999, 2002a) demonstrated that injury-induced sprouting dopaminergic fibres were intimately associated with activated microglia and macrophages as they navigated towards the wound edge. This response was correlated with an increasing gradient of brain- and glial- derived neurotrophic factors (BDNF and GDNF, respectively), whereas sprouts were unable to penetrate into the core of the wound where trophic factors were scarce (Batchelor et al., 1999, 2000a). However, both observations from this chapter as well as findings by King et al. (2001), have demonstrated that sprouting axons were able to penetrate into neocortical lesions, infiltrated by microglia and macrophages. Furthermore, mice deficient in a variety of macrophage subtypes (osteopetrotic mice) and mice in which the expression of BDNF and GDNF were inhibited by antisense oligonucleotides, demonstrated decreased periwound sprouting following striatal lesion, relative to controls (Batchelor et al., 2000, 2002b). Collectively, these studies strongly support a role for microglia and macrophages in facilitating axonal re-growth following injury. However, Dusart et al (1999), report that axotomy induced sprouting of Purkinje cells is not correlated with the presence of activated macrophages.

4.4.4 Myelin initially degenerates following injury, but is increased within perilesion tissue at later post injury time points

In this chapter, alterations in oligodendrocytes/myelin were assessed with an

antibody specific for the myelin contained within oligodendrocyte membranes. Myelin was abundantly distributed throughout the grey matter of the neocortex, in association with axons. Although myelin degenerated from perilesion tissue following injury, it was observed at the lesion border (forming sprout-like protuberances) within a week following injury and was observed in close association with sprouting axons. Thus, in this chapter, axons were apparently able to sprout in the presence of myelin. However, specific molecules in CNS myelin, including Nogo, Myelin-Associated Glycoprotein (MAG) and Oligodendrocyte Myelin glycoprotein (OMgp), as well as other molecules secreted by reactive astrocytes and oligodendrocytes, derived from blood or within the extracellular matrix (for example tenasin and chondroitin sulphate proteoglycans as well as many other molecules) have generally been viewed as inhibitory to axonal re-growth (reviewed by Fawcett and Asher, 1999; Bandtlow and Schwab, 2000; Goldberg and Barres, 2000; Qiu et al., 2000; Watkins and Barres, 2002; McGee and Strittmatter, 2003; Domeniconi and Filbin, 2005). Interestingly, these molecules share a common receptor, NgR, which has been implicated in this process (Watkins and Barres, 2002; McGee and Strittmatter, 2003). The re-appearance of myelin at the lesion border, in addition to the formation of a dense astrocytic plexus, may have contributed to the decrease in axonal sprouting, observed several weeks following injury. Thus, although myelin may have been associated with sprouting axons initially, formation of the glial scar effectively prevented this sprouting response in the long term.

4.4.5 Brain injury induces vascular remodelling and restoration

Structural brain injury frequently results in deformation or tearing of blood vessels.

In the present investigation, blood vessels immediately surrounding the lesion were largely destroyed, as indicated by an absence of staining with Isolectin IB4. From seven days onwards, labelled blood vessels were again observed in and around the lesion site. Although this investigation did not quantify the alterations in brain vasculature following injury, studies by Chen et al. (2003a) have demonstrated a degree of vascular remodelling following focal brain injury, including decreased blood vessel density and increased diameter, which eventually returned to control values. A recent review by Dvorak (2005), indicates the involvement of angiogenesis, the formation of new blood vessels, as a mechanism of wound healing as well as aberrant growth, such as tumour formation. However, the author refers to this form of angiogenesis, compared to that normally occurring during development of the vascular system, as proceeding abnormally and resulting in the formation of vessels that are irregularly branched and distributed. In this chapter, vascular remodelling contributed to restoration of relatively normal vasculature within close proximity of the lesion. These finding suggest that vascular growth and re-modelling are an important feature of brain repair following injury.

4.4.6 Neurons undergo early reactive cytoskeletal alterations in response to brain injury

Brain injury results in significant neuronal degeneration and death. Consistent with previous investigations, this manifested as frank neuronal loss at the injury site, as well as degenerative alterations including neurite beading and disconnection (Chen et al., 2003a). Degenerative neuronal changes were generally restricted to a region immediately surrounding the lesion cavity. The reactive axonal, and particularly

cytoskeletal, changes observed in the present study are consistent with previous investigations and have been described as an evolving pathology potentially involving altered ionic concentrations, abnormal enzyme activation, altered neurofilament phosphorylation, impeded axonal transport, axonal swelling and eventual axonal disconnection, resulting in the formation of terminal axon bulbs incorporating abnormally compacted or accumulated cytoskeletal elements (Schalaepfer, 1987; Adams, 1992; Egeler-Peerdeman, 1993; Jafari et al., 1997, 1998; Maxwell et al., 1997; Povlishock et al., 1997; Fitzpatrick et al., 1998; Graham et al., 2000; King et al., 2000a, b, 2001; Smith and Meaney, 2000; Postmantur et al., 2000; Vickers et al., 2000; Chen et al., 2003a; Hamberger et al., 2003).

Interestingly, although abnormal neurofilament accumulation is often reported as characteristic of neuronal damage, some studies have reported a reduction in neurofilaments at sites of injury (Hoffman et al., 1984, 1985; Maxwell and Graham, 1997) and, contrary to the present investigation in which neurofilament triplet expression was found to be unaltered in response to injury, some data indicate diminished neurofilament expression in response to injury (Hoffman et al., 1987, 1993; Goldstein et al., 1988, Hoffman and Cleveland 1988; Muma et al., 1990; Nixon and Shea, 1992). However, Gervasi et al. (2003) report an increase in peripherin and NF-M mRNA, associated with increases in protein, following optic nerve injury. These discrepancies potentially reflect the use of a variety of injury paradigms involving different CNS and PNS regions (Gervasi et al., 2003).

4.4.7 Injury-induced adaptive neuronal alterations include sprouting and re-expression of important growth related proteins

Neurons have been shown to undergo a variety of molecular and cellular alterations in response to injury. Indeed, the present investigation provides data supporting an attempt by mature cortical neurons to regenerate following injury. Alterations associated with attempted regeneration included substantial axonal sprouting in addition to significant re-expression of the developmentally important growth-associated protein, GAP-43, and significantly increased expression of the cytoskeletal protein, βIII-tubulin. Alterations in gene expression, including an increase in developmentally important growth and cytoskeletal genes, have been reported following injury to both the PNS (reviewed by Gillen et al., 1997) and CNS (reviewed by Emery et al., 2000, 2003) and support the view that neurons undergo adaptive changes following injury, directed at brain repair. In general, regenerative changes following injury to the PNS, which are typically much more successful than those occurring following CNS injury, include increased synthesis of actin, tubulin and GAP-43 and decreased neurofilament expression (Bisby and Tetzlaff, 1992). Although findings from the CNS are less clear, the increases in βIII-tubulin and GAP-43 reported in the present study indicate that CNS neurons undergo at least a transient attempt at regeneration following injury.

Although the mechanisms underlying the axonal response to injury are not fully understood, several reports suggest that damaged axons undergo a stereotypic sequence of reactive and regenerative alteration, which are associated with cytoskeletal reorganisation and up-regulation of growth associated protein

(Povlishock and Becker, 1985; Christman et al., 1997; King et al., 2001). Indeed axons undergo an early swelling response, which may be associated with degenerative or regenerative alterations. For example, Povlishock and Becker (1985) and King et al. (2001) have shown that reactive axonal swellings may elaborate sprout-like protuberances. Furthermore, sprouts may have unusual features including club-like endings and partial myelination (King et al., 2001) or dystrophic growth cones (Tom et al., 2004). Interestingly, evidence of axonal sprouting has also been reported to occur following both stroke and spinal cord injury (reviewed by Carmichael, 2003a, b). However, the development of abnormal growth cones at the proximal tips of sprouting CNS neuron axons is postulated to account for regenerative failure in the CNS (Tom et al., 2004; Verma et al., 2005).

Notably, both the axonal sprouting response and concomitant gene expression alterations were generally not maintained past approximately 14 days post-injury, a time when the glial scar is likely to form (reviewed by Raivich et al., 1999). Indeed, expression of both GAP-43 and βIII-tubulin had returned to control levels by 28 days following injury and axonal sprouts were rarely observed after this time.* Although the glial scar is, therefore, likely to be impeding axonal regrowth it has been suggested that the incapacity for re-establishment of neuronal connections following injury may be a means of protection, ensuring that sprouting axons do not integrate maladaptively into neural architecture (Larner, 1995; McKinney et al., 1999; Ravivich et al., 1999; Jacobs et al., 2000). These findings provide interesting avenues for future research, particularly regarding the opportunistic window in which appropriate axonal sprouting may be facilitated.

* Sprouting axons appear to require a substrate upon which to grow. Microglia/macrophages, which have infiltrated the injury site, are likely to provide this substrate and, additionally, may produce molecules that promote axonal growth. As the glial scar develops and microglia/macrophages disperse from the injury site it is plausible that axonal sprouts alter their growth paths, are retracted, degenerate and/or are incorporated into the glial scar.

4.5 Conclusion

Consistent with previous investigations, this study demonstrates substantial alterations in key neural cell populations and components, including astrocytes, microglia/macrophages and neurons as well as oligodendrocytes/myelin and brain vasculature in response to acute focal brain injury. Importantly, astrocyte and microglia/macrophage cell populations underwent a stereotypical activation response following injury, which was associated with substantial alterations in the damaged neuronal population. Neurons underwent alterations indicative of attempted regeneration, including reactive sprouting and significant increases in the expression of important cytoskeletal and growth-associated genes. Collectively, the reactive and adaptive alterations observed in the present study, involving the co-ordinated response of both neuronal and glial cell populations, are indicative of substantial neural and vascular re-modelling.

These changes presumably reflect the extent of endogenous brain repair possible following acute focal brain injury and demonstrate an adaptive and sustained attempt at re-establishment of brain cytoarchitecture. Importantly, the neuro-glial alterations observed in this study reflect, to a large extent, the stereotypical wound-healing response, seen in tissues such as the skin, following physical trauma. In this regard, brain healing may be viewed as a general response, involving tissue-specific cell populations, incorporating phases of coagulation, inflammation, proliferation and re-modelling, resulting in the formation of a glial scar.

Although this investigation did not extend to measuring the functional outcome of

the cellular, morphological and molecular outcomes observed, it does suggest that the damaged brain re-models to the best of its ability. Brain injury generally proceeds as an evolving sequence of alterations, involving both primary and delayed secondary changes, which may be adaptive or deleterious in nature. Importantly, the timing of degenerative and regenerative phases may afford windows of opportunity, in which deleterious reactions can be inhibited and adaptive changes promoted. In this regard, this study highlights several aspects of the neural response to injury that may be therapeutically targeted to promote effective recovery, including both the promotion of neuronal/axonal re-growth as well as therapies aimed at enhancing the potentially facilitative influences glial cell populations may play in this response.

Axotomy of cortical neurons grown *in vitro* evokes cytoskeletal and morphological alterations directed at intrinsic axonal sprouting

5.1 Introduction

TBI in humans and experimental animal models frequently results in widespread axonal disruption, or DAI, in the absence of other brain parenchymal alterations (Povlishock and Becker, 1985; Christman et al., 1997; Maxwell et al., 1997; Graham et al., 2000; Smith and Meaney, 2003; Povlishock and Katz, 2005). In this regard, axons are damaged when the brain undergoes rapid acceleration/deceleration or rotational forces resulting in axonal severing, shearing or stretching (Smith and Meaney, 2000). Axotomy, or axonal disconnection, may occur by primary or delayed mechanisms; axons may be torn at the time of injury or, more commonly, the axonal cytostructure is damaged by injury-induced alterations in the local internal and/or external environment that evoke a complex cascade of detrimental alterations, ultimately disrupting the axonal cytoskeleton, impeding axonal transport and culminating in axonal swelling and disconnection (Schalaepfer, 1987; Adams 1992; Yamaguchi and Povlishock, 1992; Egeler-Peerdeman, 1993; Povlishock and Jenkins, 1995; Jafari et al., 1997; 1998, Maxwell et al., 1997; Povlishock et al., 1997; Fitzpatrick et al., 1998; Postmantur et al., 2000; Smith and Meaney, 2003; Chung et al., 2005) – events which may or may not result from disturbances in the axonal membrane and subsequent abnormal enzyme activation (Maxwell et al., 1997; Chung et al., 2005). These changes may result in neuronal death, atrophy, or an attempt at regeneration (Tuszynski and Gage, 1995; Singleton et al., 2002; Selzer, 2003).

Although injury to the CNS is associated with poor axonal regenerative ability (Fawcett and Asher, 1999; Schwartz et al., 1999; Domeniconi and Filbin, 2005), several studies have demonstrated striking examples of axonal plasticity and sprouting following injury and indicate that these changes involve substantial cytoskeletal reorganisation (Povlishock and Becker, 1985; Kristt, 1987; McHale et al., 1995; Dusart et al., 1999; Christman et al., 1997; Deller and Frotscher, 1997; McKinney et al., 1997; Dickson et al., 2000; King et al., 2001; Tom et al., 2004; Kerschensteiner et al., 2005). However, injury-induced sprouting in the mature CNS has generally been viewed as a transient abortive process and, in instances where functional injury-induced sprouting has been observed, this has largely been attributed to collateral sprouting from local undamaged axons (Deller and Frostcher, 1997). Thus, whether an injured axon can sprout from its surviving proximal tip remains to be fully elucidated. In this regard, complex interactions between components of the neuronal cytoskeleton, are crucial for axonal outgrowth, branching and pathfinding during neuronal development and maturation (Tanaka and Sabry, 1995; Brandt, 1998; Szebenyi et al., 1998; Dent et al., 1999; Gallo and Letourneau, 2000; Kalil et al., 2000; Korey and VanVactor, 2000; Suter and Forscher, 2000; Dent and Kalil, 2001, 2003; Dent et al., 2003). However, it is unknown whether similar cytoskeletal mechanisms underlie axonal sprouting.

Promoting and enhancing post-injury axonal sprouting is the primary goal of many therapeutic repair strategies (Selzer, 2003). However, uncontrolled axonal growth and aberrant synapse formation may ultimately be maladaptive and has been implicated in the development of epileptiform activity following brain injury

(Larner, 1995; Salin et al., 1995; McKinney et al., 1997) and the pathogenesis of Alzheimer's disease (Masliah et al., 1991, 1992; Vickers et al., 2000; Arendt, 2001). In this regard, it may not always be appropriate to promote post-injury axonal growth and synaptic re-establishment unless the precise and correct targets of newly growing axons can be defined and growing axons can effectively reach these targets.

Due to the inherent complexity and relative inaccessibility of the adult mammalian brain it is difficult to assess the reaction of damaged axons, particularly in the presence of confounding glial factors. *In vitro* experimental models are, therefore, important for assessing neuronal responses to trauma as well as providing a platform for assessing potential therapeutic agents (Dickson et al., 2000; Fayz and Tator, 2000; Chung et al., 2005) and, despite their simplified nature, are capable of reproducing important aspects of *in vivo* brain injury (Morrison et al., 1998). As discussed above, axonal injury is a frequent universal consequence of TBI. To assess the response of individual axons to injury, this chapter utilised an *in vitro* model of axotomy, in which axons of relatively mature neurons were directly transected (Dickson et al., 2000). This study explored the intrinsic regenerative potential of CNS axons and whether this response recapitulates developmental patterns of axonal growth. Agents that disrupt specific cytoskeletal components were utilised to determine whether similar cytoskeletal mechanisms underlie axonal growth and regeneration and determine if cytoskeletal disruption is a viable therapy for preventing maladaptive axonal sprouting responses.

5.2 Experimental procedures

5.2.1 Primary dissociated neocortical neuron culture preparation and *in vitro* axonal injury

Primary dissociated neocortical neuron cultures were prepared and axonal injuries were performed as described in sections 2.2.2 and 2.2.3, respectively. Neuronal cultures were fixed during development at 3-5 days *in vitro* (DIV) or at 4, 14 and 24 hours following axonal injury at 21 DIV.

5.2.2 Assay of *in vitro* cell proliferation

To determine the approximate birth dates of cortical neurons *in vitro*, cultures were incubated with 10μM of BrdU, for 72 hours at either 7 or 17 DIV. Cultures underwent a complete media change to remove any unincorporated BrdU and were grown to 21 DIV. Following fixation in 4% PFA/0.001M PBS, DNA denaturation was performed by incubating cultures in 2M HCl for 1 hour at 37°C. Dividing cells were visualised by immunolabelling with an antibody against BrdU (Sigma, Table 2.1).

5.2.3 Analysis of Cell Death

The nucleic acid-binding vital dye, propridium iodide (PI), was used to analyse cell death. PI is excluded from cells with intact cell membranes, however, is able to penetrate the cell membrane and label the nuclei of dead and dying (late apoptotic and necrotic) cells, where membrane integrity is compromised. A volume of 10μl of

PI (at 50μg/ml) was added to cultures and they were gently agitated to evenly distribute the PI and examined under a Leica DM IRB inverted microscope.

5.2.4 Administration of cytoskeletal disrupting agents

To determine the effects of cytoskeletal disruption during neurite development and post-injury events, cytoskeletal agents that disrupt the dynamic properties of the most motile cytoskeletal components, namely microtubules and microfilaments, were utilised. Specifically, taxol (Amersham Biosciences, San Francisco, CA, USA) was used to stabilise microtubules and promote microtubule formation and bundling, nocodazole (Sigma, St. Louis, MO, USA) was utilised to cause microtubule destabilisation and dissolution and latrunculin A (Molecular Probes, Eugene, Oregon, USA) was utilised as an actin depolymerising agent. Taxol was reconstituted in sterile 0.01M PBS and added to the subsequent media of cultures at a concentration of 10μg/ml. Nocodazole was reconstituted in sterile 12% DMSO/0.01M PBS and added to cultures at a final concentration of 1μg/ml. Latrunculin A was reconstituted in sterile 10% DMSO/0.01M PBS and added to cultures at a final concentration of 1μg/ml. Vehicle-treated cultures were processed concurrently to serve as controls.

To determine the effect of taxol-induced microtubule stabilisation, nocodazole-induced microtubule destabilisation and latrunculin A-induced actin depolymerisation during neurite development, cultures were incubated in the presence of each agent for 48 hours between three and five DIV. Additionally, to determine the effect of cytoskeletal disruption during injury-induced axonal

sprouting events, cytoskeletal disrupting agents were administered to the subsequent media of cultures immediately following localised axotomy at 21 DIV and cultures were re-incubated for 4, 14 or 24 hours post-injury. Studies to determine whether the effects of taxol were reversible were performed using 1μg/ml taxol (as higher concentrations caused substantial cell death with prolonged exposure). To investigate the permanency of taxol-induced microtubule stabilisation, taxol was washed out of developing cultures at 5 DIV and cultures were maintained as normal for up to 21 DIV and compared to vehicle-treated cultures as well as cultures which were constantly exposed to taxol. To determine whether the effects of taxol could be reversed following axonal injury, taxol was washed out of injured cultures after four hours and cultures were allowed to recover for a further 20 hours prior to fixation.

5.2.5 Live cell digital imaging

Time-lapse digital imaging was utilised to investigate the effect of taxol on growth cone motility and morphology, as well as the dynamic response of neurons to localised axotomy. 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 "imaging buffer" (mM: NaCl, 124; KCl, 5; CaCl2, 0.2; MgCl2, 1; dextrose, 30; HEPES, 25; pH 7.3, Zhang and Benson, 2001) over a time course of 1 hour prior to imaging, to promote neuronal survival when imaging over several hours. Using differential interference contrast ('Normaski') microscopy, a time series of images were captured from a Leica DM IRB inverted microscope, equipped with a heated stage (37°C).

5.2.6 Transmission and scanning electron microscopy

For scanning electron microscopy studies, cultures growing on glass coverslips were fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) for 20 minutes at room temperature, post-fixed with 0.1% osmium tetraoxide in phosphate buffer, at room temperature for 15 minutes and dehydrated through a graded ethanol series. Specimens were critical point dried with CO_2, mounted onto copper stubs and sputter coated with gold for observation with a JEOL JSM-840 scanning electron microscope. For transmission electron microscopy studies, cortical cultures were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer, at 37°C for 10 minutes. Cultures were post-fixed in 1.5% osmium tetroxide in 0.1M phosphate buffer at room temperature for 30 minutes, followed by incubation in 2% uranyl acetate in 70% ethanol for 5 minutes. Cultures were then dehydrated through a graded ethanol series and embedded in 100% epon. Specimens were sectioned at 70nm, stained with 5% uranyl acetate (10 minutes) and Reynolds lead citrate (10 minutes) and examined on a Philips EM-410 transmission electron microscope.

5.2.7 Immunocytoochemistry and staining of cultured neocortical cells

Following fixation in 4% PFA/0.01M PBS, indirect immunofluorescence labelling was utilised to visualise the distribution of specific components (see table 5.1), including βIII-tubulin, tau and phosphorylated neurofilaments, in developing neurites and injured sprouting axons, as described in section 2.2.3. Additionally, to label for filamentous actin, cultures were incubated with AlexaFluor 488 phalloidin (Molecular Probes, Eugene, OR, USA, dilution 1:200), for 30 minutes following immunohistochemistry. Cultures were also labelled for a range of markers indicative

of neuronal maturity (see Table 5.1). Specific antibody details are provided in Table 2.1.

Table 5.1. Antibodies used to identify different neuronal compartments

Cellular compartment	Marker
Neurons	NSE
Neurites	NF-M
	βIII-tubulin
Axons	SMI312
	Tau
Dendrites	MAP-2
Synapses	Synaptophysin
Post-mitotic neuronal nuclei	NeuN
Proliferating cells	BrdU

5.2.8 Quantitative and statistical analysis

The effect of taxol on neurite development was quantitatively analysed by determining the proportion of neurites tipped by a growth cone. A growth cone was defined as a fan-shaped structure at the tip of a neurite, with a width three times greater than its neurite of origin. The effect of taxol treatment on neurite length was also determined. Digital images of immunofluorescence labelled specimens were captured pre-treatment and post-vehicle or post-taxol treatment, and the lengths of 100 neurites from each category were measured.

The effect of taxol on post-injury axonal sprouting was determined in terms of sprout number and sprout length, four hours after injury. Cultures injured by axonal

transection were incubated in taxol or vehicle for four hours immediately following injury, followed by PFA fixation and immunofluorescence labelling. Digital images of several individual injury sites were captured. To quantitate the effect of taxol on sprout elongation, the length of 100 post-injury axonal sprouts were measured from vehicle- and taxol-treated fellow cultures. Additionally, to determine the effect of taxol on sprout elaboration, the number of post-injury axonal sprouts was calculated from 15 individual transection injury sites from vehicle- and taxol-treated fellow cultures. This value was expressed as the number of sprouts per 100μm of cut site length.

For quantitation, taxol-related experiments were repeated five times (i.e. on cultures derived form five individual litters) and studies were performed by an investigator blind to the treatment of the cultures under investigation. NIH Image (version 1.61) software was used to capture digital images and perform measurements. Statistical analysis was performed using StatView (version 5.0) software. As appropriate, paired t-tests or ANOVA were performed on quantitative data. *post-hoc* comparisons (Fisher's test) were performed following ANOVA.

Preliminary investigations to determine the effects of nocodazole-induced microtubule destabilisation and latrunculin A-induced actin depolymerisation during neurite development and regeneration were qualitatively assessed.

5.3 Results

5.3.1 *In vitro* cortical neuron development

In the days following plating, dissociated cortical neurons formed aggregates, adhered to the coverslip substrate and elaborated several slender neurites. Analysis of the morphological and cytoskeletal characteristics of developing cortical neurons was performed at three DIV. Figure 5.1 illustrates the localisation of specific cytoskeletal components in developing neurites at three DIV. Both βIII-tubulin and tau were localised throughout entire neurons, extending into the tapered extremities of dendrites and splayed extremities of axons, as well as the growth cone remnants (Szbenyi et al., 1998; Kalil et al., 2000), spikes and small branches located along the lengths of some neurites (Figure 5.1A and B). Both βIII-tubulin and tau were more abundant in neurite shafts and the central growth cone domain than in the proximal domain of growth cones. Filamentous actin was distributed unevenly throughout developmental neurites, but was particularly enriched within growth cones and extended into filopodia and lamellipodia (Figure 5.1C). Phosphorylated neurofilaments were, however, only localised in a sub-set of neurites and labelling was absent in growth cones (Figure 5.1D).

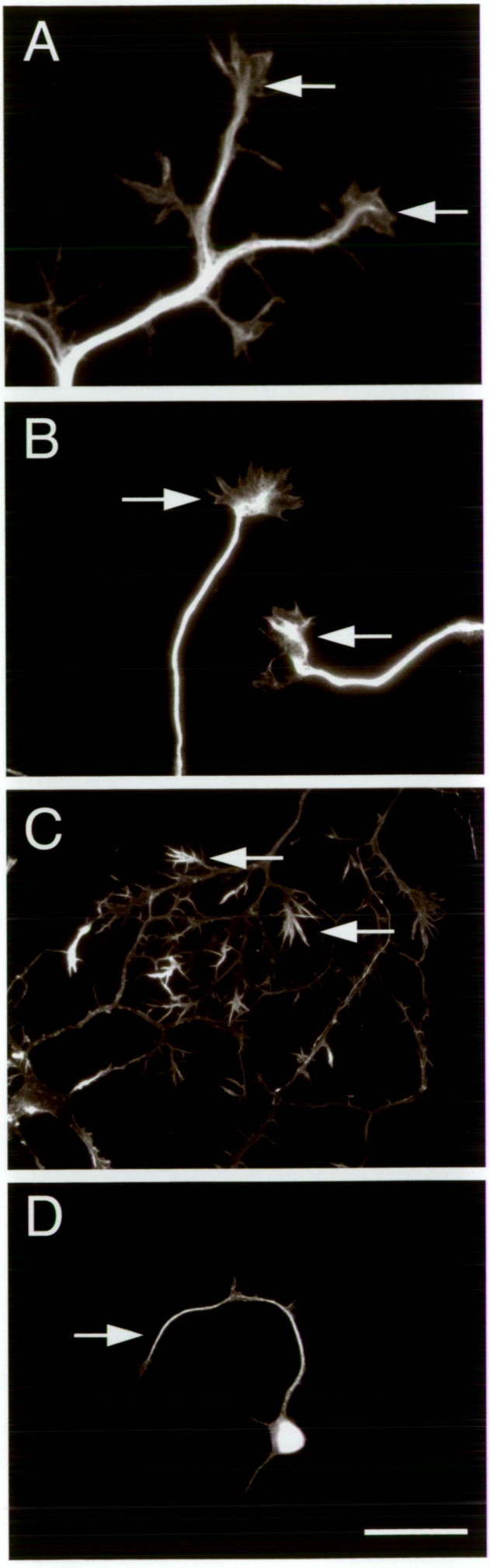
5.3.2 Cytoskeletal disruption during neurite development

The effects of cytoskeletal disruption were examined by exposing developing cultures to the microtubule stabilising and destabilising agents, taxol and nocodazole, respectively as well as the actin depolymerising agent, latrunculin A. To determine the effect of microtubule stabilisation on neuronal development, developing cultures

Figure 5.1 Cytoskeletal components demonstrated specific distribution in developing neurites

Developing neurites were characterised by a slender neurite shaft tipped by an expanded fan-shaped structure, the growth cone. Components of the microtubule cytoskeleton, such as βIII-tubulin (A) and tau (B), were distributed throughout neurite shafts, extending into the proximal and sometimes distal regions of developmental growth cones (arrows denote examples). Filamentous actin (as visualised by phalloidin labelling) was predominantly localised to typically motile structures such as developmental growth cones and the spikes and small branches extending from neurite shafts (C, arrows denote examples). Phosphorylated neurofilaments were generally restricted to neurite shafts and rarely extended into growth cones or small neurite branches (D, arrow denotes examples). All images were taken at 3DIV.

Scale bar: A and B = 10μm; C and D = 20μm


A
B
C
D

were exposed to taxol and examined for morphological and cytoskeletal alterations. Initially, neurites were tipped by characteristically splayed growth cones, however, in the presence of taxol the neurites rapidly formed bulbous end-structures, many of which lacked the filopodial protrusions characteristic of growth cones (Figure 5.2).

Developing cultures, incubated in taxol between three and five DIV demonstrated substantially altered morphology compared to vehicle treated controls (Figure 5.3). Vehicle-treated neuronal aggregates elaborated numerous slender neurites, which extended radially and often in defined bundles. These neurite bundles splayed out distally into individual neurites, many of which were tipped by a growth cone (Figure 5.3A). In contrast, the neurites of taxol-treated neurons remained localised around their neuronal aggregates, often curling and looping back towards the aggregates from which they originated (Figure 5.3B). Scanning electron microscopy demonstrated that compared to vehicle-treated neurites, which demonstrated elaborate growth cones at their distal tips (Figure 5.4A), taxol-treated neurites were thickened, often swollen into bulbous structures at their distal tips and lacked elaborate morphological structure. Furthermore, some neurites retained aberrant growth cones with filopodial-like protrusions distally, while others completely lacked protuberances at their distal tips (Figure 5.4B and C respectively).

Neuron specific βIII-tubulin was distributed throughout neurite shafts, but was also localised to club- and loop-like structures in the distal tips of taxol-treated neurites (Figure 5.5A), whereas tau (Figure 5.5B) was evenly distributed throughout the distended neurites. Filamentous actin was most abundant in neurite tips, where it

Figure 5.2 Disruption of microtubule dynamics with taxol resulted in growth cone collapse and inhibition of neurite growth

Live cell imaging, utilising differential interference contrast (Normaski) optics, demonstrated that taxol-induced microtubule stabilisation lead to the rapid formation of bulbous structures at neurite tips and cessation of neurite growth (arrows denote examples).

Scale bar: 20μm

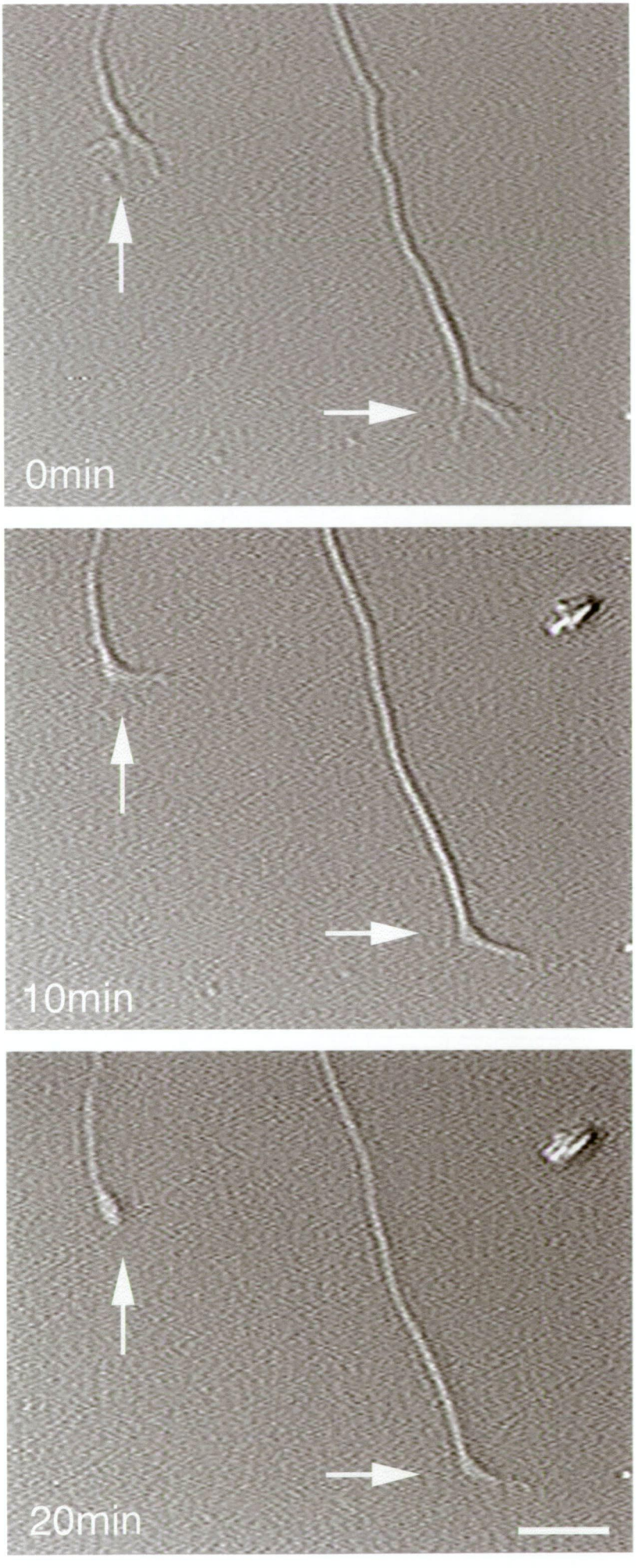

0min
10min
20min

Figure 5.3 Disruption of microtubule dynamics with taxol caused neurite distension and altered neurite path-finding

By 5 DIV, developing cortical neurons had aggregated and elaborated numerous radially extending fasiculated neurite bundles (A, arrow denotes example). In contrast, taxol-induced microtubule disruption between 3 and 5 DIV (B) resulted in the formation of swollen neurites (arrowhead), which predominantly curled and looped back upon themselves (arrow).

Scale bar: 60μm

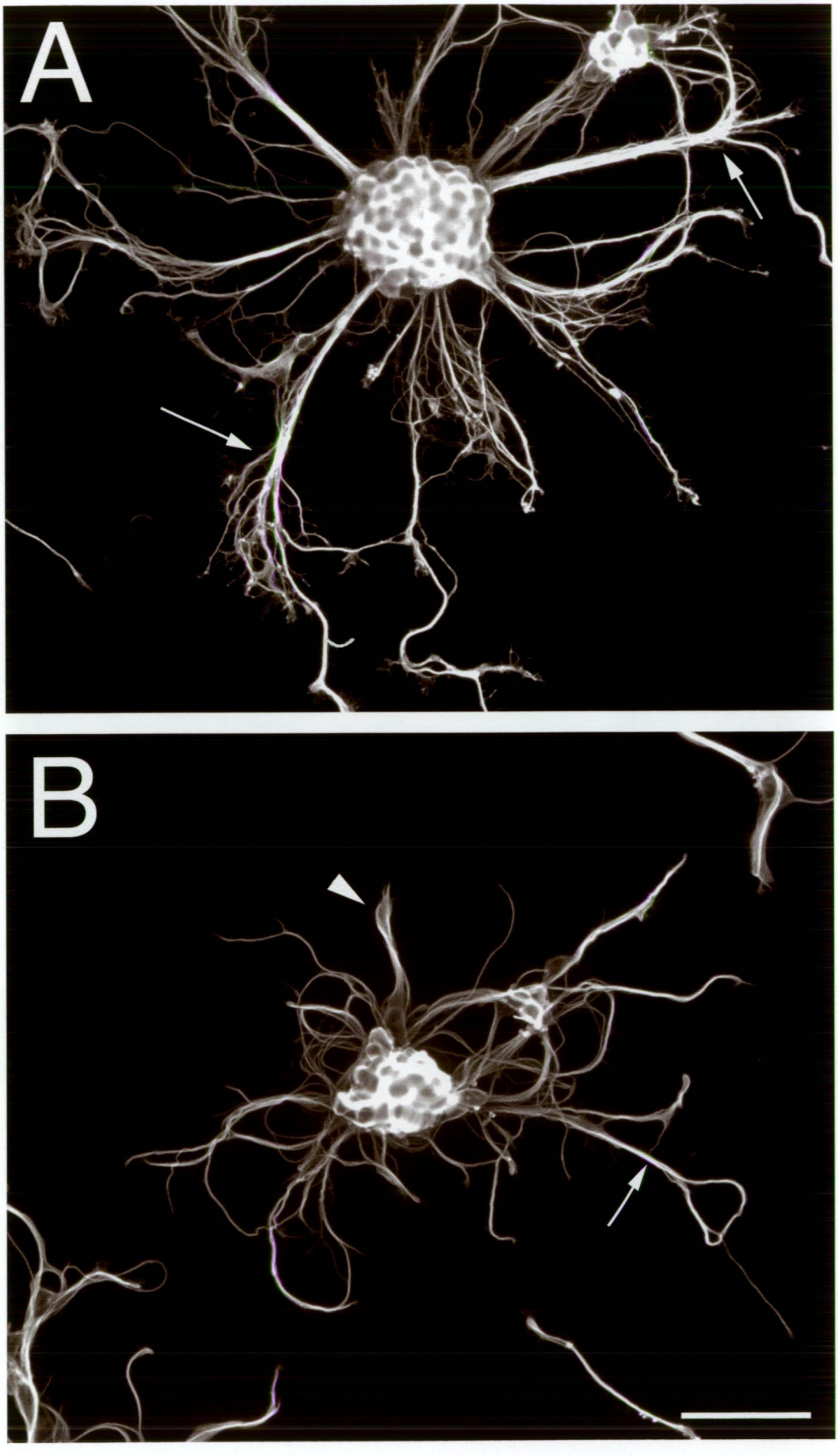
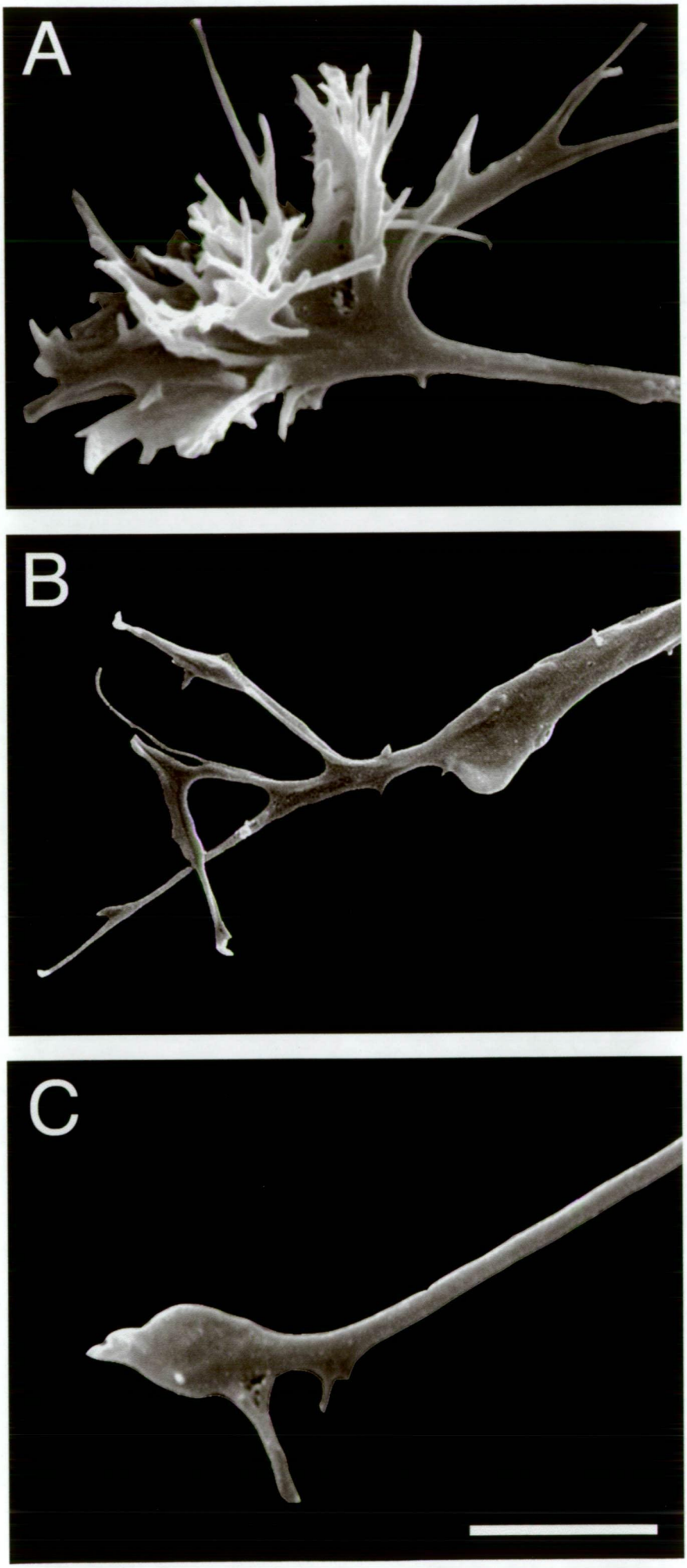

A
B

Figure 5.4 Microtubule disruption induced marked morphological alterations in the growth cones of developing neurites

Scanning electron microscopy was utilised to demonstrate alterations surface morphology in response to taxol-induced microtubule disruption relative to vehicle-treated controls. Vehicle-treated developing neuronal cultures exhibited growth cones with distinct 3-dimensional morphological complexity (A). Taxol-induced microtubule disruption, however, resulted in a loss of morphological complexity, defined by growth cone collapse and near (B) or complete (C) loss of filopodia and lamellipodia.

Scale bar: 2μm


A
B
C

often formed ball-like accumulations and defined filopodia could no longer be distinguished (Figure 5.5C). Additionally phosphorylated neurofilaments, although uniformly distributed in neurite shafts, were often accumulated into bulb- and ring-like structures at neurite tips (Figure 5.5D). Filamentous actin, βIII-tubulin and tau were distributed throughout the filopodial-like structures emanating from the swollen tips of some taxol-treated neurites; presumably these structures correspond to growth cones, in which normal morphology has been disrupted by microtubule stabilisation.

Quantitative analysis demonstrated that taxol significantly ($p < 0.05$) reduced the proportion of neurites tipped by a growth cone from 53.2% ± 7.8 (SE) in vehicle-treated controls, to 18.2% ±3.7 (SE) following taxol treatment (Figure 5.6A). Additionally, taxol inhibited neurite elongation between three and five DIV (Figure 5.6B). At three DIV, mean neurite length for untreated cultures was 64.0μm ± 3.9 (SE). By five DIV, vehicle-treated neurites had elongated significantly ($p < 0.05$) to 90.9μm ±2.1 (SE), however, taxol-treated neurites did not extend significantly ($p > 0.05$) from pre-treatment and were significantly shorter ($p < 0.05$) than vehicle-treated controls, with a mean neurite length of 59.8μm ± 1.4 (SE).

Taxol washout demonstrated that the effects of taxol were long lasting, with stunted neuronal morphology persistent at 21 DIV. However, the effects of taxol were partially reversible as increased neurite growth was observed in cultures from which taxol was removed, as compared to cultures grown in the continued presence of taxol (cell death was high in the latter cultures). Moreover, some of the neurites from which taxol was removed regained their growth cones (Figure 5.7).

Figure 5.5 Cytoskeletal components were abnormally distributed within taxol-treated neurites

Although βIII-tubulin was distributed relatively uniformly throughout the distended neurite shafts and bulbous neurite tips of taxol-treated cultures (A, arrow), it was also localised to abnormal loop-like structures in some neurite tips (A, arrowheads). Tau was also distributed uniformly throughout distended taxol-treated neurite shafts, but also occasionally extended into their filopodial extremities (B, arrow). Unlike the elaborate and extensive labelling of developmental growth cones for filamentous actin, phalloidin labelling of taxol-treated growth cones demonstrated bulb-like actin accumulations within neurite tips (C, arrows). As observed in vehicle-treated cultures, phosphorylated neurofilaments were localised to neurite shafts, but also extended into the distended end structures of taxol-treated neurites (D, arrow) and were localised to abnormal bulb- and loop-like structures within neurite tips (D, arrowheads).

Scale bar: 20μm

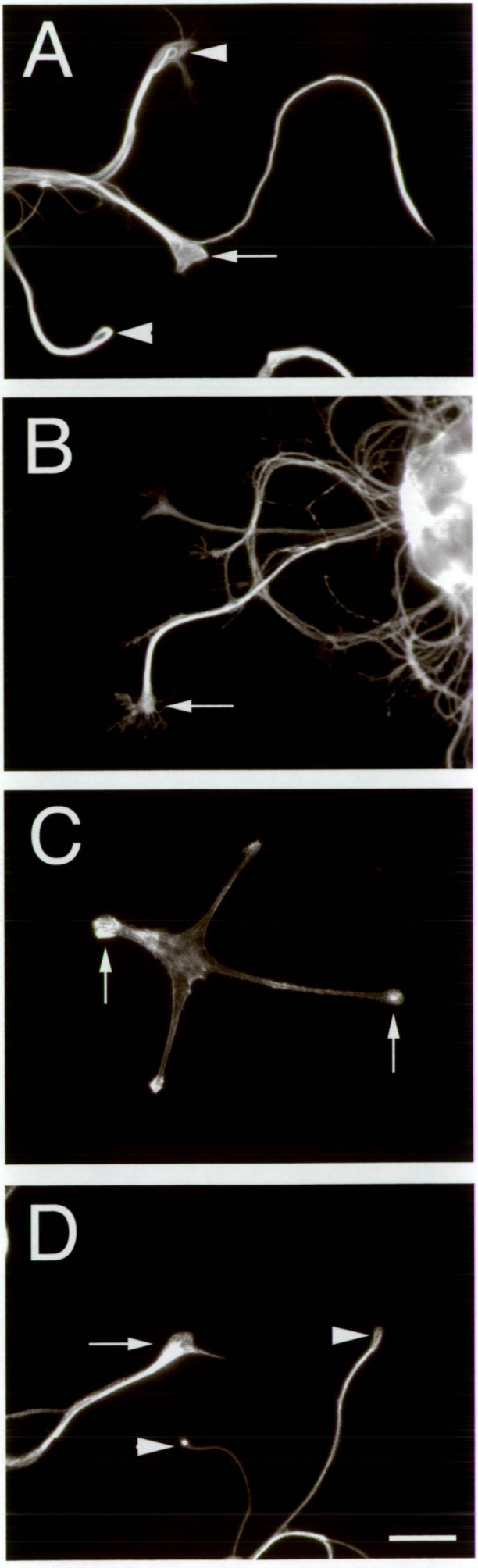
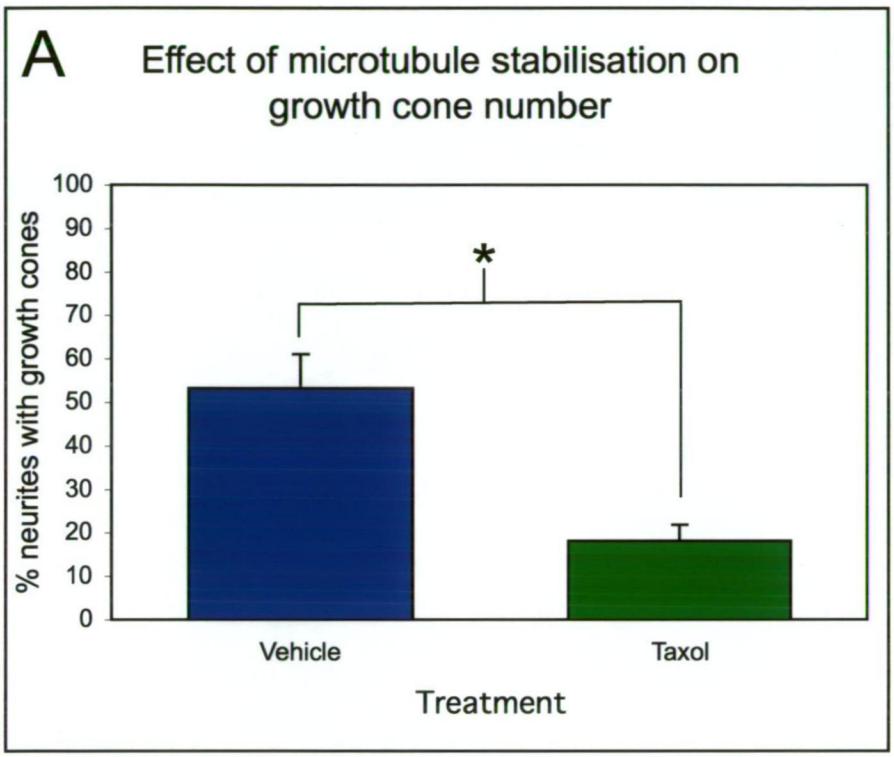

A
B
C
D

Figure 5.6 Quantitative analysis demonstrated that taxol-induced microtubule stabilisation resulted in a statistically significant reduction in the number of neurites tipped by growth cones and inhibition of neurite growth

Quantitative analysis demonstrated that taxol-treatment between 3 and 5 DIV resulted in a substantial reduction in growth cone number relative to vehicle-treated controls (A). Likewise, quantitation of neurite length at 3 DIV and following 48 hours of either vehicle- or taxol-treatment demonstrated vehicle-treated cultures underwent statistically significant neurite growth between 3 and 5 DIV, relative to baseline measurement, whereas taxol-treatment significantly inhibited neurite growth (B).

* $p < 0.05$. Error bars are standard error of the mean


A
Effect of microtubule stabilisation on growth cone number
% neurites with growth cones
100
90
80
70
60
50
40
30
20
10
0
*
Vehicle
Taxol
Treatment

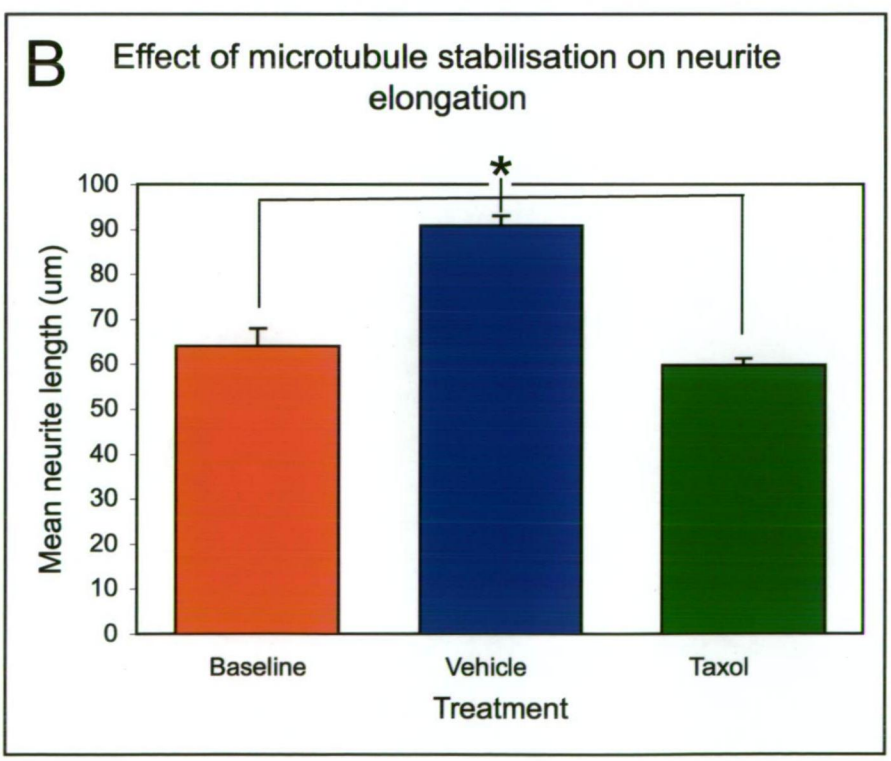
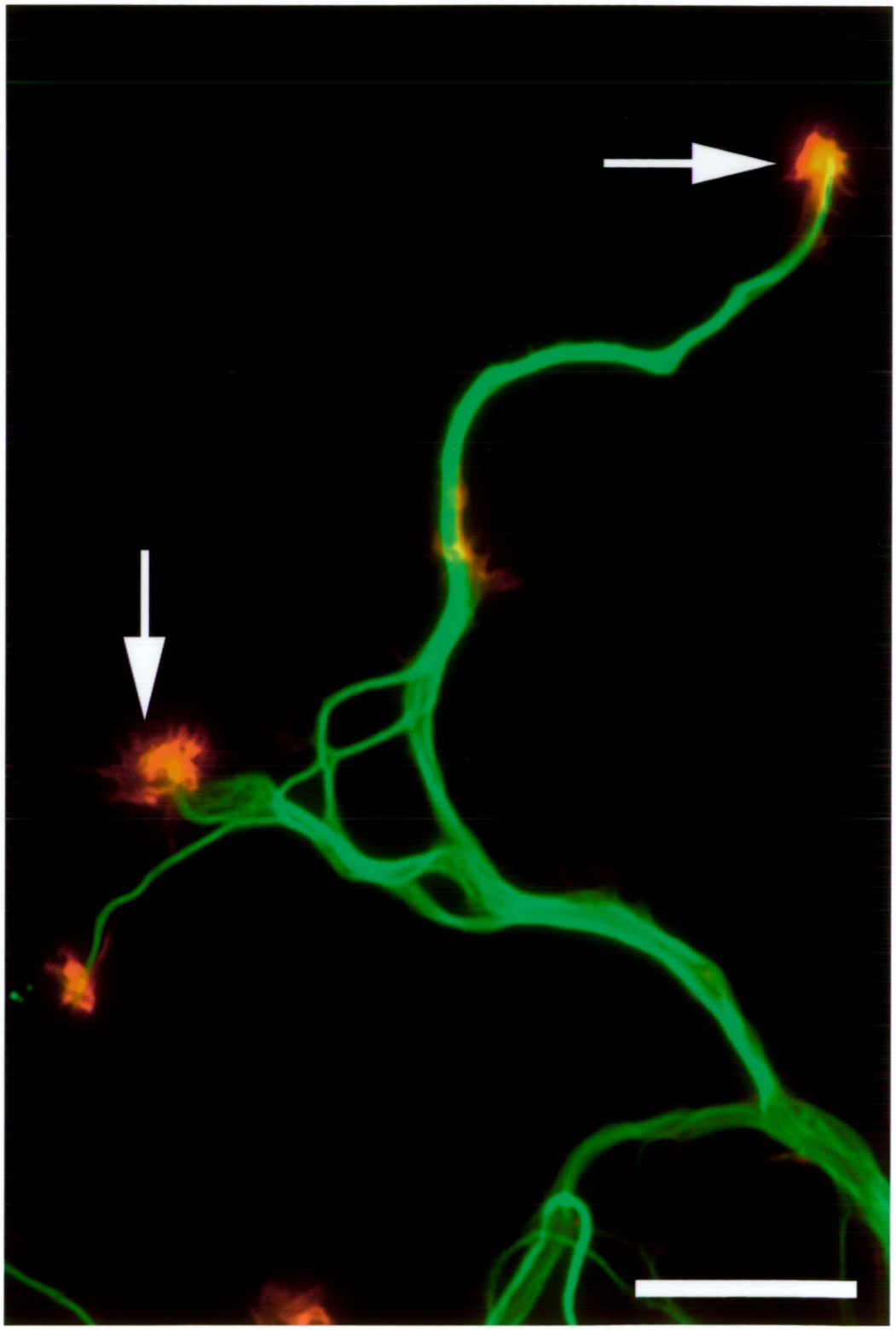

B
Effect of microtubule stabilisation on neurite elongation
Mean neurite length (um)
100
90
80
70
60
50
40
30
20
10
0
*
Baseline
Vehicle
Taxol
Treatment

Figure 5.7 The effects of taxol-induced microtubule disruption were partially reversible during neurite development

Following taxol-treatment between 3 and 5 DIV, taxol was removed from the media and cultures re-incubated for several days prior to double immunofluorescence labelling for βIII-tubulin (green) relative to filamentous actin (red, phalloidin labelling). Although the effects of taxol were relatively long lasting, indicated by prolonged neurite distension, expanded growth cone-like structures were restored at the tips of several neurites (arrows denote examples).

Scale bar: 20μm



Preliminary investigations were carried out with nocodazole (Figure 5.8) and latrunculin A (Figure 5.9) to determine the effects of microtubule destabilisation and actin depolymerisation during neurite development. Both nocodazole and latrunculin A exposure for 48 hours between three and five DIV resulted in profound morphological alterations and aberrant cytoskeletal arrangement compared to control cultures. Nocodazole-induced microtubule destabilisation resulted in stunted neurite growth. Additionally, these cultures exhibited neuronal cell bodies and neurites that were often bordered by sections of lamellipodial-like membrane, which were not observed in vehicle-treated cultures by this stage of development, while many growth cones lacked obvious lamellipodia. The presence of abnormally abundant filopodial-like structures was confirmed with labelling for βIII-tubulin, tau, filamentous actin and phosphorylated neurofilaments (Figure 5.8A, B, C and D respectively). Alternatively, latrunculin A-induced actin depolymerisation resulted in a marked loss of growth cone-like structures at neurite tips. Furthermore, other typically actin-rich structures such as the spikes and small branches emanating from neurite shafts were also absent from latrunculin A treated cultures. Again these finding were confirmed by labelling for βIII-tubulin, tau, filamentous actin and phosphorylated neurofilaments (Figure 5.9A, B, C and D respectively).

5.3.3 Characterisation of neuronal maturity by 21 DIV

A main aim of these investigations was to determine the role of the neuronal cytoskeleton in the reactive and regenerative events characterising the response of mature neocortical neurons to injury. Thus, the degree of neuronal maturity of neurons grown *in vitro* for several weeks was determined. By 21 DIV, cultures were

Figure 5.8 Nocodazole-induced microtubule disruption resulted in an abundance of filopodia and lack of lamellipodia on developmental growth cones

Nocodazole treatment of developing neuronal cultures between 3 and 5 DIV, followed by labelling for βIII-tubulin (A), tau (B), filamentous actin (C, phalloidin) and phosphorylated neurofilaments (D, SMI312), resulted in a loss of lamellipodia from developmental growth cones, revealing a distinctly filopodial morphology (arrows in A-C denote examples). Moreover, spikes and filopodial-like structures were abundant along neurite shafts (arrowheads in A-C denote examples). Similar to vehicle-treatment, phosphorylated neurofilaments were restricted to neurite shafts (arrows in D).

Scale bar: A = 5μm; B = 20μm; C and D = 10μm

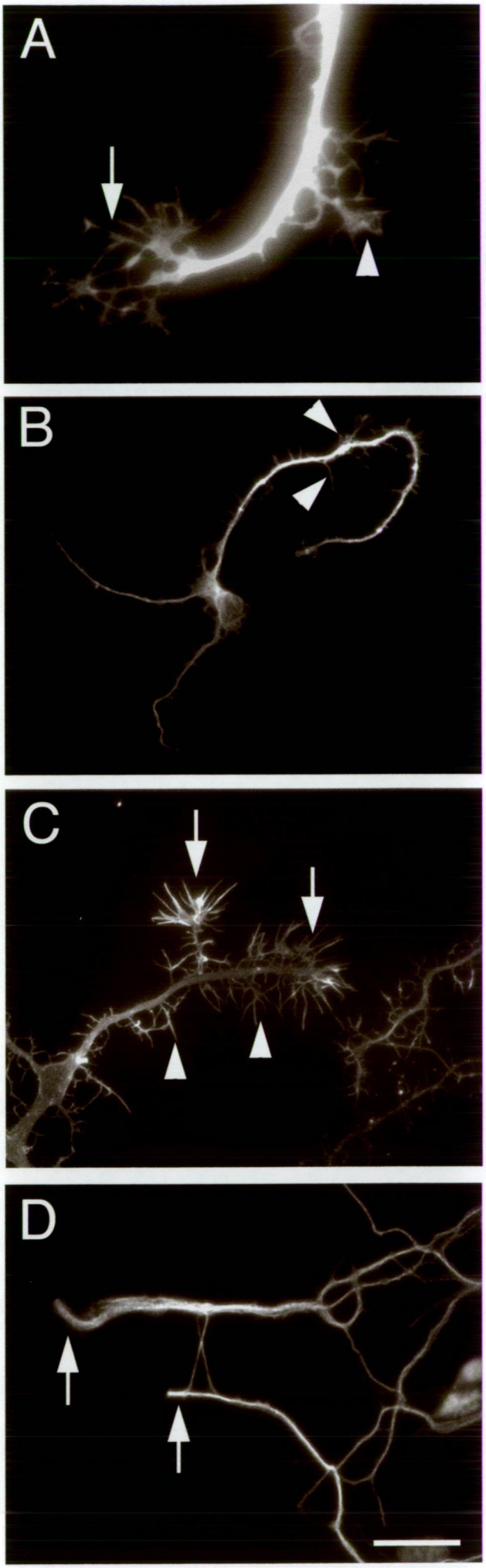
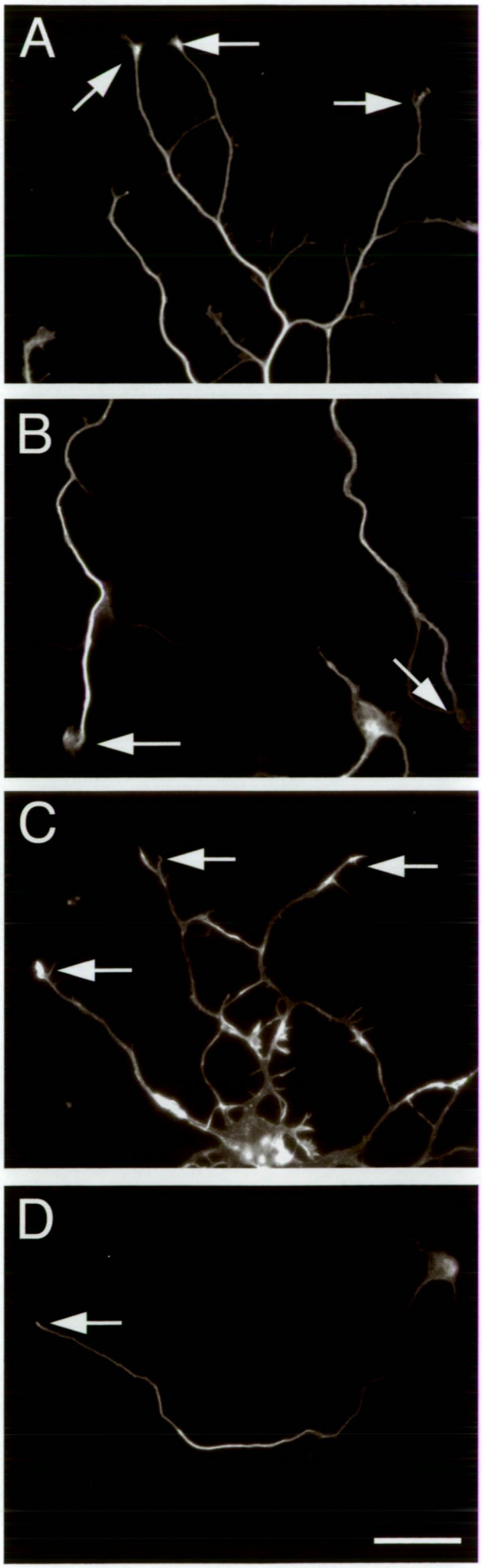

A
B
C
D

Figure 5.9 Latrunculin A-induced actin depolymerisation resulted in a loss of growth cones and other typically motile structures from developing neurites

Cultures treated with latrunculin A between 3 and 5 DIV had vastly reduced growth cone numbers and lacked spikes and small branches along neurite shafts, as indicated by labelling for βIII-tubulin (A), tau (B), filamentous actin (C) and phosphorylated neurofilaments (D) (arrows denote examples).

Scale bar: 20μm


A
B
C
D

characterised by large, multi-cellular aggregates (Figure 5.10A), from which elaborate networks and bundles of neuronal processes extended, forming interconnections with other aggregates (Figure 5.10B). BrdU was administered to cultures at seven (Figure 5.11A) or 17 DIV (Figure 5.11B) to determine whether any neurons were actively proliferating. The nuclei of all neurons lacked immunoreactivity for BrdU and neuronal nuclei were positive for NeuN, a marker of post-mitotic neurons (Figure 5.11C).

By 21 DIV, neurons were immunoreactive for a range of markers indicative of neuronal maturity. Specifically, neuronal populations or cellular compartments were found to be immunoreactive for neurofilament triplet proteins (Figure 5.12A, B), MAP-2 (Figure 5.12C) and tau (Figure 5.12D). Extensive tau and phosphorylated neurofilament labelling demonstrated the abundance of axons within the neurite bundles interconnecting neuronal aggregates (MAP-2 labelled dendrites were rarely localised within these neurite bundles). Moreover, both pyramidal neuron and non-pyramidal/interneuron populations were present within cultures, as indicated by positive labelling for markers of these different neuronal types, such as neurofilament and calretinin, respectively. Calretinin labelling demonstrated the presence of a subpopulation of cortical interneurons (Figure 5.12E). The extensive presence of synaptic contacts was demonstrated by labelling for the pre-synaptic marker, synaptophysin (Figure 5.12F). Transmission electron microscopy (Figure 5.13) confirmed the presence of synapses (Figure 5.13A) and demonstrated that axons within axonal bundles were not myelinated (Figure 5.13B). Collectively, morphological and immunohistochemical studies demonstrated that embryonically-

Figure 5.10 By 21 DIV neocortical neuron cultures were characterised by the formation of multi-cellular aggregates interconnected by radially extending bundles and networks of neuronal processes

Scanning electron microscope images demonstrating the morphological makeup of neocortical cultures by 21DIV. On initial plating, cells were rounded and lacked processes. Over the course of 21 days in culture, cells aggregated to form large multi-cellular clusters (A, arrows), which elaborated numerous neurites neurite bundles (B, arrows).

Scale bar: A and B = 20μm

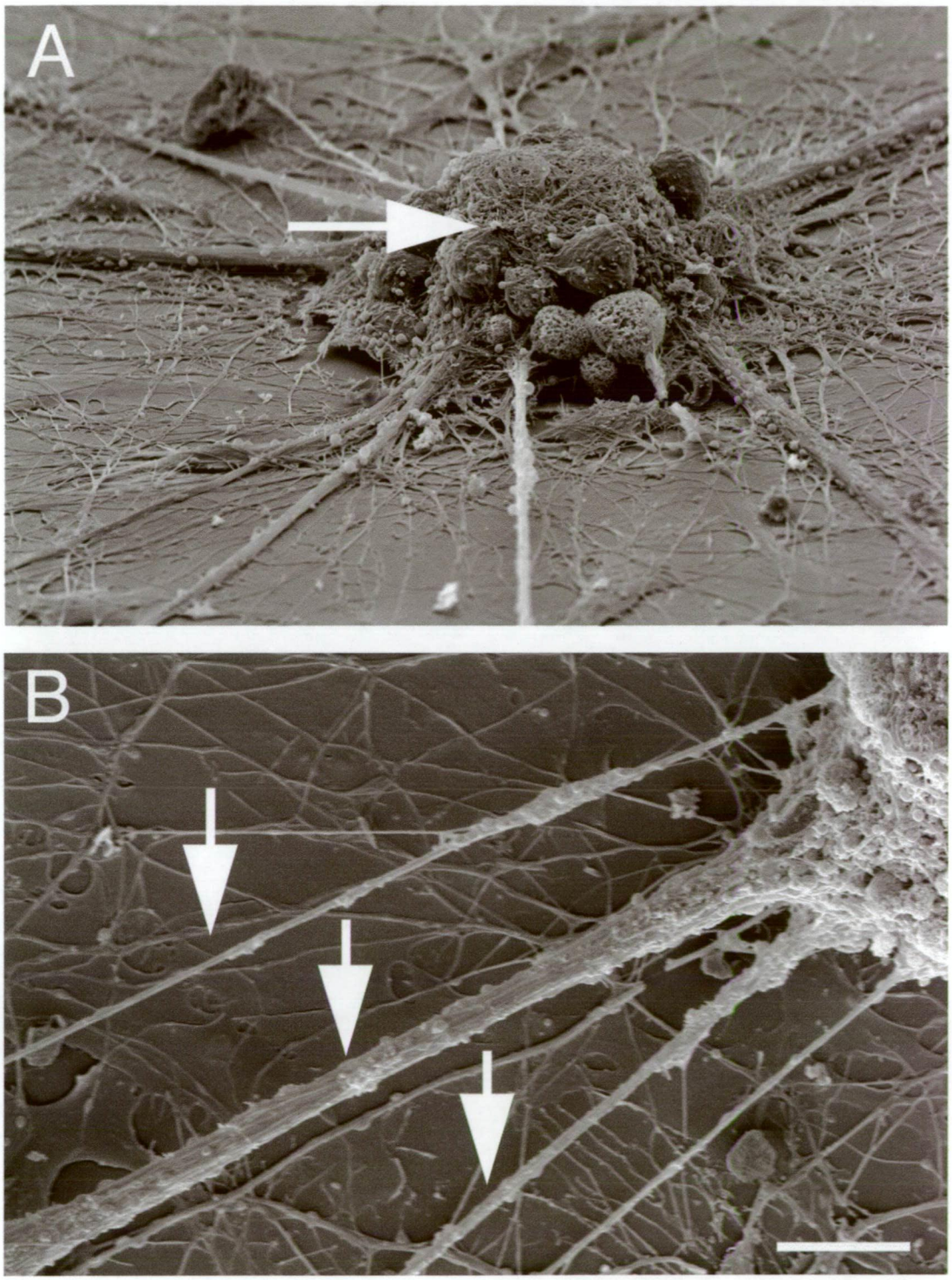

A
B

Figure 5.11 Dissociated neurons grown in culture for 21 days were no longer mitotically active

Neocortical neuronal cultures were incubated in the presence of BrdU for 3 days at either 7 (A) or 17 (B) DIV. Cultures were grown to 21 DIV and double labelled with neuron specific enolase (NSE, red). Double labelling demonstrated that neurons had not incorporated BrdU (green) and were therefore not mitotically active. Arrows in A and B denote examples of neurons labelled for NSE, arrowheads denote examples of BrdU-labelled nuclei, at no stage were these markers localised to the same cells. The post mitotic state of cultured neocortical neurons was confirmed by positive labelling for the post-mitotic neuronal nuclei marker, NeuN (green), relative to tau (red) and numerous NeuN-labelled nuclei were located throughout neuronal aggregates by 21 DIV (C, arrows denote examples).

Scale bar: A, B and C = 30μm

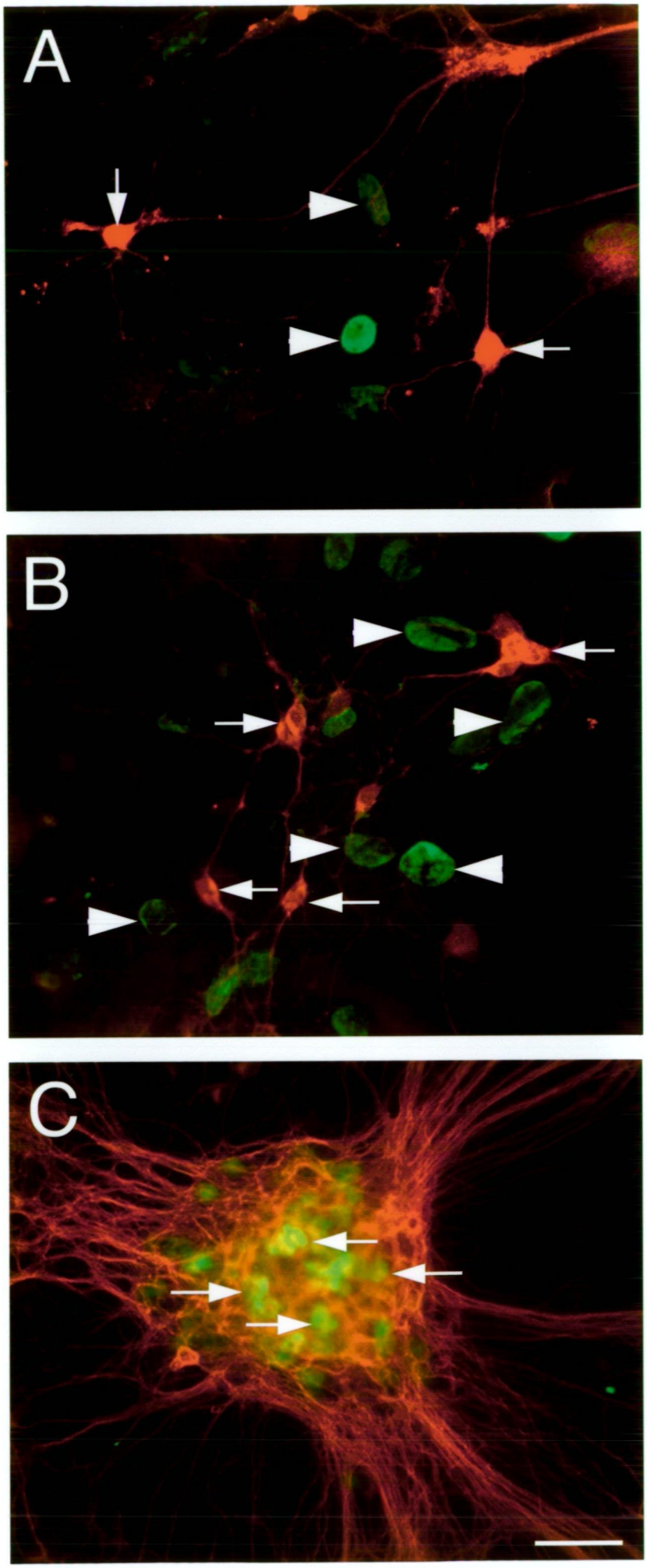

A
B
C

Figure 5.12 Dissociated neurons grown in culture for 21 days demonstrated labelling for a variety of markers indicative of mature neuronal phenotypes

By 21 DIV, cultured cortical cultures contained both pyramidal and non-pyramidal neurons and neurons were positive for a range of maturity-related markers including phosphorylated neurofilaments (as indicated by SMI312 labelling, A), NF-M (B), MAP2 (C), tau (D) and calretinin (E). Neurons had also made numerous synaptic contacts as indicated by extensive labelling for synaptophysin (F).

Scale bar: 40μm

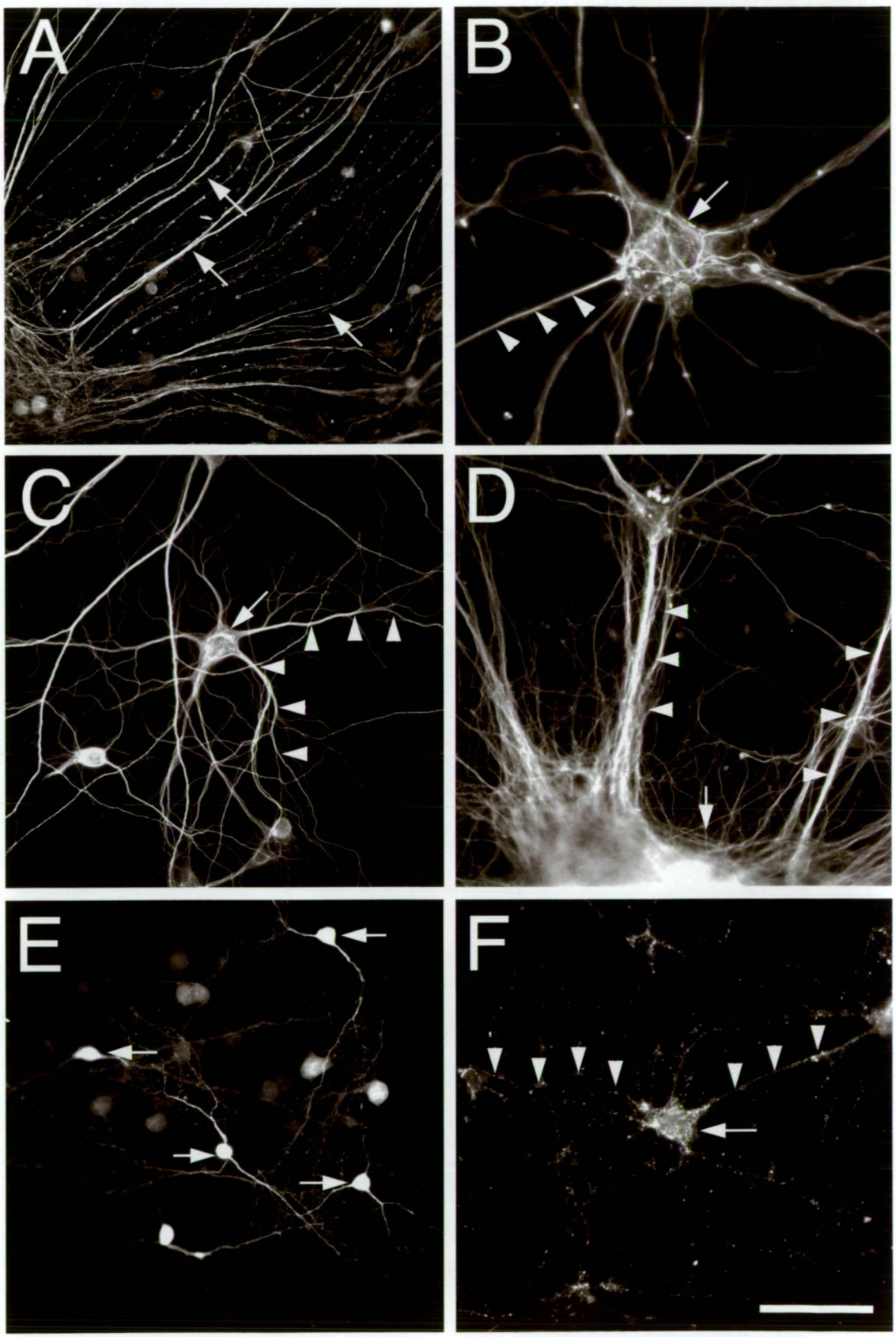
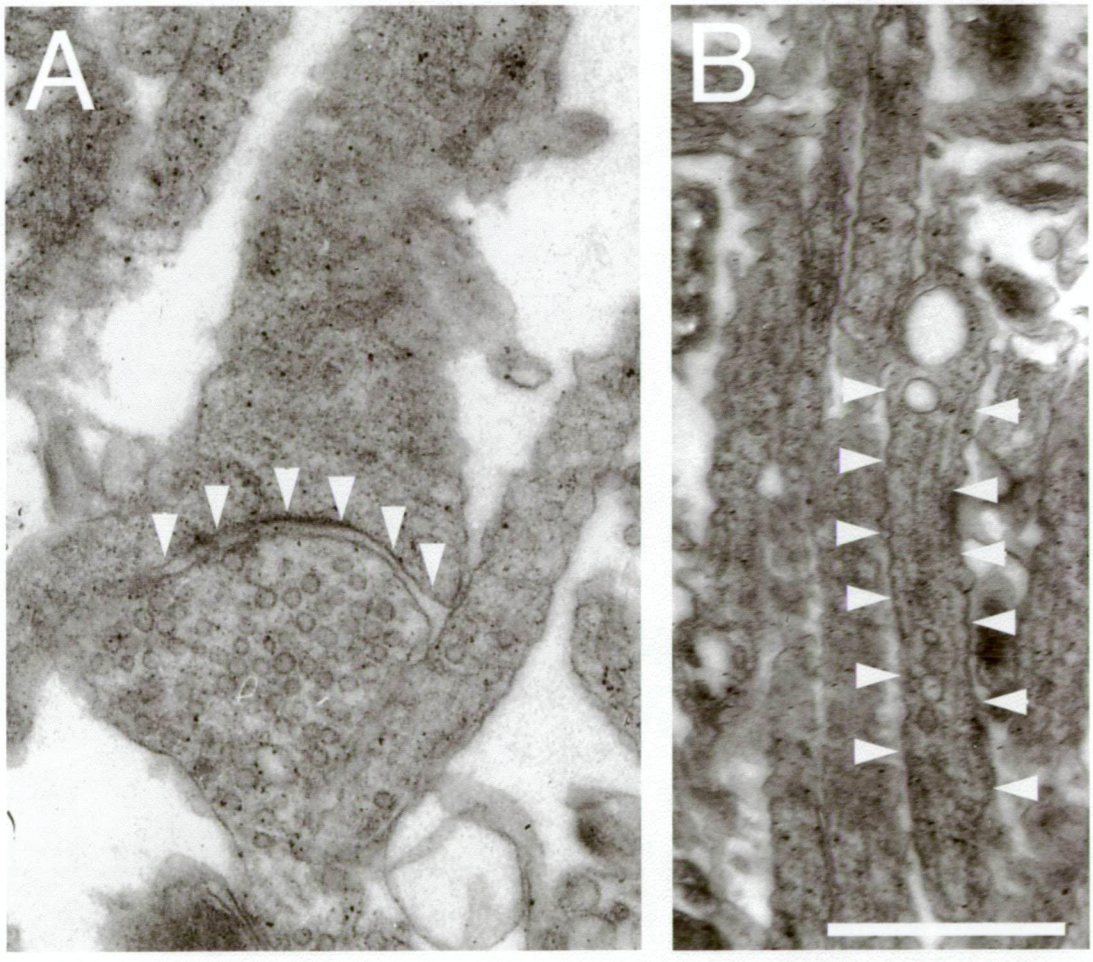

A
B
C
D
E
F

Figure 5.13 Dissociated neurons grown in culture for 21 days made synaptic contacts and were not myelinated

Transmission electron microscopy confirmed the presence of synaptic contacts within neocortical cultures (A, arrowheads denote an example of a synapse) and demonstrated the absence of axonal myelination by oligodendrocytes (B, arrowheads denote an example of an unmyelinated axon).

Scale bar: A and B = 2μm


A
B

derived neocortical neuron cultures, grown for 21 days, developed neurochemically defined sub-populations of neurons, which established polarity, were post-mitotic, formed synapses and possessed a range of markers indicative of neuronal maturity. Furthermore these cells were growing in relative isolation, free of their normal myelin sheaths and supporting astrocytic network. Collectively, these findings indicated that culturing embryonic neocortical cells for 21 days is adequate to achieve a mature neuronal phenotype.

5.3.4 Characterisation of the response of mature cortical neurons to localised axonal transection

Cultured cortical neurons were injured by localised neurite transection at 21 DIV, when they had reached relative maturity (as discussed in Section 5.3.3). Time-lapse digital imaging demonstrated that neurons respond to transection with a specific, highly dynamic sequence of changes (Figure 5.14). Immediately following injury, neuronal processes began to retract away from the site of injury (Figure 5.14A). This retraction response persisted for up to 4-6 hours after injury. Within 2-6 hours following injury, an extensive axonal sprouting response was initiated, manifested by the elaboration of fine protrusions into the injury site from the lesion boarder (Figure 5.14B). Live cell imaging demonstrated that these sprouting processes were extremely dynamic and grew both directly across the lesion site as well as back towards the transected axonal bundle from which they originated. Post-injury axonal sprouts were generally characterised by slender sprout shafts tipped by expanded growth cone- or club-like end structures (Figure 5.15), although some sprouts tapered distally.

Figure 5.14 Axonal transection injuries induced a dynamic response including neurite retraction and sprouting

Axonal injury at 21 DIV evoked a rapid and highly dynamic sequence of alterations within the proximal segment of severed neurites, characterised by an early phase of retraction away from the injury border (A), followed by the elaboration of fine sprout-like protuberances into the lesion site within several hours following injury (B).

Scale bar: 40μm

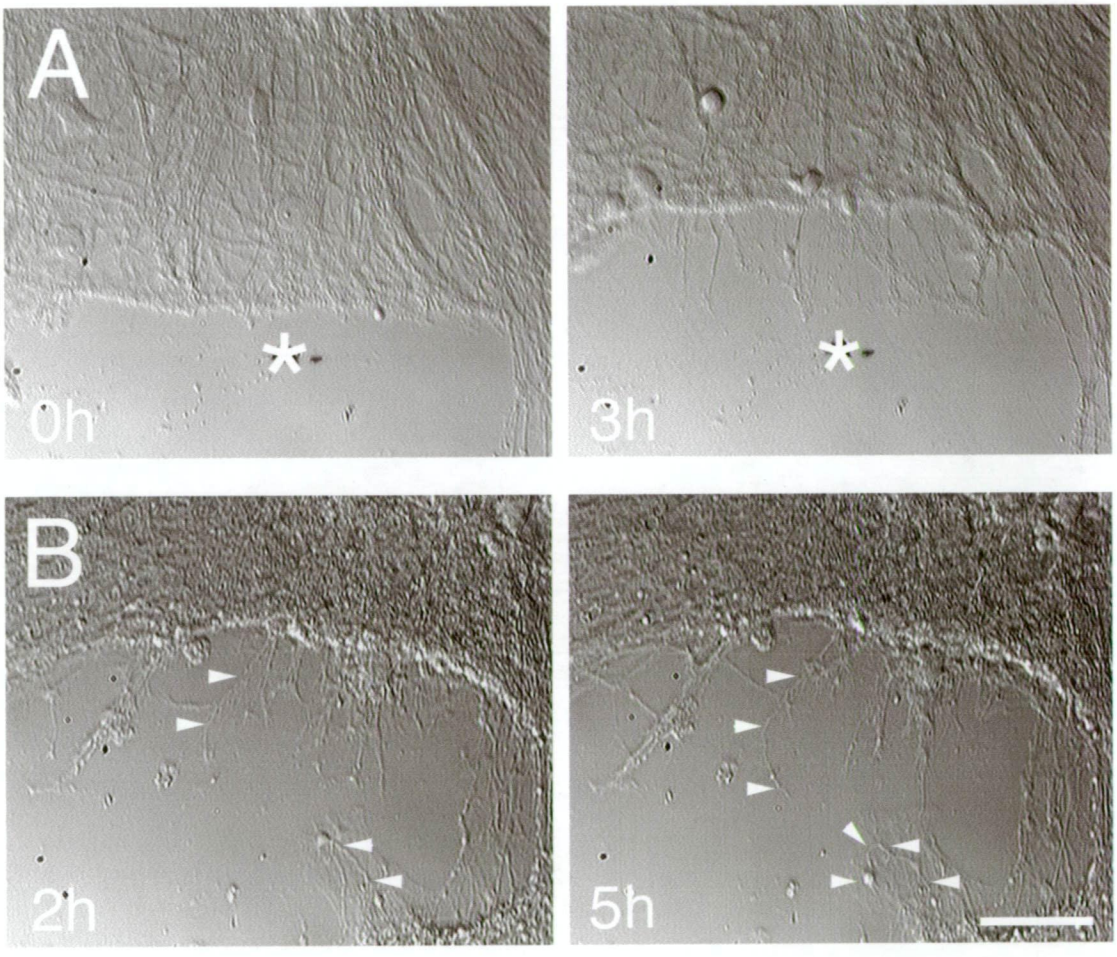
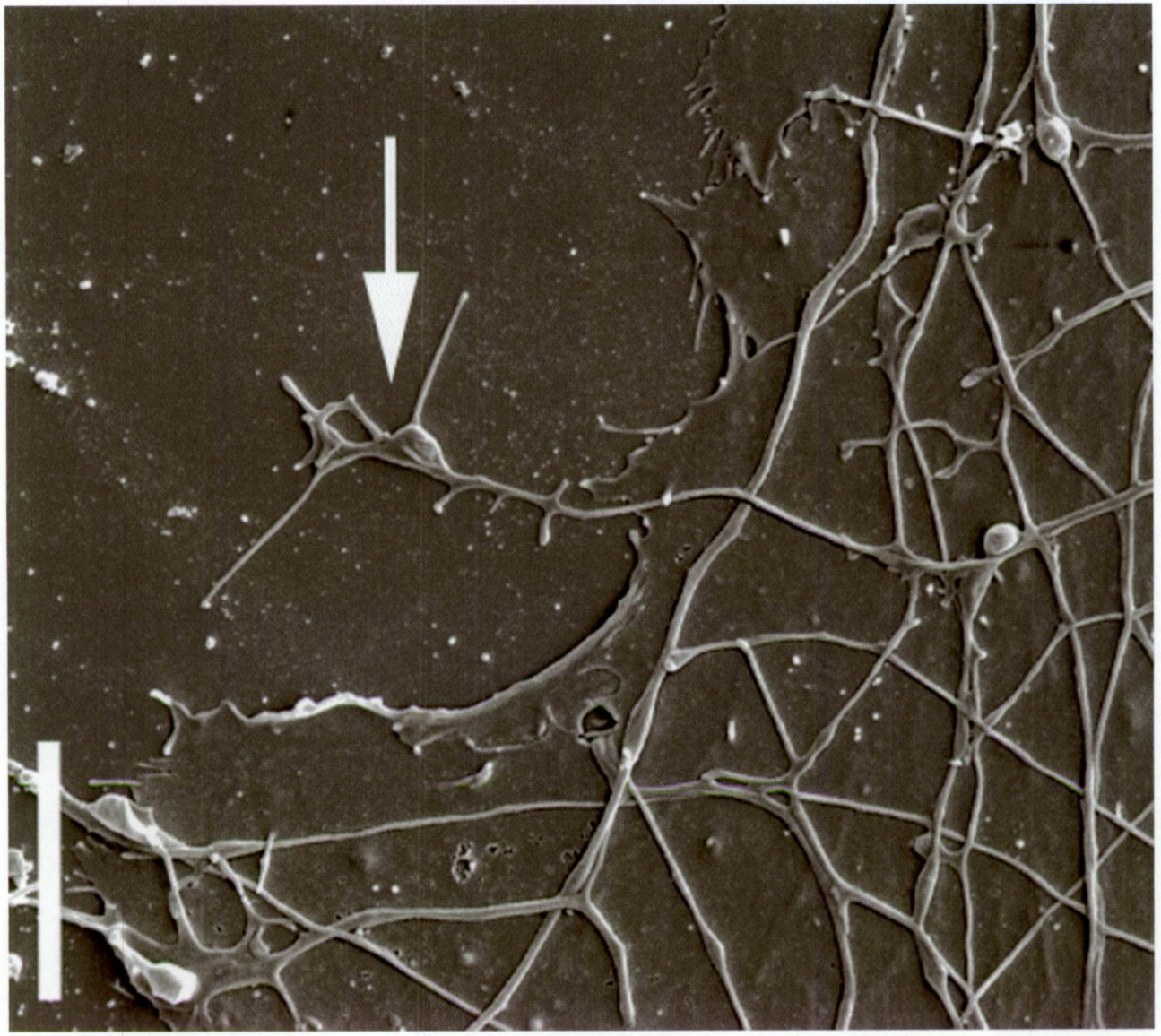

A
0h
3h
B
2h
5h

Figure 5.15 Post-injury axonal sprouts were characterised by slender sprout shafts and expanded distal tips, a similar morphology to developmental growth cones

Scanning electron microscopy of injury sites at 4 hours following axonal transection demonstrated that injury-induced sprouts exhibited similar morphological features to those observed in developing neurites, including prominent filopodial-like structures (arrow). Other sprout morphologies were also observed, including sprouts with bulbous end structures, which extended filopodial-like structures.

Scale bar: 5μm



Immunofluorescent labelling was utilised to determine the distribution of cytoskeletal components in post-injury neurite sprouts at 4, 14 and 24 hours following injury (Figure 5.16). Post-injury analysis demonstrated that the alterations occurring in response to axonal transection were consistent across all cultures at each time point investigated. Double labelling studies demonstrated that MAP-2 labelled processes (dendrites) only extended short distances from the neuronal aggregates and were rarely transected during neuronal injury. A prominent feature marking the injury site border was the accumulation of neurofilament triplet proteins into ring- and bulb-like structures (Figure 5.16) as has been described previously in both *in vivo* and *in vitro* models of neuronal injury (King et al., 1997, 2001; Dickson et al., 2000) as well as the previous investigations in this thesis. Ring and bulb-like structures were apparent at all time points examined, existing either in isolation or as a continuum of the severed axonal tips.

βIII-tubulin (Figure 5.17A) and tau (Figure 5.17B) were consistently distributed throughout post-injury axonal sprouts at all time points investigated. At four hours post-injury, numerous axonal sprouts had extended into the injury site. By 14 hours post-injury, sprouts had continued to elongate, with net extension towards the opposite side of the injury site from their origin. At 24 hours following injury, sprouting was extensive and many sprouts had crossed the injury site. Phosphorylated neurofilaments became distributed in post-injury axonal sprouts by 14 hours post-injury, as opposed to either βIII-tubulin or tau, which were distributed throughout sprouts at all post-injury time points examined (Figure 5.17C). Both βIII-tubulin and tau were uniformly distributed throughout sprout shafts and their

Figure 5.16 Axonal transection induced the abnormal accumulation of neurofilament triplet proteins into bulb- and ring-likes structures

A prominent characteristic of the injury site, following axonal transection, was the aberrant accumulation of neurofilament triplet proteins. Neurofilaments accumulated into bulb-like structures (small arrows) as well as ring-likes structures, which were either continuous with (large arrows), or in isolation of (arrowheads), severed axonal tips. These structures were immunoreactive for all neurofilament triplet sub-units, as well as phosphorylated neurofilaments. Labelling is for NF-M. Scale bar: 100μm

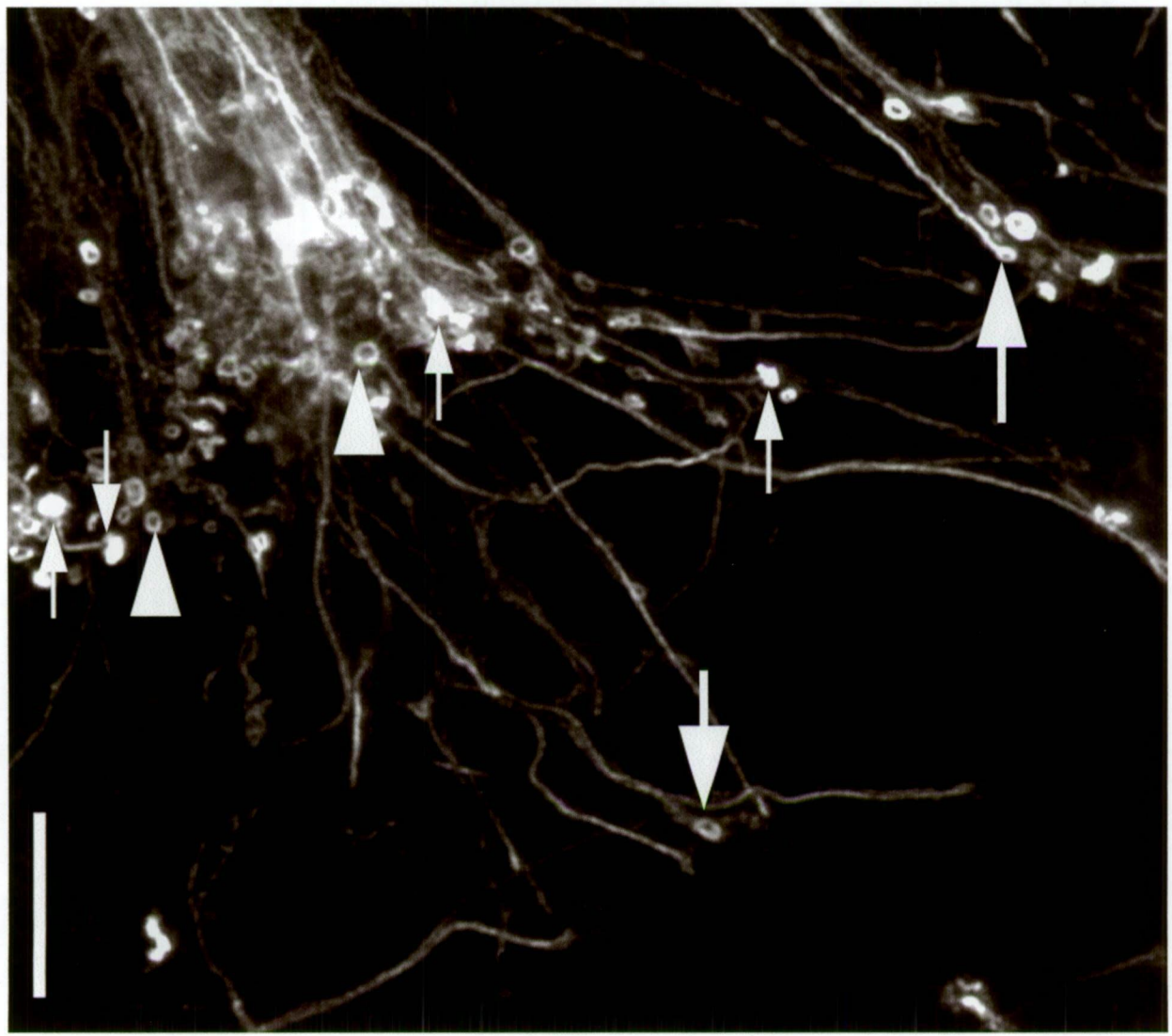
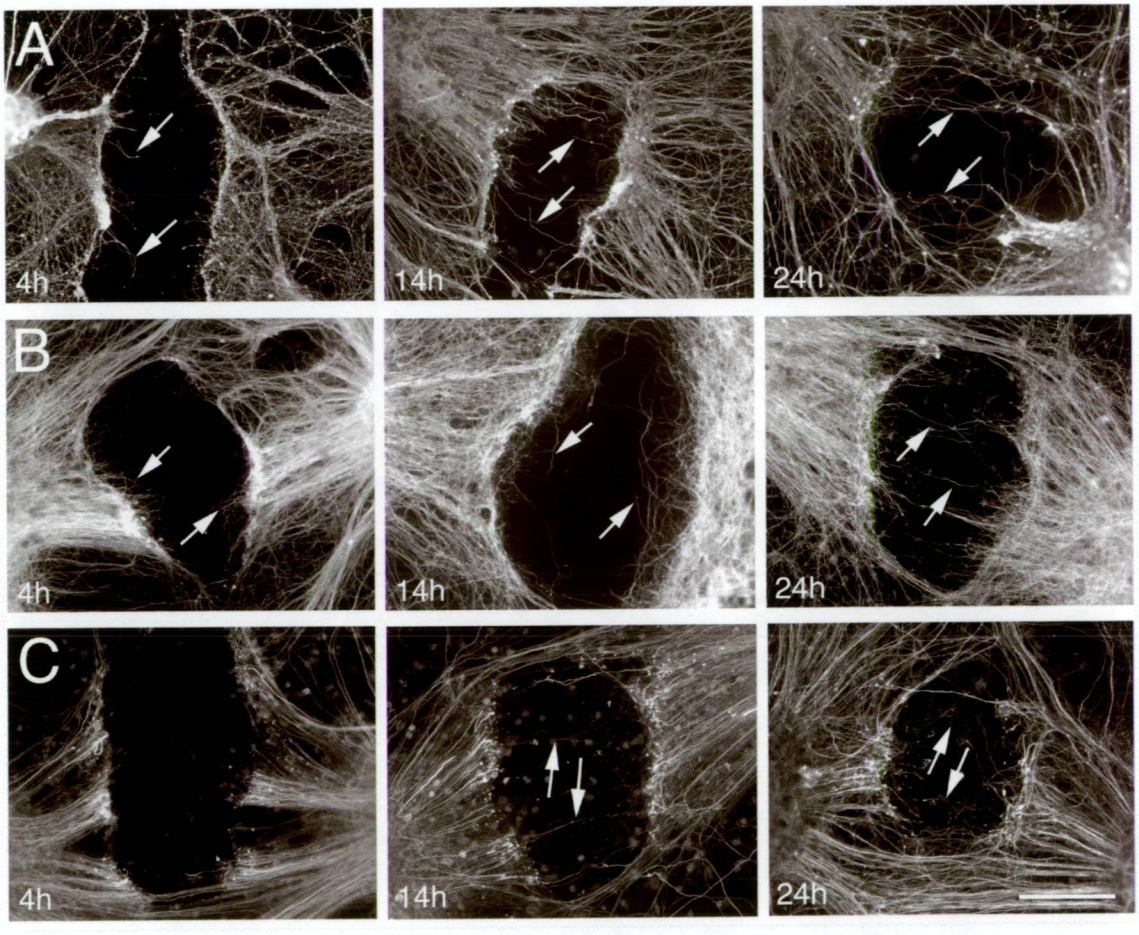


Figure 5.17 βIII-tubulin and tau were localised to axonal sprouts at all time points examined, whereas neurofilament triplet proteins were expressed later in the sprouting response

Analysis of injured neuronal cultures at 4, 14 and 24 hours post-injury demonstrated that the injury-induced sprouting response was progressive and sustained resulting in extensive re-growth across the lesion site by 24 hours following injury. Sprouts were immunopositive for the microtubule related markers βIII-tubulin (A) and tau (B) and were labelled for NF-M by 14 hours following injury (C). Arrows in each image denote examples of injury-induced sprouts.

Scale bar: 120μm


A
4h
14h
24h
B
4h
14h
24h
C
4h
14h
24h

splayed end-structures (Figure 5.18A and B). Filamentous actin was abundant in sprout growth cones (Figure 5.18C), however phosphorylated neurofilaments were restricted to the sprout shafts (Figure 5.18D).

5.3.5 Cytoskeletal disruption during attempted axonal regrowth

The effect of microtubule stabilisation on post-injury axonal sprouting was investigated by exposing cultures, grown for 21 DIV, to taxol immediately following axonal transection. Taxol exposure had a substantial effect on post-injury axonal sprouting (Figure 5.19). In vehicle-treated cultures the degree of sprouting increased as time progressed resulting in extensive sprout growth across the injury sites by 24 hours post-injury (Figure 5.19A). However, post-injury axonal sprouting was comparably limited in taxol-treated cultures (Figure 5.19B). In addition, taxol exposure resulted in substantial alteration in sprout morphology (Figure 5.20). Injured, vehicle-treated cultures elaborated sprouts that were generally tipped by expanded growth cone-like structures (Figure 5.20A). Conversely, injured taxol-treated cultures elaborated sprouts tipped by bulbous end-structures (Figure 5.20B). Taxol wash-out at least partially reversed the inhibitory effects of microtubule stabilisation on sprout growth, with increased sprouting observed in cultures in which taxol had been removed as compared to those continually exposed to taxol following injury at 21 DIV. Additionally, some sprouts regained growth cone-like structures at their tips following taxol washout (Figure 5.20C).

Quantitative analysis demonstrated that taxol substantially influenced sprout outgrowth and elongation (Figure 5.21). Sprout outgrowth was significantly ($p <$

Figure 5.18 Cytoskeletal components demonstrated specific distribution within sprouting axons

Similar to developing neurites, injury-induced axonal sprouts expressed βIII-tubulin (A) and tau (B) throughout the sprout shaft and expanded distal tip (arrows) whereas filamentous actin was primarily localised to growth cone-like structures at sprout tips (C, arrow) and phosphorylated neurofilaments were restricted to sprout shafts (D, arrows).

Scale bar: 20μm

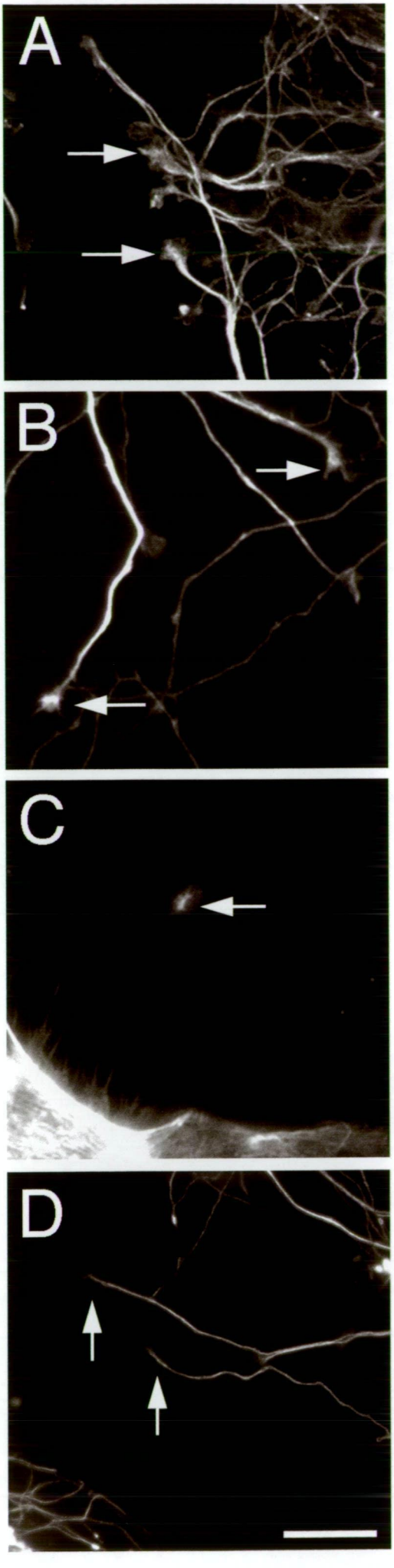

A
B
C
D

Figure 5.19 Taxol-induced cytoskeletal stabilisation resulted in fewer sprouts crossing injury sites by 24 hours post-injury

When mature injured cultures were exposed to taxol, the sprouting response was substantially altered and, relative to vehicle-treated cultures (A), few sprouts were observed to have crossed injury sites by 24 hours post-injury (B). Arrows denote examples of sprouts.

Scale bar: 60μm

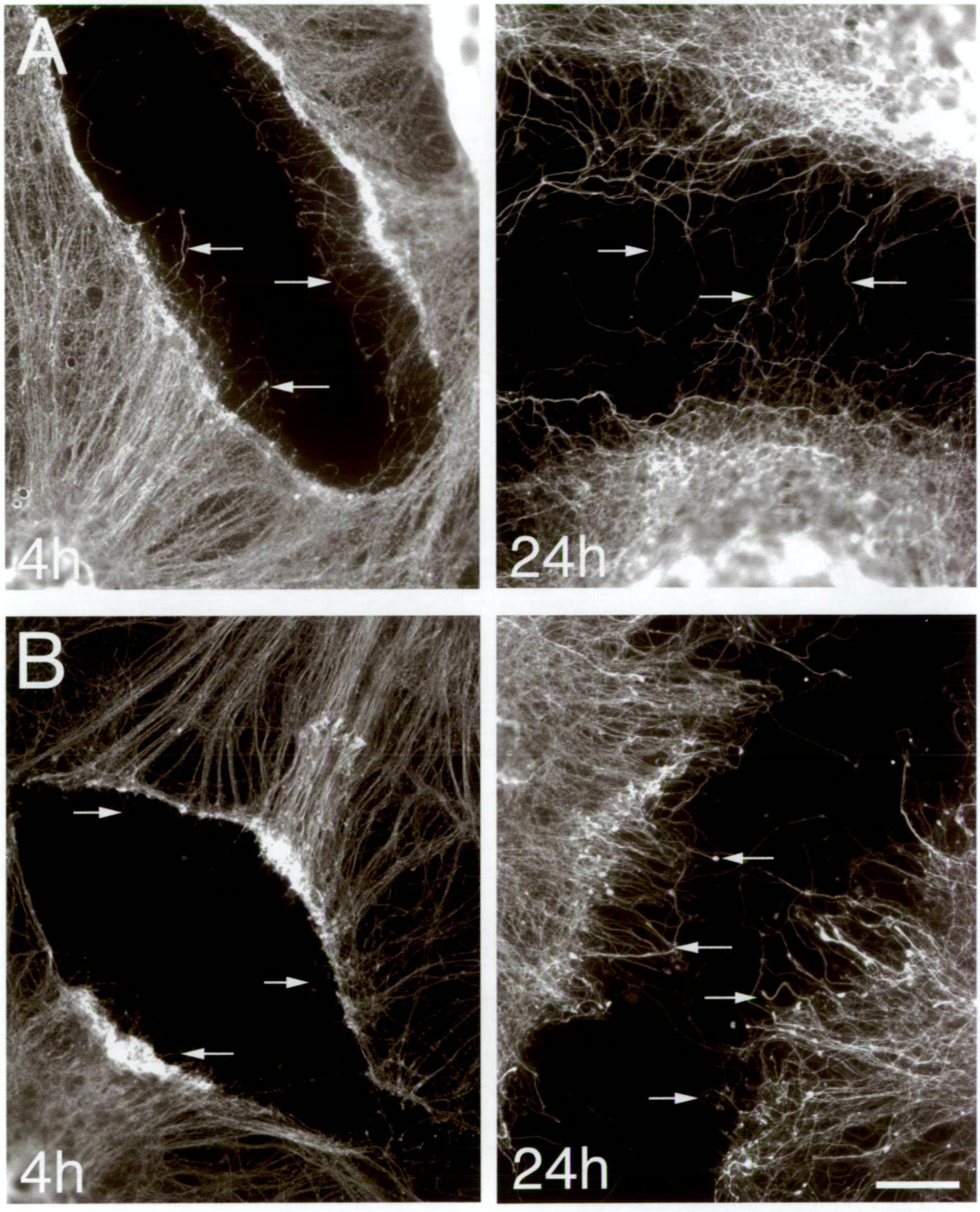
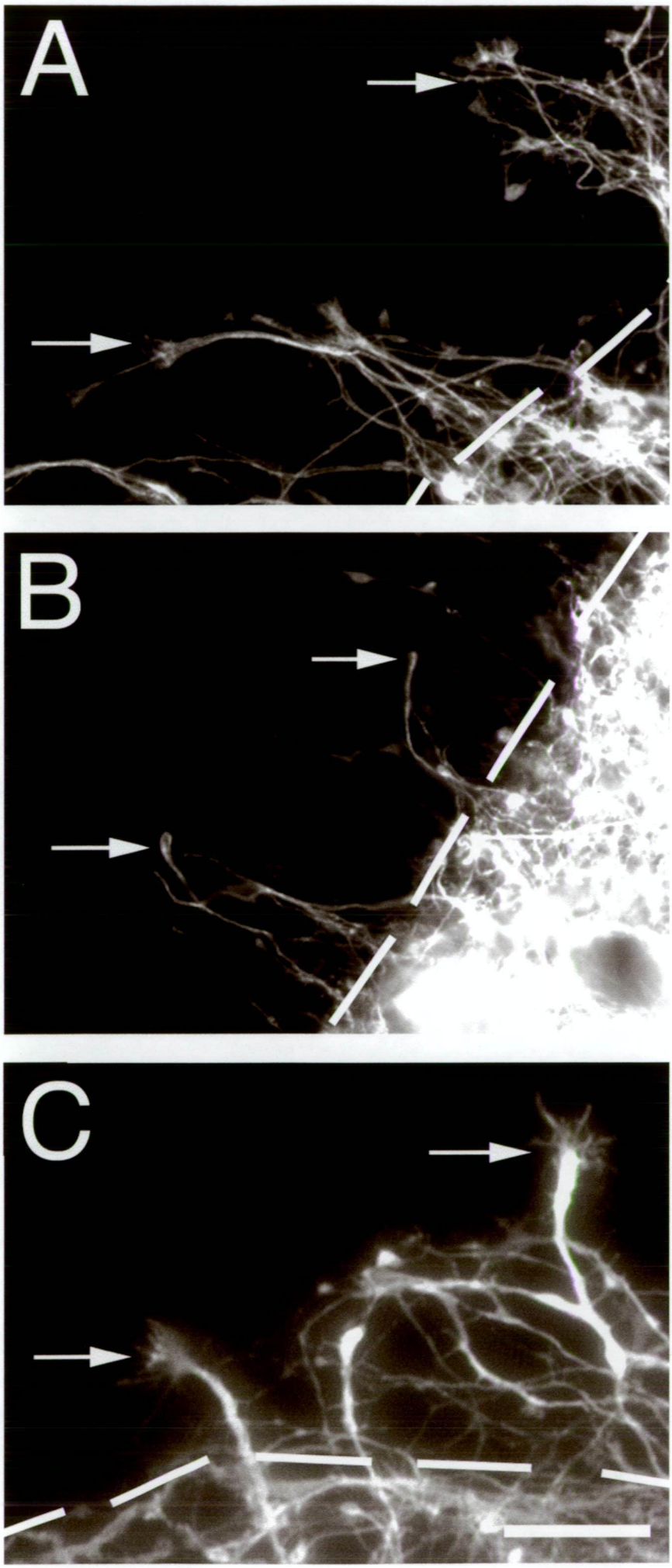

A
4h
24h
B
4h
24h

Figure 5.20 Taxol-induced microtubule stabilisation resulted in substantially altered sprout morphology, but these effects were partially reversible

Although the sprouts of injured vehicle-treated cultures demonstrated expanded growth cone-like structures at their distal tips (A), the distal tips of neurites from taxol-treated cultures displayed bulb-like morphology (B). Taxol washout from injured sprouting cultures resulted in the restoration of lamellipodial like structures on growth cone-like structures at sprout tips, but sprouts remained distended (C). Arrows denote examples of sprouts. Dotted lines define injury site borders in each image.

Scale bar: A and B = 20μm; C = 10μm


A
B
C

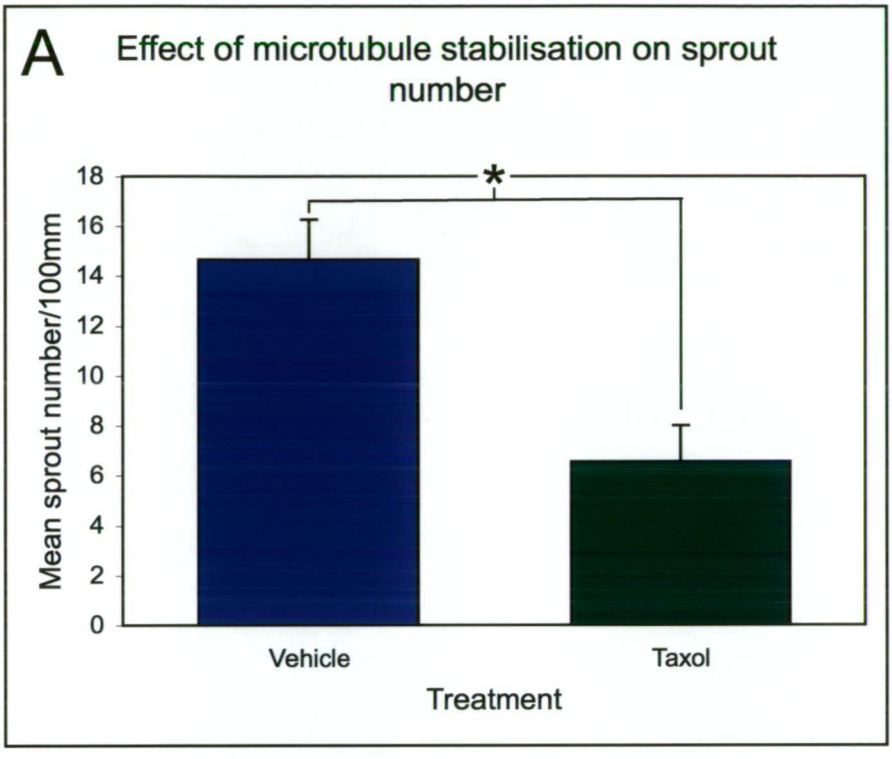
0.05) inhibited by taxol, with vehicle-treated cultures elaborating an average of 14.7 ± 1.6 (S.E) sprouts per 100μm of cut site length compared to taxol-treated cultures, which had elaborated on average only 6.6 ± 1.5 (S.E) sprouts per 100μm of cut site length, four hours following injury (Figure 5.21A). Similarly, statistical analysis indicated a significant ($p < 0.05$) difference between mean sprout length in injured, taxol-treated (21.6μm ± 0.8 (S.E)) and vehicle-treated (42.6μm ± 0.5 (S.E)) cultures at four hours post-injury, demonstrating that taxol profoundly inhibited sprout elongation (Figure 5.21B). Cell death analysis, using the vital dye propridium iodide, demonstrated the localisation of dead and dying cells along injury site boarders. Taxol exposure did not affect the level of cell death up to 24 hours following axotomy. However, increased cell death was observed when cultures were exposed to taxol for greater than 72 hours.

Injured sprouting cultures were also exposed to the nocodazole and latrunculin A. Nocodazole exposure substantially inhibited post-injury axonal sprouting, with little evidence of sprouting observed by four hours post-injury (Figure 5.22A). Moreover, when present these sprouts were often tipped by growth cones elaborating numerous filopodial-like protrusions, which lacked obvious lamellipodia, similar to the growth cones observed following nocodazole treatment of developing neuronal cultures (Figure 5.22B). Interestingly, latrunculin A treatment of injured cultures had no obvious affect on the axonal sprouting response and cuts sites were often repopulated by numerous new sprouts several hours following injury (Figure 5.23A). However, these sprouts often lacked obvious growth cone-like structures observed in vehicle-treated cultures (Figure 5.23B).

Figure 5.21 Quantitative analysis indicated that taxol-induced microtubule stabilisation significantly reduced sprout outgrowth and elongation

Quantitation of sprout number/100μm cut site length demonstrated a significant reduction in injury-induced sprouts between vehicle- and taxol-treated cultures (A). Additionally, sprout length was significantly reduced in response to taxol-treatment relative to vehicle-treated controls (B).

* $p < 0.05$. Error bars are standard error of the mean


A
Effect of microtubule stabilisation on sprout number
Mean sprout number/100mm
18
16
14
12
10
8
6
4
2
0
*
Vehicle
Taxol
Treatment

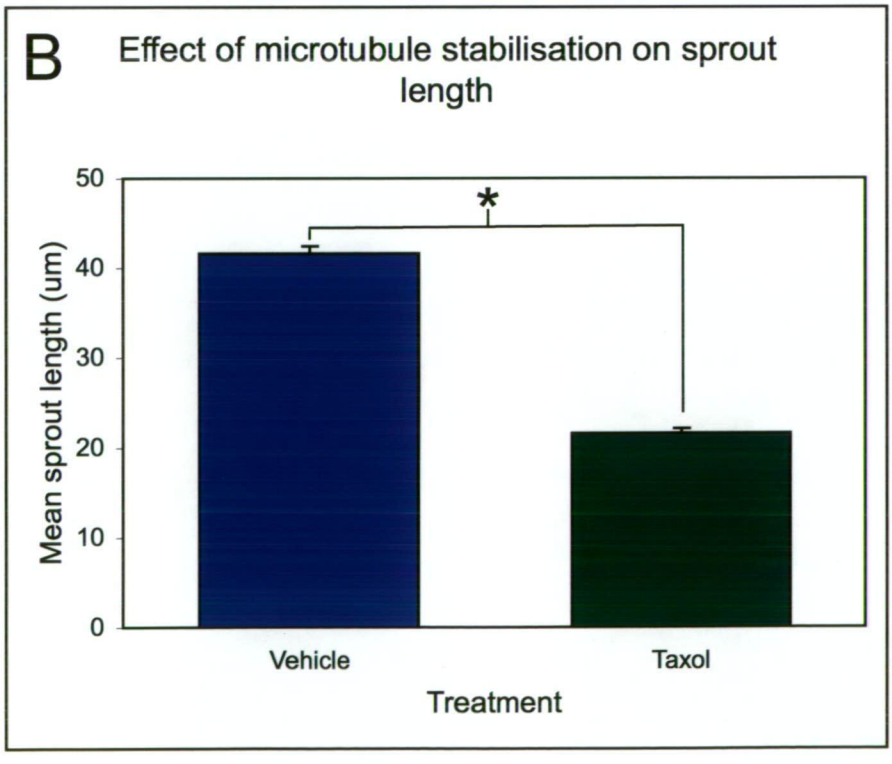

B
Effect of microtubule stabilisation on sprout length
Mean sprout length (um)
50
40
30
20
10
0
*
Vehicle
Taxol
Treatment

Figure 5.22 Nocodazole-induced microtubule disruption substantially reduced post-injury axonal sprouting

By 4 hours following injury, cultures exposed to nocodazole exhibited relatively few sprouts per injury site (A, dotted line denotes injury border). Moreover, the growth cone-like structures at sprout tips elaborated numerous defined filopodia, but lacked lamellipodia (B, arrow).

Scale bar: A = 30μm, B = 10μm

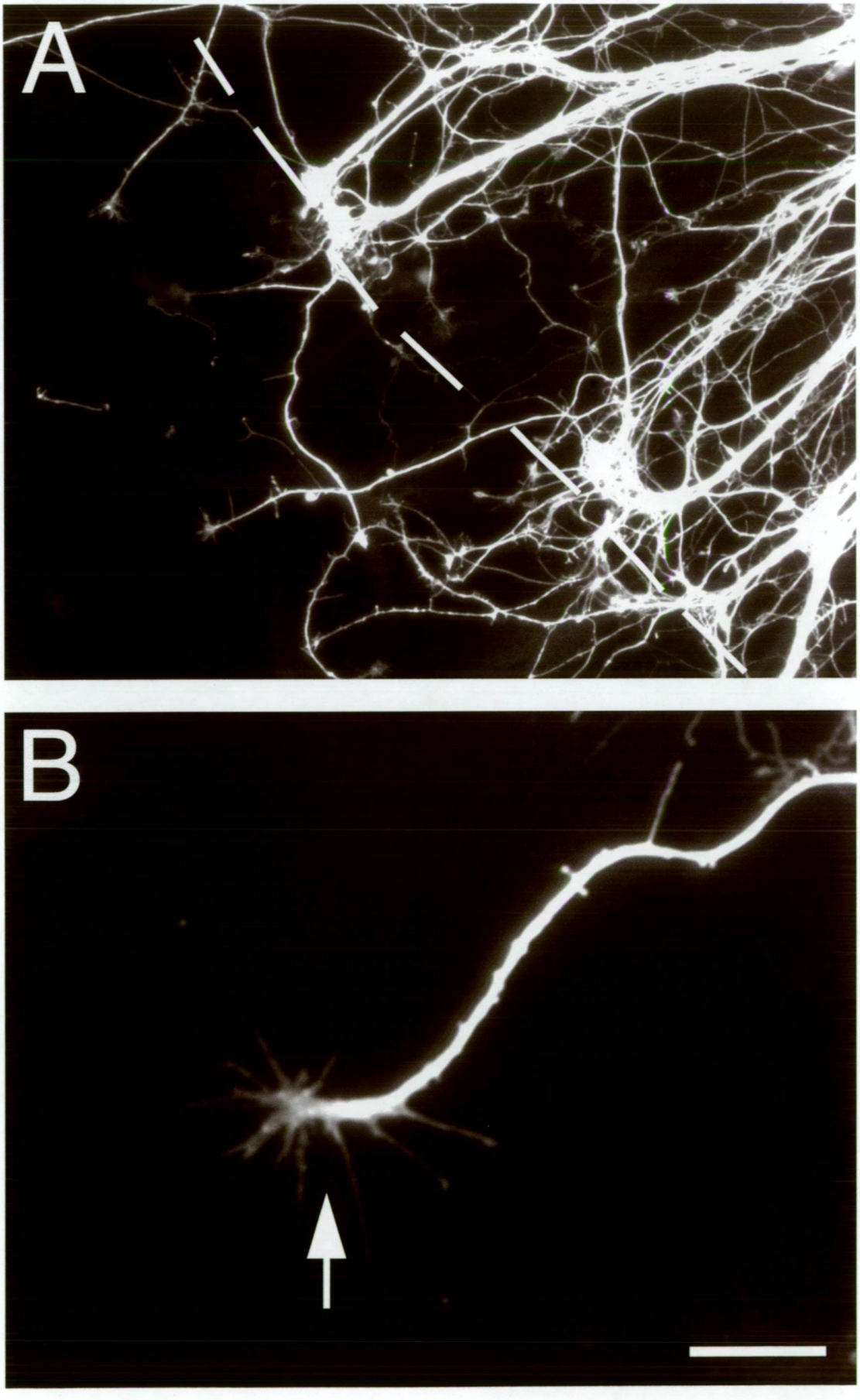
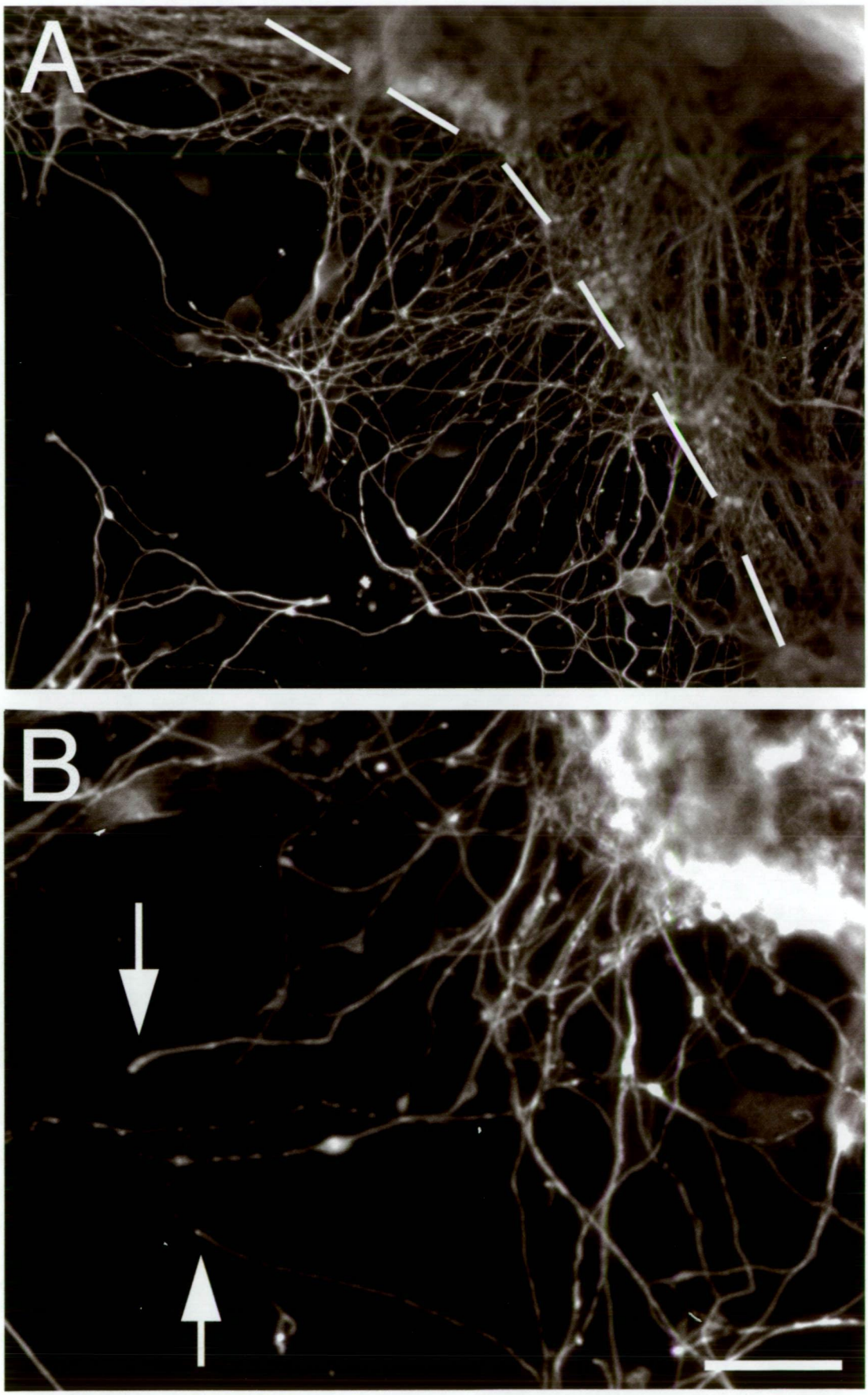

A
B

Figure 5.23 Latrunculin A-induced actin disruption altered sprout growth-cone morphology

Although axonal sprouting was profuse in the presence of the actin depolymerising agent, latrunculin A (A, dotted line denotes injury border), the expanded end-structures frequently observed on neurite tips in control cultures, were abolished in latrunculin A-treated cultures (B, arrows denote examples of sprouts lacking growth cones).

Scale bar: A = 30μm, B = 15μm


A
B

5.4 Discussion

The previous chapters in this thesis have demonstrated that a variety of adaptive alterations are evoked in the mature injured brain including the extensive induction of neural progenitor cells, adaptive changes in various glial populations and substantial axonal sprouting, associated with adaptive alterations in gene expression. Importantly, the intrinsic mechanisms underlying the potential for axons of mature central neurons to regrow in response to injury are not fully understood and are difficult to appreciate in the complexity of the CNS milieu and confounding glial responses. This study has, therefore, utilised live digital imaging, scanning electron microscopy and immunofluorescence-labelling techniques to investigate the intrinsic axonal sprouting response exhibited by relatively mature neurons in a simplified axonal injury model, in which neurons are grown in isolation. Furthermore injury-induced axonal sprouting was compared to initial neurite development to determine whether similar dynamic cellular and cytoskeletal events underlie these processes. Importantly, comparing how axons attempt to regenerate following injury with initial axonal development will provide important clues regarding the mechanisms employed by regenerating axons and allow therapeutic strategies to more effectively target specific aspects of the regenerative response.

In this study, a substantial regenerative attempt was indicated by the elaboration of sprout-like protuberances into injury sites. The overall morphology, motility and cytoskeletal composition of these injury-induced axonal sprouts was highly homologous with developing neurites. This was further highlighted by the demonstration that exposure to microtubule disrupting agents had similar effects on

initial neurite growth and injury-induced axonal sprouting. Additionally, disruption of the cytoskeleton substantially affected the morphology and cytoskeletal makeup of the growth cone-like structures tipping both developmental and regeneratively sprouting axons. Collectively, these results indicate that similar cytoskeletal dynamics may underlie both developmental and regenerative growth and contribute to clarifying the dynamic cytoskeletal mechanisms utilised by regeneratively sprouting axons.

5.4.1 *In vitro* experimental models of neuronal/axonal injury are important for determining intrinsic degenerative and regenerative responses

Although much research on neural repair is currently directed at understanding the mechanisms of degeneration and regeneration utilising *in vivo* models of experimental brain injury, which aim to simulate numerous aspects of human brain trauma, it is often difficult to gauge the response of individual neurons/axons to injury and separate intrinsic neuronal responses from confounding glial factors. In this regard, the poor regenerative ability of the adult brain, and CNS in general, has been attributed to a variety of factors encompassing a non-permissive environment as well as an intrinsic inability of neurons to re-initiate growth (Fawcett, 1992, 1997). This study was, therefore, designed to assess the potential for intrinsic axonal regeneration, utilising a culture system in which neurons can be grown to maturity in relative isolation (Dickson et al., 2000).

Importantly, a frequent consequence of TBI is diffuse axonal injury and subsequent widespread axonal disconnection (Maxwell et al., 1997). This disruption typically

occurs in central white matter and includes both myelinated and unmyelinated axons (Reeves et al., 2005). A variety of *in vitro* experimental models have been developed to determine the effects of mechanical injury on cultured neuronal populations and although these models are able to replicate many aspects of brain injury (reviewed by Morrison et al., 1998), few are able to specifically injure axons. Moreover, specifically analysing the axons of CNS neurons utilising *in vitro* systems has been particularly difficult due to the challenges involved in culturing CNS neurons, relative to PNS neurons, and in examining the axonal component independent of the somato-dendritic component (Taylor et al., 2005).

In this regard, specialised culture chambers have been developed in which axonal and somato-dendritic neuronal compartments can be assessed individually. These chambers, known as compartmentalised, or Campenot, chambers utilise growth factors to direct axonal growth to specific regions of the chamber (for example Campenot, 1982; Eng et al., 1999). However, this system has predominantly been used for investigating PNS neurons and is less useful for investigating CNS neurons, which differ in their growth requirements and do not efficiently grow under such conditions (Taylor et al., 2005). The neuronal culture system utilised in the present chapter resulted in the formation of discrete axonal bundles, interconnecting neuronal aggregates, which could be focally injured (for example transected or stretched) to determine the potential for intrinsic axonal regeneration in the absence of confounding glial factors. In this regard, individual bundles of axons were injured and immediately manipulated and/or imaged or examined by scanning electron microscopy and cytochemical techniques.

A similar culture system has previously been utilised, in which axonal bundles were transiently stretched by pressurised fluid deflection (Chung et al., 2005). However, this system produced a delayed axotomy response and, although useful for elucidating the evolving pathology of axonal disconnection, was less appropriate for examining potential regenerative events. Importantly, studies utilising defined axonal transection models (for example Dickson et al., 2000; Chung et al., 2002; Haas et al., 2004), similar to that utilised in the present investigation, have demonstrated the usefulness of this model system in examining axonal regeneration and determining the effects of inhibitory and facilitative molecules during regenerative attempts. Moreover, these studies were also able to compare neurite developmental and post-injury sprouting events. Recently, Taylor et al. (2005), have developed a specialised microfluidic culture system in which hydrostatic pressure is utilised to fluidically isolate axonal from somato-dendritic compartments. Importantly, this system is amenable to direct axonal manipulation.

Collectively, these studies highlight the role of *in vitro* neuronal injury models as economical, accessible systems, which can be easily controlled to study the intrinsic response of neurons to various forms of extrinsic chemical, molecular and/or mechanical manipulation. *In vitro* axonal injury models are useful for understanding the similarities and discrepancies occurring between axonal development and regeneration and provide a solid platform for the initial testing phase of agents that may translate into potential clinical therapies. As such, the following discussion compares injury-induced axonal sprouting events with initial axonal development and examines the role of the axonal cytoskeleton in these processes.

5.4.2 Cytoskeletal disruption during neuronal development highlights the importance of cytoskeletal dynamics during neurite elongation

To determine the role of the cytoskeleton during neurite growth and regenerative sprouting, the distribution of various cytoskeletal components was firstly confirmed by immunohistochemistry. As has been described extensively by other investigators, typical distribution of cytoskeletal components was observed in developing neurites in the current study. Notably, filamentous actin was localised to the most motile periphery of developmental growth cones, tubulin and microtubule-associated proteins such as tau were predominantly distributed within neurite shafts and growth cone central domains, occasionally extending into filopodia, and neurofilaments were restricted to neurite shafts and excluded from growth cones. The relative localisation of these cytoskeletal components may reflect their relative importance in axonal growth. For instance, microtubules have been demonstrated to be essential in axonal outgrowth (Okabe and Hirokawa, 1990; Walker, 2001), whilst axonal growth can proceed in the absence of neurofilaments (Jiang et al., 1996; Zhu et al., 1997; Levasseur et al., 1999), albeit at a slower rate (Walker, 2001). Importantly, neuronal intermediate filament expression has been associated with periods of rapid growth, particularly in axons where neurofilament triplet proteins become highly phosphorylated (Benson et al., 1996)

As described above, the culture system utilised in this study was particularly useful for studying exclusively neuronal alterations in response to extrinsic manipulation and was therefore amenable investigating the effect of bath-applied cytoskeletal disrupting agents. In this regard, early stage cultures (three DIV) were exposed to

taxol to investigate the effects of microtubule stabilisation on neurite development. Taxol, widely used in the treatment of various cancers, promotes the polymerisation of tubulin monomers and the formation of stable, non-functional microtubules (Gotaskie and Andreassi, 1994). The microtubule network is a particularly motile component of the axonal cytoskeleton within developing neurites (for reviews see Brandt, 1998; Kalil et al., 2000; Kalil and Dent, 2005). Exposure of cortical neurons to taxol in the current study resulted in neurite distension, cessation of neurite elongation and a significant reduction in growth cone formation. Similar effects of taxol have been observed in cultured chick sensory neurons (Letourneau and Ressler, 1984; George et al., 1988).

These studies also demonstrated that, following taxol treatment, loops of tubulin immunoreactivity were localised within the deformed end-structures that replaced developmental growth cones at neurite tips, with ring- and bulb-like accumulations of phosphorylated neurofilaments often observed within the central core of the microtubule loops. The segregation of microtubules and neurofilaments is unusual as these cytoskeletal components are normally intermixed (Letourneau and Ressler, 1984). The dilated bulbous structures that formed at neurite tips following taxol exposure frequently lacked filopodia and lamellipodia, and thus probably lacked the ability to effectively transduce guidance cues - a factor likely to account for the curling and looping of neurites around their aggregates of origin.

Abnormalities occurring after taxol exposure are likely to reflect the bundling and proliferation of microtubules within neurites as well as altered microtubule spacing

and interaction with microtubule associated proteins (Black, 1987). Taxol-promoted microtubule proliferation may exhaust the local pool of tubulin monomers available for utilisation in neurite elongation and presumably causes steric hindrance of axonal transport, which may prevent the delivery of constituents necessary for neurite extension. Indeed, Theiss and Meller (2000) demonstrated that taxol inhibits anterograde axonal transport. Furthermore, although results from this chapter demonstrated that some taxol-treated neurites regained growth cones and neurite growth increased following taxol washout, many neurites remained distended, indicating that taxol induced microtubule stabilisation is relatively permanent.

Although nocodazole specifically acts on microtubules, its affects oppose those of taxol; nocodazole binds to tubulin and causes microtubule depolymerisation (Dinter and Berger, 1998). Preliminary investigations demonstrated that exposure of developing cortical cultures to nocodazole inhibited neurite growth and caused substantial morphological reorganisation, which differed from the morphological aberrations induced by taxol exposure. Nocodazole-exposed neurites developed growth cones that lacked lamellipodia, as reported previously by Gallo (1998). Additionally, a proportion neurons were bordered by a lamellipodial-like fringe, structures that were not observed in taxol-treated cultures. Collectively, studies involving microtubule disruption (both stabilisation and destabilisation) highlight the importance of this cytoskeletal component in developmental neurite growth, as illustrated by severely misdirected aberrant growth or growth inhibition in the presence of such agents. In this regard, studies recently reviewed by Kalil and Dent. (2005) indicate that microtubules may play a more predominant role in

developmental axon guidance than previously appreciated, a process in which actin filaments were believed to be primarily responsible for.

Nevertheless, the complex interplay between microtubules and actin filaments was indicated in the present study, where disruption of the actin cytoskeleton also resulted in inhibited neurite growth as well as a loss of actin-rich structures, such as growth cone filopodia and lamellipodia. Each cytoskeletal disrupting agent had a dose dependant effect on neurite growth and was utilised at a sub-lethal concentration. Interestingly, each agent also disrupted a specific aspect of cytoskeletal dynamics, but ultimately prevented neurite growth, with differing effects on growth cone morphology. Collectively, these studies emphasise the significance of dynamic interactions between the various cytoskeletal components for successful neurite growth.

5.4.3 Axons undergo rapid, highly dynamic alterations in response to physical injury indicative of attempted regeneration

Numerous studies, in both *in vitro* and *in vivo* injury models as well as human brain injury cases, have documented the deleterious effects of brain and neuronal injury on the neuronal cytoskeleton, including cytoskeletal misalignment, abnormal accumulation or loss of cytoskeletal elements and cytoskeletal dissolution (reviewed by Povlishock and Christman, 1995; Maxwell et al., 1997; Fitzpatrick et al., 1998; Smith and Meaney, 2000; Povlishock and Katz, 2005). Thus, injury-induced alterations in the cytoskeleton of mature CNS neurons are implicated in resultant neuronal degeneration. Importantly, axotomy of neurons in the developing nervous

system, peripheral nervous system or nervous system of lower vertebrates and invertebrates is often followed by a robust and relatively successful or complete attempt at regeneration, whereas axonal injury in the mature CNS is met with a limited capacity for repair. As discussed, this may be due to non-permissive elements in the mature CNS environment as well as endogenous neuronal mechanisms, including factors such as a developmental loss of growth capacity (Fischer et al., 2004) and inability to rapidly seal damaged axonal membranes (Ahmed et al., 2001).

However, accumulating data now indicate that neurons from the adult brain have the capacity to mount a regenerative attempt following injury. Many of these studies indicate that these regenerative attempts are associated with cytoskeletal rearrangement and manifest as the elaboration of fine sprout-like protuberances into the vicinity of lesion sites (for example Foerster, 1982; Kristt, 1987; Salin et al., 1995; Christman et al., 1997; Dickson et al., 2000; Pastor et al., 2000; King et al., 2001; Chung et al., 2002; Haas et al., 2004). Importantly, the mechanisms underlying attempted axonal regenerative attempts are not fully understood and, whilst collateral sprouting from undamaged axons has been the largely accepted mode of axonal re-growth following axotomy, few studies have directly investigated the potential for mature CNS neurons to sprout from disconnected axonal proximal tips following axotomy. In this regard, growth cone formation at severed axonal tips has been postulated as a key mechanism in successful axonal regeneration (Ziv and Spira, 1997; Verma et al,. 2005), although these structures may differ significantly between axotomy in the PNS and CNS and in invertebrates as opposed to vertebrates

(as discussed below). Interestingly, the formation of dystrophic, or abnormal, growth cones at severed tips of CNS axons has been attributed to the inability of these neurons to effectively re-grow their axons following injury (Tom et al., 2004).

This study attempted to specifically investigate the response of mature mammalian CNS neuron axons to transection. Thus, utilising an *in vitro* model of axonal injury capable of eliciting a substantial axonal sprouting response (Dickson et al., 2000), this study demonstrated that axonal transection induced a characteristic and highly dynamic response, which included both reactive and regenerative sprouting events. Time-lapse imaging verified that axons retract away from injury sites immediately following, and up to several hours after, transection. Post-injury axonal retraction has been reported previously following lamprey spinal cord transection (McHale, 1995). The retraction response was accompanied by other reactive alterations, including swelling in the distal region of severed axons and the accumulation of neurofilaments into ring- and bulb-like structures, as reported previously (Povlishock and Becker, 1985; Meller, 1993: Dickson et al., 2000; King et al., 2001).

Results from this chapter demonstrated that extensive neurite sprouting was initiated within four to six hours of injury. By 24 hours post-injury, several sprouts had traversed the lesion sites and many exhibited small growth cone-like structures at their tips, suggesting that this is a guided response and not simple elongation along random pathways. Importantly, this response was maintained beyond 24 hours following injury, indicating that cortical neurons not only possess an intrinsic capacity to sprout following injury, but also an ability to sustain this response.

Moreover, although sprouting in the mature CNS has been largely viewed as an abortive response, attributed to both a non-permissive environment as well as intrinsic neuronal factors, the finding that mature neurons are able to sustain a regenerative attempt free of their normal glial matrix suggests that the poor regenerative ability of the adult brain may be more attributable to compounding glial factors, than an incapacity of the neurons themselves. Notably, the defined media utilised in this study lack a range of growth factors that might support regeneration, although it does contain insulin (Brewer et al., 1993; Brewer, 1995).

Analysis of the morphology, cytoskeletal composition and dynamic properties of post-injury sprouts indicated some notable similarities with developing neurites. For instance, developing neurites and sprouting axons were motile, slender structures. Additionally, a majority of sprouts were tipped by expanded growth cone-like structures, which exhibited immunoreactivity for tubulin, tau and filamentous actin, but not neurofilaments. However, putative regeneration-associated growth cones were generally smaller and less elaborate than developmental growth cones. Importantly, injury-induced axonal sprouts are not always tipped by growth cone like structures. For example, the 'sprouting bulbs' reported by King et al. (2001) and Povlishock and Becker (1985) elaborated finger-like protrusions, lacking defined end-structures. Moreover, studies by Jacobs et al. (1997), involving injury to lamprey spinal axons showed that, contrary to normal growth cones in the developing nervous system, the growth cones of regenerating axons lacked filopodia and lamellipodia and were filled with neurofilaments.

Interestingly, Hall et al. (1997) have demonstrated that axotomy close to the soma of neurons from the lamprey CNS results in the formation of ectopic axonal sprouts from dendrites, whereas axotomy at more distal sites results in sprouting from the proximal stump. Moreover, utilising cultured *Aplysia* neurons, Spira et al. (1996, 2001, 2003) demonstrated that axotomy resulted in the formation of a motile growth cone at the proximal axonal tip, which was associated with substantial reorganisation of cytoskeletal proteins as well as other organelles within this region and is likely to involve calcium activated processes (Gitler and Spira, 1998; Spira et al., 2001). Similarly, Goldberg and Burmeister. (1992) showed that filopodial-like structures are rapidly elaborated from transected *Aplysia* axons. Overall, these studies demonstrate that axons undergo a stereotypical sequence of changes in response to transection, including initial retraction, proximal tip swelling, with normal axonal diameter maintained up-stream from this, and finally the transformation of the swollen tip into a dynamic growth cone (reviewed by Spira et al., 2003). Stone et al. (2001), however, note that axonal injury does not culminate in swelling in all axons, with smaller calibre axons undergoing relatively little change over time. Nevertheless, similar stereotypical reactive alterations have been observed following axotomy of mammalian CNS neurons, however, the full repertoire of responses may be lacking, potentially accounting for the poorer regenerative ability of the mammalian CNS relative to lower vertebrates, invertebrates, the PNS and the developing nervous system.

Results from this study indicate that in both developing and regeneratively sprouting axons, neurofilaments are established at later time points than either microtubules or

microfilaments. These results suggest that neurofilaments are not required for initial sprout outgrowth. For instance, Zhu et al. (1997) and Levavasseur et al. (1999) have demonstrated that neurite outgrowth proceeds in the absence of neurofilaments. Neurofilaments, however, may play an important role in sprout elongation. In this regard, reduced regenerative ability of the peripheral nervous system has been reported in neurofilament deficient animals (Jiang et al., 1996; Zhu et al., 1997). Further, Meller (1993) speculates that neurofilaments may provide the propulsive force underlying sprout growth.

5.4.4 Inhibition of injury-induced axonal sprouting with taxol or nocodazole highlights the importance of microtubule dynamics in axonal regeneration

Administration of taxol immediately following axonal transection at 21 DIV resulted in distinct morphological changes, similar to those observed in developing taxol-treated developing neurites. Taxol exposure substantially inhibited post-injury axonal sprouting, causing a significant reduction in sprout length and number at four hours post-injury. Post-injury sprouting was not completely abolished by taxol, suggesting that mechanisms other than microtubule dynamics may be responsible for sprout formation and growth. For instance, the actin and microtubule cytoskeletons may act in a co-ordinated manner to dictate the cytoskeletal response underlying sprout growth, as is the case during neurite development (Letourneau et al., 1987), with disruption of one component affecting the dynamics of the other (Dent and Kalil, 2001). Similar to taxol, nocodazole also substantially inhibited axonal sprouting. Interestingly, disruption of actin did not obviously inhibit injury-induced

axonal sprouting as numerous axonal sprouts had crossed the lesion site of latrunculin A-treated cultures several hours after injury. However, sprouts in latrunculin A-treated cultures generally lacked distal growth cone-like.

Collectively, these results indicate some of the notable similarities between developing and sprouting axons, and verify that the dynamic properties of microtubules are fundamental to the processes of sprout outgrowth and elongation. However, discrepancies are also highlighted in the relative importance of actin filaments in axonal development compared to regeneration, in that actin depolymerisation inhibited developmental, but not regenerative, growth. These findings also highlight the relative importance of the growth cone during axonal regenerative attempts. For example, although regenerative sprouting axons lacking obvious growth cones were able to undergo substantial growth, this may have been misdirected.

5.4.5 Cytoskeletal disruption as a therapeutic strategy to prevent aberrant axonal sprouting following brain injury and in Alzheimer's disease

The results from this chapter, as well as numerous other investigations, indicate that cytoskeletal disruption may be an effective therapy for targeting several postulated maladaptive cytoskeletal alterations induced by both physical injury and as part of the evolving pathology of certain neurodegenerative diseases. For example, using an *in vivo* model of brain trauma, Adlard et al. (2000) demonstrated that taxol inhibits microtubule loss and neurofilament accumulation, alterations that are viewed as pathological following neuronal injury. Furthermore, injury-induced axonal

sprouting has been implicated in the development of hyper-excitable circuitry, aberrant synapse formation and post-traumatic epilepsy following head trauma (Larner, 1995; McKinney et al., 1997; Prince, 1997; Prince et al., 1997; Jacobs et al., 2000; Santhakumar et al., 2001). Post-traumatic epilepsy is a common consequence of TBI, occurring months to years after the initial injury. Notably, abnormal epileptiform activity can result from both hippocampal and cortical injury/lesion, however, post-traumatic epilepsy is particularly common following penetrating wounds of the neocortex (Prince, 1997). Results from this chapter indicate that microtubule disruption effectively prevents injury-induced axonal sprouting *in vitro*. Therefore, agents such as taxol and nocodazole may have important therapeutic implications in preventing maladaptive sprouting responses following brain trauma. Importantly, the present investigation has demonstrated that the effects of taxol were at least partially reversible, an important feature should this agent be used therapeutically in the treatment of neuronal injury.

Interestingly, disrupted neural circuitry (Phinney et al., 1999), including aberrant axonal sprouting (Arendt, 2001; Masliah et al., 1991, 1992) has been demonstrated in Alzheimer's disease, where axonal sprouts have been localised around ß-amyloid plaques. Importantly, many of the cytoskeletal abnormalities observed in both human and experimental models of brain injury are replicated in cases of Alzheimer's disease (King et al., 2000; Vickers et al., 2000; Woodhouse et al., 2005). This has led to the proposal that ß-amyloid deposition may cause chronic physical injury to surrounding axons, physically compressing and potentially severing them, which results in an attempt by the afflicted axon to regenerate

(reviewed by Vickers et al., 2000; Woodhouse et al., 2005). Thus, the formation of aberrant, or dystrophic neurites, has also been interpreted as a mechanism of regenerative failure (DeWitt and Silver, 1996), in which plaques induce a 'mass-effect' on surrounding neurites causing them to attempt to regenerate, but this is ultimately compounded by the chronic, evolving nature of ß-amyloid plaque formation (Vickers et al., 2000; Woodhouse et al., 2005).

Cytoskeletal-stabilising agents such as taxol may have important implications in curbing cytoskeletal abnormalities and aberrant sprouting associated with the evolving pathology of Alzheimer's disease as well as associated disorders (reviewed by Trojanowski et al., 2005). For instance, taxol has been demonstrated to reduce ß-amyloid toxicity in Alzheimer's disease (Michaelis et al., 1998, 2005) as well as inhibit detrimental alterations in intracellular calcium levels that may result in neuronal death (Burke et al., 1994), aberrant changes in tau antigenicity (Mattson, 1992) and excitotoxicity (Furukawa and Mattson, 1995). At low doses taxol has also been shown to promote recovery of function following spinal cord injury (Perez-Espejo et al., 1996). However, Figueroa-Masot et al. (2001) have reported that taxol potently induces apoptosis in cultured cortical neurons in a dose and exposure-time dependent manner. Accordingly, the present study recorded relatively little cell death following taxol exposure for 24 hours, but cell death was substantially increased following 72 hours of taxol treatment. Trojanowski et al. (2005) review work indicating that, utilised as a therapy for Alzheimer's disease and related conditions, taxol may be used at much lower doses and different dose regimes than when used as a chemotherapeutic agent, to prevent toxicity.

5.5 Conclusion

The inability of axons to successfully regenerate following brain trauma has been attributed to several factors within the CNS milieu as well as an intrinsic incapacity for regeneration by damaged neurons themselves (Berry et al., 1994; Fawcett, 1997; Bandtlow and Schwab, 2000; Golberg and Barres, 2000; Qui et al., 2000; Batchelor and Howells, 2003; Rhodes and Fawcett, 2004). The current study demonstrates that mature CNS cortical neurons can respond rapidly to injury by sprouting. Importantly, the microtubule disrupting agents, taxol and nocodazole, inhibited both initial neurite development and injury-induced axonal sprouting, indicating that common mechanisms of microtubule dynamics are likely to underlie both processes.

Although taxol and nocodazole have opposing effects on microtubule structure (polymerisation and depolymerisation, respectively), both agents essentially act to suppress microtubule dynamics, which are crucial to normal neurite growth and post-injury axonal sprouting. Interestingly, disruption of microfilaments had differential effects on developing and regeneratively sprouting axons, indicating the relative importance of the different cytoskeletal components in these processes. Collectively, this study has highlighted the remarkable intrinsic capacity of relatively mature cortical neurons to undergo a sustained axonal sprouting response after injury. These finding therefore suggest that the poor regenerative ability of central axons *in vivo* is likely to be more attributable to the non-permissive environment of the CNS rather than an intrinsic incapacity of the neurons themselves.

Finally, conditions such as the development of post-traumatic epilepsy following acquired brain injury (Larner, 1995; Prince, 1997; Prince et al., 1997; Jacobs et al., 2001) as well as the evolving pathology of Alzheimer's disease and other neurodegenerative disorders (Trojanowski et al., 2005) may include episodes of abnormal reactive neurite sprouting and detrimental cytoskeletal changes. Thus, agents that specifically target particular cytoskeletal components may be of therapeutic use in curbing the pathological evolution of these conditions. Importantly, the study of axonal degeneration and regeneration *in vitro* will be useful for studying the mechanisms underlying these processes as well as testing and developing therapeutic agents to prevent detrimental alterations and enhance adaptive alterations following brain injury and in various neurodegenerative disorders.

α-internexin undergoes identical reactive alterations following physical injury and in the evolving pathology of Alzheimer's disease

6.1 Introduction

Abnormalities in the neuronal cytoskeleton are common to physical injury and a variety of neurodegenerative diseases (Lariviere and Julien, 2004). In this regard, the neurofilament triplet proteins, unique to neurons, undergo striking alterations in response to physical injury and the evolving pathology of Alzheimer's disease (AD) (King et al., 1997, 2000a, b; Vickers et al., 2000; Woodhouse et al., 2005). Indeed, abnormalities in neurofilament triplet proteins are a key hallmark of neuronal trauma (Hoffman et al., 1984, 1985, Maxwell and Graham, 1997; Jafari et al., 1997, 1998; Maxwell et al., 1997; Povlishock 1997; Nixon, 1998; Dickson et al., 2000; King et al., 2000a, b, 2001; Zhang et al., 2000), where they accumulate into bulb- and ring-like formations at sites of axonal damage and/or become localised to regenerative axonal sprouts in both *in vitro* (Dickson et al., 2000) and *in vivo* (King et al., 2001) models of experimental neuronal injury.

Intriguingly, abnormal accumulations of neurofilament triplet proteins and aberrantly sprouting neurites, bearing striking morphological and neurochemical resemblance to physically injured neurons undergoing reactive and regenerative events, have been observed in AD (King et al., 1997, 2000a, b; Vickers et al., 1997, 2000; Woodhouse et al., 2005). AD is a chronic neurodegenerative disorder in which a specific pattern

of pathological changes result in extensive synapse loss and neuronal death, culminating in memory decline, impaired cognitive ability and profound dementia (Vickers et al., 2000; Morrison and Hof, 2002; Spires and Hyman, 2004). Pathological hallmarks of AD include the extracellular presence of ß-amyloid plaques and associated intracellular neurofibrillary pathology (Vickers et al., 2000; Woodhouse et al., 2005). However, the mechanism linking ß-amyloid deposition with neuronal abnormalities remains elusive. Interestingly, a sub-set of cortico-cortical projection, neurofilament triplet-rich pyramidal neurons demonstrates selective vulnerability to AD pathology, whereas interneurons usually remain un-altered (Hof et al., 1990; Hof and Morrison, 1990; Vickers et al., 1992, 1994, 2000; Vickers, 1997; Sampson et al., 1997; Morrison et al., 1998; Morrison and Hof, 2002; Bussiere et al., 2003).

AD is a progressive disorder and develops through a defined sequence of stages involving a specific distribution of pathological alterations (Braak and Braak, 1991). In end-stage AD, both tau and neurofilament triplet proteins accumulate within neuronal cell bodies and dendrites to form neurofibrillary tangles (NFTs) and neuropil threads, respectively. Additionally, dystrophic neurites (DNs) are associated with a sub-population of ß-amyloid plaques, termed neuritic plaques. DNs encompass neurites that have undergone a variety of morphological and/or neurochemical alterations. The pathological changes contributing to end-stage AD pathology may be initiated in the brain many years before the clinical symptoms are apparent. Thus, a preclinical stage of AD is also recognised, characterised by minor cognitive impairments and a lack of tau-labelled neurofibrillary pathology, but

presence of plaques, in neocortical areas (Braak and Braak, 1991; Morris et al., 1996; Vickers et al., 2000). Changes in neuronal intermediate filament proteins may, therefore, constitute the initial cytoskeletal disruption in DNs in preclinical cases of AD, preceding classical NFT formation, tau pathology and overt dementia (Masliah et al., 1993; Vickers et al., 1994; Su et al., 1996, 1998; Nakamura et al., 1997; Dickson et al., 1999). In this respect, neurofilament triplet immunopositive ring- and bulb-like structures, particularly associated with dense fibrillar ß-amyloid plaques, are the first cytoskeletal alteration observed in preclinical AD cases (Dickson et al., 1999; Vickers et al., 1996). Analysis of the distribution of these cellular alterations across aged-control, preclinical and end-stage AD cases provides an opportunity to investigate the development of AD pathology as a continuum.

As discussed above, the neurofilament triplet proteins have been widely studied, both in the neuronal response to injury and the evolving pathology of AD. Conversely, α-internexin, also a member of the neuronal intermediate filament family of proteins, has largely been regarded as an important developmental protein (Kaplan et al., 1990; Fleigner et al., 1994). Accordingly, few studies have investigated the normal distribution of α-internexin throughout neuronal development and within the adult brain. Furthermore, α-internexin has received little attention with regard to neuro-degenerative and neuro-regenerative research, although recent studies indicate that this protein may indeed be involved in such events (for example McGraw et al., 2002). Moreover, with respect to AD, the discrete localisation of neurofilament triplet containing neurons suggests that they alone cannot account for all the cell loss occurring throughout the disease process. In this regard, Shepherd et al. (2001)

demonstrated that neurons containing neurofilament triplet proteins are spared in AD. Additionally, recent studies regarding neuronal intermediate filament inclusion disease (NIFID) have indicated that abnormalities in α-internexin are a major feature of the disease pathogenesis (Cairns et al., 2004a, b, c; Momeni et al., 2005; Mosaheb et al., 2005). Thus, intermediate filament proteins, such as α-internexin, may be implicated in the evolving pathology of the AD.

Chapters 4 and 5 of this thesis have highlighted the cytoskeletal alterations characteristic of neurons undergoing reactive and regenerative events following injury, particularly the early formation of reactive neurofilament triplet protein bulb- and ring-like structures, which are also characteristically associated with ß-amyloid plaques in early AD. Utilising *in vitro* and *in vivo* experimental neuronal injury models, as well as brain tissue from individuals in preclinical and end-stages of AD, this study was designed to determine the developmental localisation of α-internexin, as well as the distribution of this protein within the adult brain. Additionally, to further elucidate the cytoskeletal alterations characterising neuronal injury relative to the evolving pathology of AD, this study also comparatively analysed the involvement of α-internexin in injury-induced neuronal alterations and the neuritic changes associated with ß-amyloid plaque formation in AD. Results from this study are discussed with regard to the 'mass-effect' variant of the Amyloid Cascade hypothesis, proposing that ß-amyloid deposition in AD causes physical damage to surrounding neurons (reviewed by Vickers et al., 2000; Woodhouse et al., 2005).

6.2 Experimental procedures

6.2.1 Primary dissociated neocortical neuron culture, axonal transection injuries and immunohistochemistry

Primary dissociated neocortical neuron cultures were prepared as described in section 2.2.1. To determine the developmental distribution of α-internexin, cultures were fixed between 1 and 21 DIV. The effect of physical injury on α-internexin distribution was assessed following axonal transection injuries performed on cultured neocortical cells at 21 DIV, as described in Section 2.2.2. Injured cultures were fixed at 6 or 12 hours following injury as well as at 1, 2, 3, 4, or 5 days post-injury. Cultures were labelled with antibodies directed against α-internexin relative to either NF-M or tau as illustrated in Table 6.1 (full antibody details are provided in Table 2.1).

As described in Chapter 5 and the introduction above, aberrant accumulation of neurofilament triplet proteins into ring-like formation, characterises an early reactive changed following axonal injury. The proportion of α-internexin, relative to NF-M ring-like structures was calculated at various time points following axonal injury. For quantitation of α-internexin ring-like structures, at least 100 rings were counted across several injury sites of cultures derived from three separate litters. From this, the proportion of α-internexin only rings, NF-M only rings and co-localisation within ring-like structures was calculated.

6.2.2 *In vivo* structural brain injuries

6.2.2.1 Immunohistochemistry

In vivo structural brain injuries were performed as described in Section 2.1.2, Figure 2.1. Animals were transcardially perfused at 1, 7, 14, 21, 42 and 84 days following injury. Non-injured, age-matched controls corresponding to the 1, 42 and 84 days post-injury time points were also perfused. Tissue was processed and sectioned for immunohistochemistry, as described in Section 2.1.4, utilising antibodies directed against α-internexin relative to either NF-M, parvalbumin or calretinin as indicated in table 6.1 (full antibody details are provided in Table 2.1).

Table 6.1. Antibodies used to identify cell populations/compartments/structures within physically injured neurons as well as preclinical and end-stage AD cases

Cell type/structure	Marker
Pyramidal neurons/triplet protein	NF-M
Interneurons	Calretinin
	Parvalbumin
Neuronal intermediate filament	α-internexin
Axons	Tau
ß-amyloid plaques	ß-amyloid*

*See antibody description in section 6.2.3.3 above

6.2.2.2 Quantitative one-step reverse transcriptase RT-PCR for mRNA analysis

To determine potential alterations in α-internexin gene expression in response to injury, tissue immediately medial and lateral to the lesion site, or equivalent regions from non-injured brains, was harvested and processed from post-injury time intervals as described in Section 3.1.2. Quantitative one-step real-time RT-PCR was

performed, as described in Section 2.1.6, using the α-internexin and GAPDH primer pairs displayed in Table 2.2. Statistical analysis indicated that the two groups of control animals were not statistically different and the data for these animals was therefore pooled to form one control group (n = 6).

6.2.3 Human brain tissue samples

To determine the similarities and discrepancies induced in α-internexin in response to experimental models of physical injury and AD pathology, brain tissue was sourced and processed from end-stage AD cases, in addition to preclinical (pathologically aged) and control cases. The end-stage of AD is characterised by profound dementia and the accumulation of ß-amyloid plaques, tau-rich NFT formation as well as extensive but selective neuronal death in the hippocampus and cortex. Whereas, the preclinical phase of AD is defined by the presence of ß-amyloid plaques, but not tau-immunoreactive neurofibrillary pathology in neocortical areas and may be associated with minor cognitive deficits (Braak and Braak, 1991; Mirra et al., 1991; Morris, 1996; Vickers et al., 2000; Morrison and Hof, 2002).

6.2.3.1 Tissue categorisation

Brain tissue samples included seven end-stage AD cases, confirming to the CERAD criteria (Mirra et al., 1991) with Braak stages V and VI distribution of hallmarks (Braak and Braak, 1991), seven preclinical AD cases and four control cases (Table 6.2). Preclinical cases were defined by the presence of widespread ß-amyloid immunoreactive plaques in the neocortex, with the distinction that these plaques

could not be defined as "neuritic" based on thioflavine S staining or immunolabelling with PHF-tau antibodies (Saunders et al., 1998; Vickers et al., 1996). Theses cases, therefore, do not conform to the CERAD criteria for diagnosis of clinical AD (Mirra et al., 1991) and are classified, pathologically, as Braak stage III (Braak and Braak, 1991). Control cases demonstrated no AD-associated pathology, but were of similar ages and post-mortem interval to preclinical and end-stage AD cases.

6.2.3.2 Tissue sourcing

Human brain tissue was obtained from multiple sources including the Institute of Biogerontology Research (USA), the National Health and Medical Research Council Brain Bank (Adelaide, Australia), and the Department of Pathology, University of Sydney (Australia). Brains had been either perfusion-fixed with 2% picric acid/4% PFA, or blocks of cerebral cortex were immersion-fixed in 4% PFA.

Table 6.2. Tissue sources for human control preclinical and end-stage AD cases

Type	Age (yrs)	Gender	Post-mortem interval (hrs)	Pathological diagnosis
AD	65	M	3	AD
AD	66	M	2.8	AD
AD	72	F	4	AD/Pneumonia
AD	74	F	2	Pneumonia
AD	76	F	26	AD
AD	84	F	3	AD
AD	88	M	7	Dementia
Preclinical AD	62	M	20	Thrombosed coronary artery
Preclinical AD	71	M	32.5	Cardiac arrhythmia
Preclinical AD	78	M	2.25	Post-operative
Preclinical AD	81	F	3	Cardiac arrest
Preclinical AD	84	M	3	Cardiopulmonary arrest
Preclinical AD	90	M	2.16	Respiratory arrest
Preclinical AD	91	M	3	Cardiac failure
Control	58	F	28	Coronary Disease
Control	65	M	16	Cardiac infarction
Control	67	M	32	Cardiac infarction
Control	83	F	8.5	Bowel disease

6.2.3.3 Tissue processing

Samples of inferior temporal gyrus and superior frontal gyrus from all case types were sectioned on a cryostat at 40-50μm and tissue sections were immunolabelled for α-internexin relative to tau, NF-M, parvalbumin or calretinin as shown in table 6.1 (specific antibody details are provide in Table 2.1, excluding anti-ß-amyloid antibody, as detailed subsequently). The antibody utilised in the current study, directed against ß-amyloid, was a rabbit antibody recognising all ß-amyloid peptides (1:500, QCB, Hopkinton, MA, USA). For the quantitation of co-localisation between α-internexin and NF-M, 100 ring-like DNs were counted across seven preclinical cases. No differences were observed in the immunolabelling profile for the various antibodies across varying post-mortem to fixation intervals or different fixation protocols.

6.3 Results

6.3.1 α-internexin distribution during development

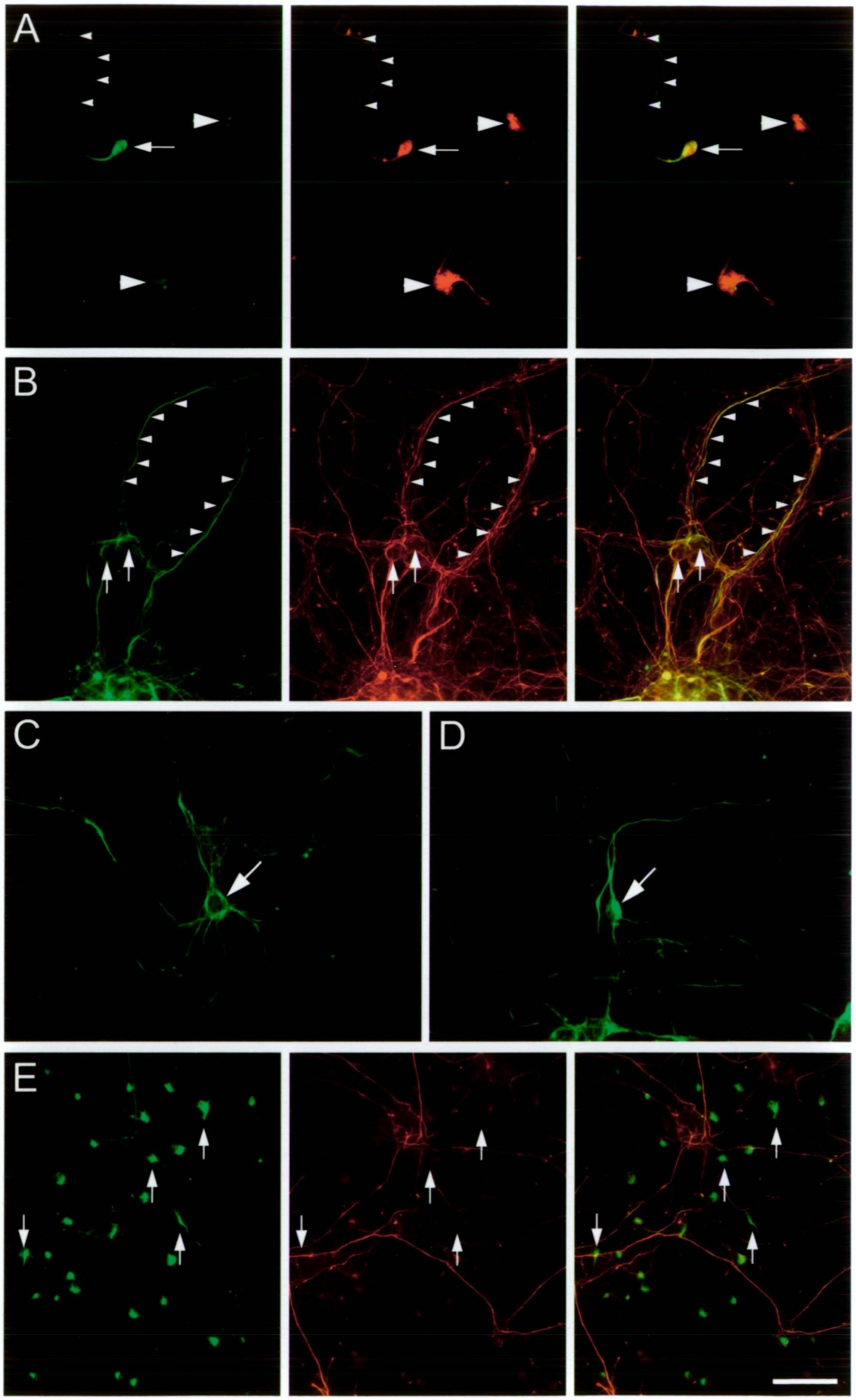
To determine the developmental cellular localisation of α-internexin relative to NF-M, rat neocortical cultures were fixed at a range of time points between 1 and 21 DIV (Figure 6.1). At early time points (1-8 DIV), NF-M immunoreactivity was more abundant than α-internexin both in terms of number of labelled cells and distribution within individual cells (Figure 6.1A). In this regard, α-internexin immunoreactivity was generally not observed independent of NF-M. Additionally, NF-M immunopositive cells often lacked α-internexin immunoreactivity even at time points as early as 1 to 2 DIV.

In older cultures (14-21 DIV), α-internexin immunoreactivity was more abundant than at earlier time points and was generally co-localised with NF-M (Figure 6.1B). In these cultures, α-internexin was located within particular neuronal types, but was variably distributed within these cells. In some cells, α-internexin was distributed in the form of slender filaments surrounding the nuclei and emanating along the neurites (Figure 6.1C). Alternatively, in other cells, labelling was uniformly distributed within the neurites and demonstrated polarised distribution within the soma (Figure 6.1D). Interestingly, after 14 DIV, a distinct population of small cells was observed, which labelled for α-internexin independent of NF-M (Figure 6.1E). This class of α-internexin immunopositive cells generally extended two to four neurites and α-internexin was distributed more abundantly on one side of the cell body than the other.

Figure 6.1 α-internexin is expressed throughout the development of neocortical neurons grown *in vitro*, and is localised to specific cell types

At time points as early as 1-2 DIV α-internexin (A, green) was not observed to precede the appearance of NF-M (A, red), and NF-M labelling was more abundant than α-internexin, both in terms of proportion of cells labelled and distribution within individual neurons (A, small arrowheads denote neurite more extensively labelled for NF-M than α-internexin, large arrowheads denote NF-M only labelled neurons, arrow denotes neuron co-labelled for α-internexin and NF-M). As neuronal cultures matured (14-21 DIV), α-internexin (B, green) became more abundant, but was generally co-located with NF-M (B, red). Arrows in B denote examples of cell bodies co-labelled for α-internexin and NF-M, arrowheads denote examples of neurites demonstrating co-labelling for these markers. Within individual neurons α-internexin demonstrated variable distribution. In cells with pyramidal-like morphology, α-internexin localised as discrete segments around the nuclei and along the neurites (C, arrow). In other cells, α-internexin was distributed relatively uniformly throughout the neurites and demonstrated polarised distribution within the cell body (D, arrow). From 14 DIV onwards, cultures were characterised by the appearance of a new population of neurons labelled exclusively for α-internexin (E, green), which had an immature phenotype, extended few short neurites (arrows denote examples) and did not co-label for NF-M (E, red). Arrows in E denote examples of cells labelled for α-internexin and not NF-M.

Scale bar: A – F = 40μm; G, H = 80μm


A
B
C
D
E

6.3.2 Alterations in α-internexin associated with the reactive and regenerative changes resulting from localised axonal injury *in vitro*

In vitro axonal transection injuries were performed to determine the localisation of α-internexin relative to the reactive and regenerative responses of physically injured axons at 6 and 12 hours following injury as well as at 1, 2, 3, 4 and 5 days following injury. Axonal transection injuries were performed at 21 DIV, when neocortical cultures had reached relative maturity (as described in Chapter 5). Within one day following injury, a variety of reactive and regenerative cellular alterations were observed in and immediately surrounding the lesion sites (Figure 6.2 and Figure 6.3).

Axonal transection elicited the prominent accumulation of both α-internexin and NF-M into abnormal ring- and bulb-like formations. Ring-like structures demonstrated three neurochemical constitutions based on their immunolabelling profile; α-internexin-only, NF-M-only and co-localisation for α-internexin and NF-M (Figure 6.2). Analysis of the presence of ring-like structures demonstrated an absence of rings after four days post-injury with the proportion of all three ring types remaining relatively constant at all time points prior to this. Specifically, of the total ring-like structures observed, 28.8± 3.21% (SE) were labelled for α-internexin only, 35.3±2.19% (SE) were labelled for NF-M only and 35.91±3.38% (SE) were labelled for both α-internexin and NF-M. Importantly, the reactive alterations observed in transected axons were followed by the elaboration of α-internexin immunoreactive neurites into the lesion site (Figure 6.3). These sprout-like processes emanated from the lesion border and were evident as early as 12 hours following injury, persisting until the latest post-injury time point examined.

Figure 6.2 α-internexin and NF-M underwent similar reactive alterations in response to axonal transection *in vitro*

Within 6 hours following axonal transection injuries, reactive cytoskeletal alterations were observed at the lesion border. These alterations included the formation of ring-like structures, labelled for α-internexin (green) and/or NF-M (red), which were present either independently or as a continuum of damaged axons. Double immunofluorescence labelling demonstrated 3 labelling patterns of ring-like structures; α-internexin only (small arrowhead), NF-M only (arrow), co-labelling for α-internexin and NF-M (large arrowhead).

Scale bar: 30μm

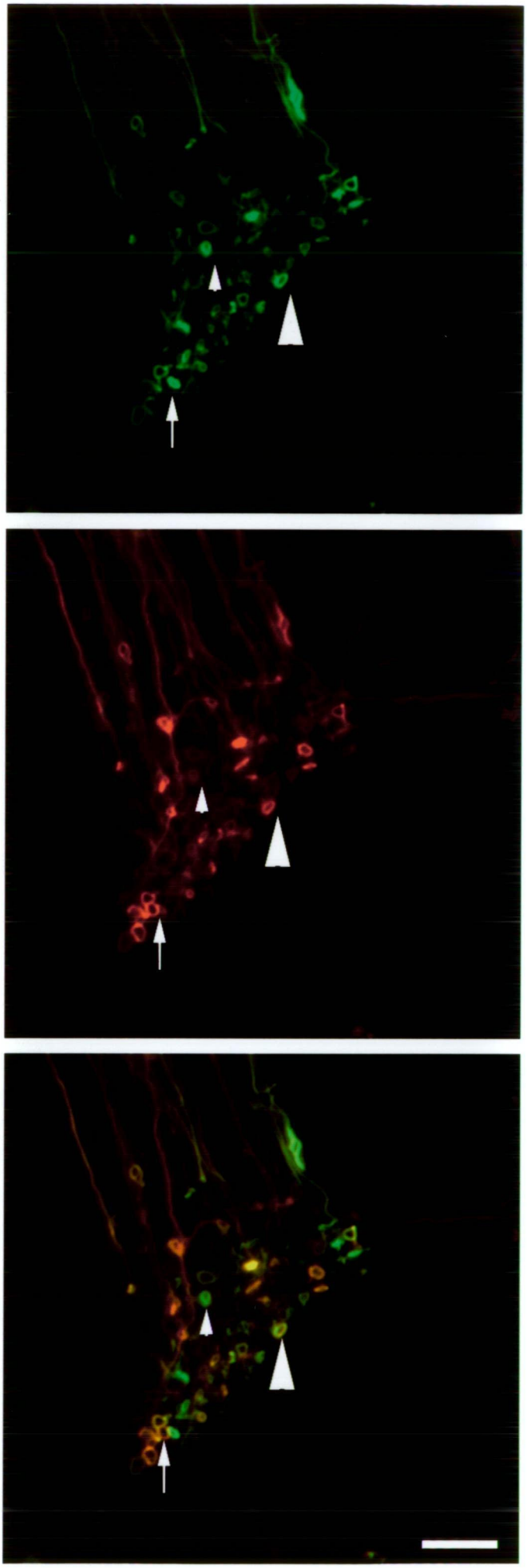
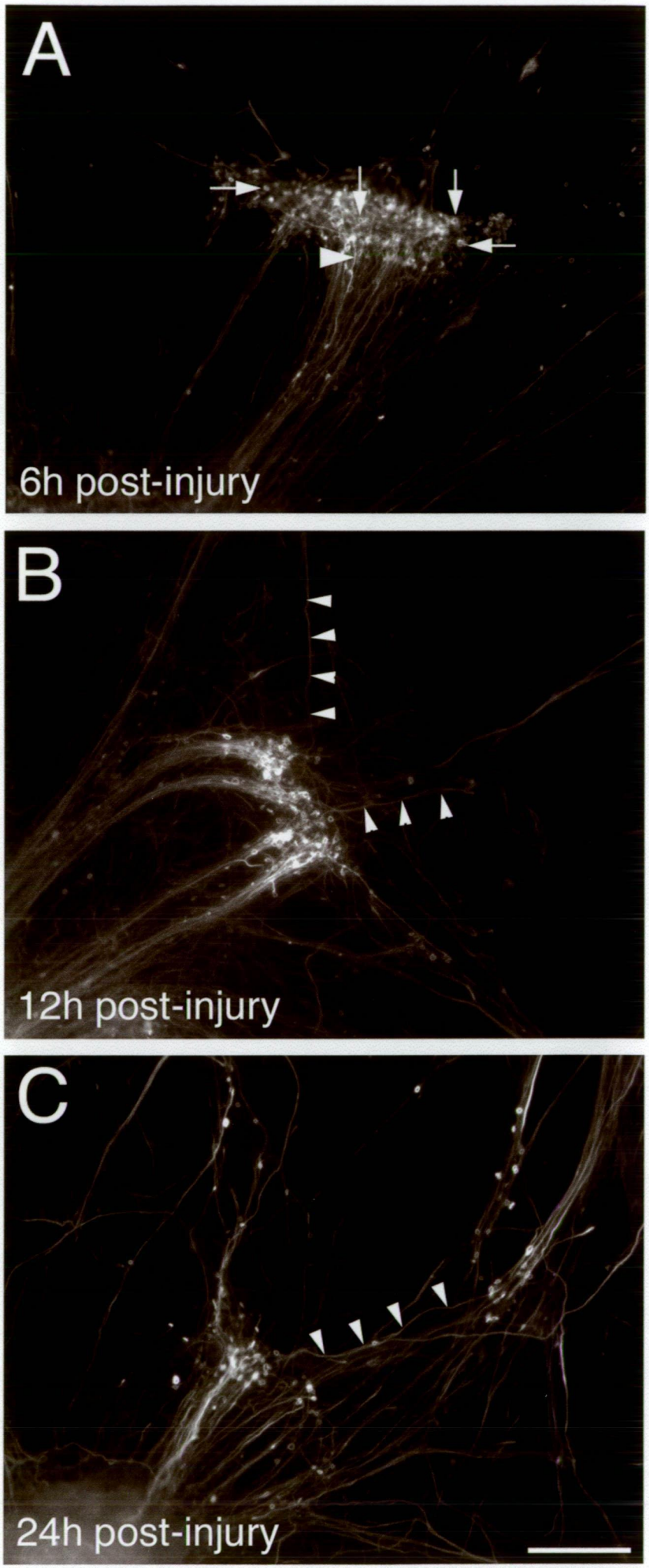


Figure 6.3 α-internexin contributed to the reactive and regenerative changes elicited by axonal transection injury

At 6 hours following axonal transection injury α-internexin had abnormally accumulated into ring-like structures at severed axonal tips (arrows in A denote examples) and was also localised to the swollen tips of severed neurites (arrowhead in A denotes an example). By 12 hours following injury numerous α-internexin positive ring-like structures littered the injury site and α-internexin was also localised to sprout-like structure within the injury site (arrowheads B denote examples). By 24 hours post-injury α-internexin-labelled axons had crossed the injury site (arrowheads in C denote an example).

Scale bar: A, B and C = 40μm


A
6h post-injury
B
12h post-injury
C
24h post-injury

6.3.3 α-internexin distribution in the adult rat neocortex

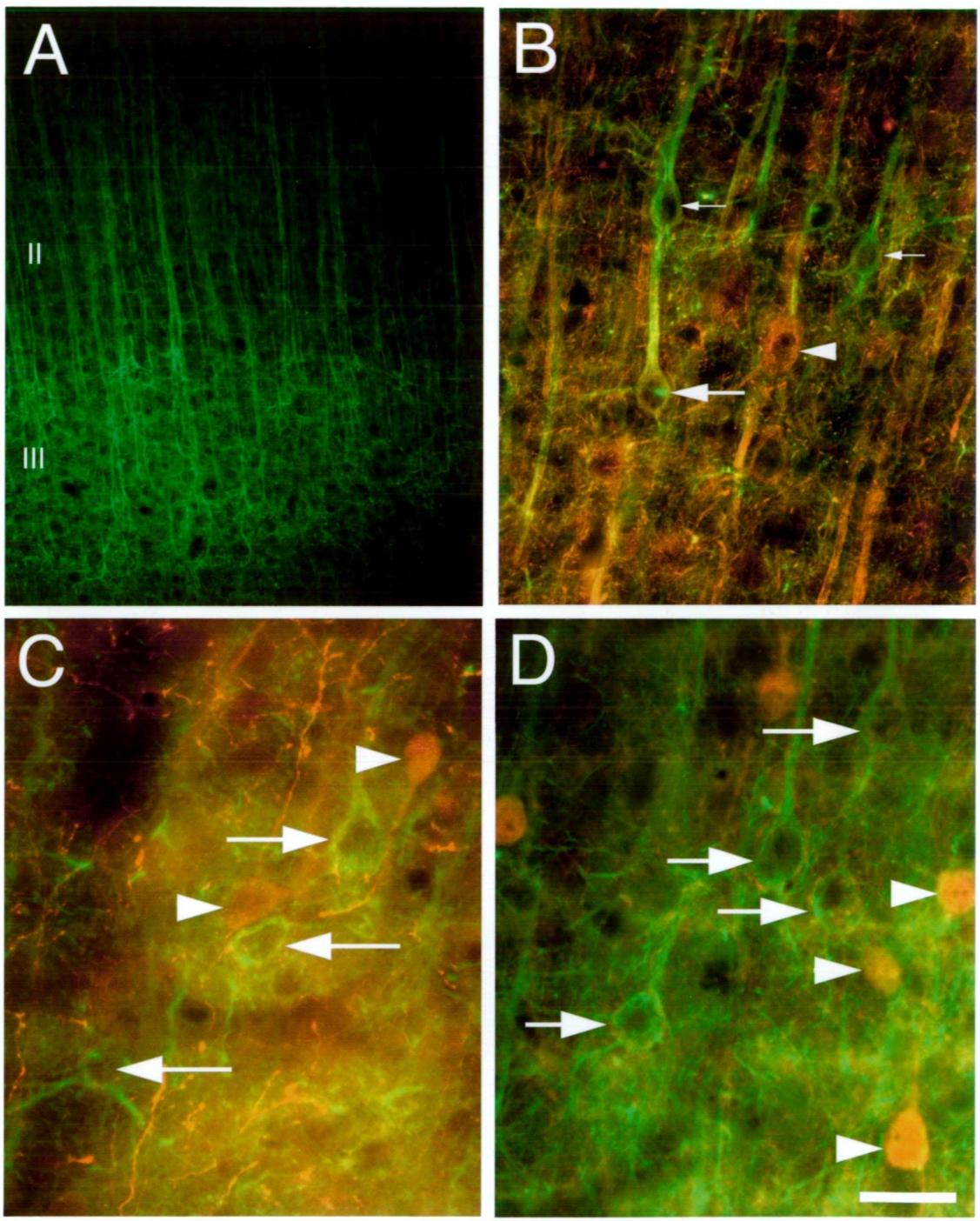
Utilising double labelling immunohistochemistry, the normal cellular localisation and laminar distribution of α-internexin relative to NF-M was determined in the adult rodent neocortex. Double labelling of brain sections from adult control non-injured rats demonstrated a distinct laminar labelling pattern of α-internexin and NF-M (Figure 6.4). Specifically, layer I revealed comparable labelling patterns for both intermediate filament types, with fine processes distributed throughout the layer. Strikingly, layer II/III contained a distinct and previously undescribed band of α-internexin positive cells (Figure 6.4A). Within individual cells, α-internexin was distributed evenly throughout the soma. Moreover, the orientation and morphology of these cells was indicative of a subclass of pyramidal neurons, in that they each possessed a pyramidal-shaped soma, clearly defined apical dendrite directed to the pial surface and laterally extending basal dendrites. Although a proportion of α-internexin immunoreactive cells were co-labelled for NF-M (Figure 6.4B), the great majority were exclusively labelled for α-internexin.

Layer V of the rat neocortex predominantly contained larger NF-M immunoreactive pyramidal cells, however, some α-internexin-labelled cells, similar to those observed in layer II/III were also contained within this layer. Double labelling for α-internexin relative to the markers calretinin (Figure 6.4C) and parvalbumin (Figure 6.4D), confirmed that α-internexin was not localised to the cell body of processes of interneurons. Collectively, these findings confirmed that α-internexin is localised to a distinct subclass of pyramidal neurons in the adult rat neocortex, with only partial overlap with neurofilament triplet containing pyramidal neurons.

Figure 6.4 α-internexin was primarily localised to a population of layer II/III cortical pyramidal neurons in the normal rodent cortex

Immunofluorescence labelling revealed that α-internexin was localised to a distinct population of neurons within layer II/III of the adult rodent cortex, which demonstrated pyramidal-like morphology (A). Double immunofluorescence labelling demonstrated that α-internexin immunoreactive neurons were likely to be a distinct class of cortical pyramidal neurons. For instance, cells in the a α-internexin-labelled population displayed distinctly pyramidal neuron morphological features. Moreover, a majority of these neurons labelled for α-internexin (green, small arrows in B) independently of NF-M (red, arrowhead in B), although co-localisation was demonstrated in a small portion of these cells (large arrow in B). Additionally, in all cortical laminae, α-internexin cells (green) were not immunoreactive for the interneuron calcium-binding proteins, calretinin (C) or parvalbumin (D). Similarly, calretinin and parvalbumin-labelled cells did not contain α-internexin labelling. Arrows in C and D denote examples of α-internexin-labelled cells and arrowheads denote examples of calretinin and parvalbumin cells, respectively.

Scale bar: A = 100μm; B = 45μm; C and D = 30μm


A
II
III
B
C
D

6.3.4 Alterations in α-internexin associated with the reactive and regenerative changes resulting from *in vivo* structural brain injury

To compare and contrast the reactive and regenerative alterations occurring in the *in vitro* axonal injury model with those occurring *in vivo*, adult rats underwent acute structural neocortical injuries and brain tissue was examined at a range of post-injury time intervals. Consistent with observations from the *in vitro* model of axonal injury, ring-like structures were a prominent feature of the lesion site and demonstrated three labelling profiles (α-internexin only, 18.0± 9.0% (SE), NF-M only, 22.5±4.5% (SE), co-labelling for α-internexin and NF-M, 59.5±4.5% (SE)) (Figure 6.5). Injury to the rat neocortex resulted in the formation of a distinct cavity that, between 1-7 days post injury, was surrounded by abnormal ring- and bulb-like accumulations of α-internexin (Figure 6.6A). By seven days post-injury, ring-like structures were still present but a substantial increase in the number of bulb-like structures was also observed (Figure 6.6B). However, from 14 days post-injury onwards both ring and bulb-like structures were absent from the lesion border.

Importantly, concomitant with the resolution of abnormal accumulations of α-internexin, alterations characteristic of attempted regeneration were demonstrated within the injured tissue from seven days post injury onwards. Notably, these alterations included a substantial number of neurites, with similar morphology to the sprouting neurites observed following both *in vitro* axonal injury (Section 6.3.2 this chapter and Chapter 5) and *in vivo* structural brain injury (Chapter 4), elaborated into the lesion cavity (Figure 6.6C). Sprouting neurites were either co-labelled for both α-internexin and NF-M or labelled independently for these intermediate filament

Figure 6.5 α-internexin and NF-M underwent similar reactive alterations in response to acute structural brain injury

Within a week following structural injury to the rat neocortex, abnormal accumulations of α-internexin (green) and NF-M (red) were observed surrounding the lesion site. Similar to the alterations observed following *in vitro* axonal injury, these alterations included the formation of ring-like structures, which demonstrated 3 labelling patterns; α-internexin only (small arrowhead), NF-M only (arrow) and co-labelling for α-internexin and NF-M (large arrowhead).

Scale bar: 20μm.

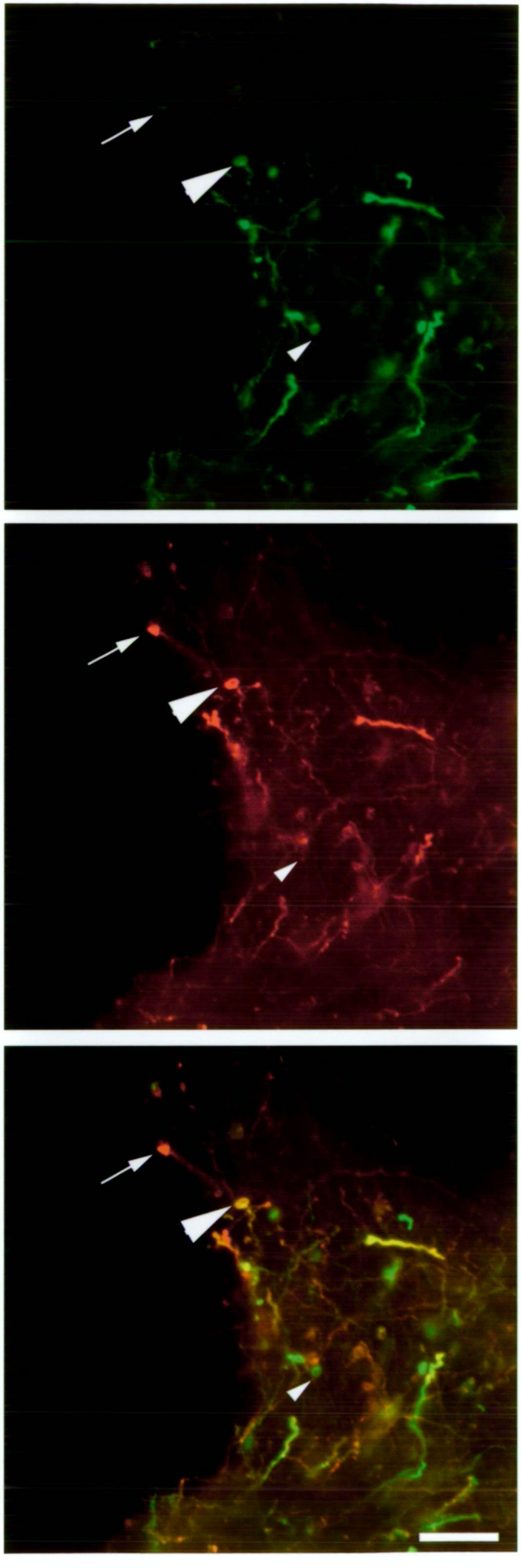
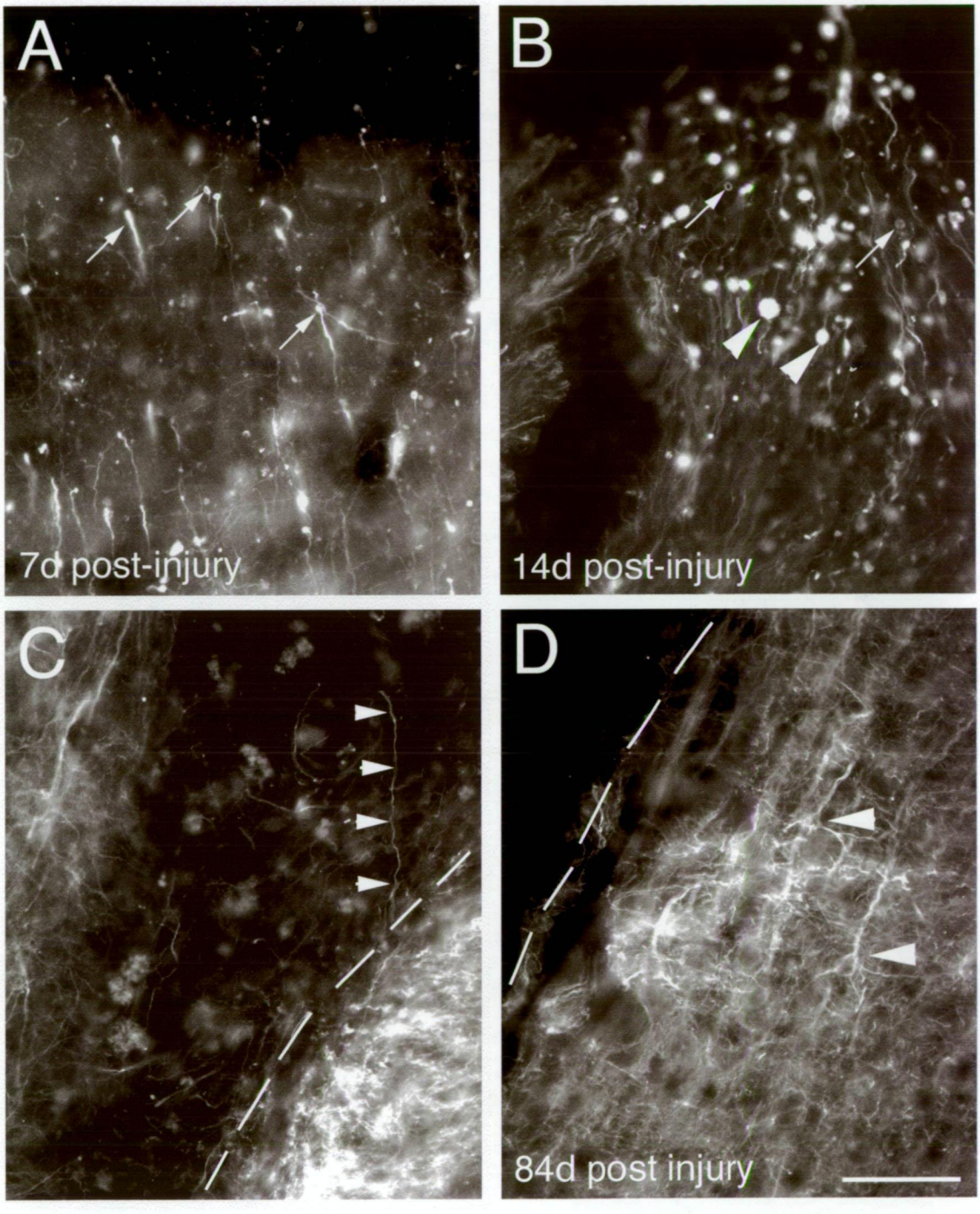


Figure 6.6 α-internexin was involved in both the reactive and regenerative changes evoked by acute structural neocortical injury

Focal injury to the rat cortex resulted in substantial destruction of layers I-IV and evoked a stereotypical sequence of reactive responses involving aberrant accumulation of α-internexin into ring- and bulb-like structures between 1 and 14 days post-injury. At 7 days post-injury, α-internexin-labelled ring-like structures were abundant in injured cortical tissue (arrows in A), whereas α-internexin-labelled bulb-like structures became more predominant by 14 days post-injury (arrows in B denote α-internexin ring-like structures, arrowheads denote α-internexin bulb-like structures). By 7 days post-injury distinct α-internexin-labelled sprout-like structures were observed within the injury site (arrowheads in C denote an example), and by 84 days following injury bulb- and ring-like pathology had resolved, axonal sprouts generally rarely observed within the injury site and relatively normal cytoarchitecture was restored surrounding the lesion border (arrowheads in D denote examples of α-internexin-labelled cells within close proximity to the lesion border). Dotted lines in C and D denote the lesion border.

Scale bar: A, B and C = 60μm; D = 80μm


A
7d post-injury
B
14d post-injury
C
D
84d post injury

proteins. Further tissue healing was indicated by the restoration of relatively normal tissue architecture by 84 days post-injury, as indicated by the presence of non-compromised α-internexin-labelled cells within close proximity of the lesion border (Figure 6.6D).

In addition to immunohistochemical analysis, quantitative real-time RT-PCR was utilised to examine whether any of the alterations in α-internexin distribution, particularly those related to regenerative events, such as injury-induced sprouting, were reflected by changes in α-internexin gene expression following injury. Indeed, quantitative mRNA analysis at 1, 7, 14 and 28 days post-injury indicated significant ($p < 0.05$) increases in α-internexin mRNA levels at 14 days post-injury, which had returned to control levels by 28 days following injury (Figure 6.7).

6.3.5 α-internexin distribution in the adult human neocortex

Interestingly, immunolabelling demonstrated the same distribution pattern of α-internexin in all human cases compared to that in the rat neocortex (Figure 6.8). Consistent with the rat neocortex, a prominent band of α-internexin-labelled cells populated layer II/III of the human neocortex, with orientation and morphology indicative of pyramidal cells (Figure 6.8A). Double labelling confirmed that these α-internexin-labelled cells constituted a distinct class of pyramidal neurons, which did not demonstrate immunoreactivity for NF-M (Figure 6.8B), or interneuron markers, parvalbumin and calretinin (Figure 6.8C). NF-M and α-internexin were co-localised within a small proportion of cortical pyramidal cells. Layer V of the human neocortex was more prominent than in the rat neocortex and was comprised

Figure 6.7 α-internexin expression was significantly increased in response to acute structural neocortical injury

Quantitative real-time RT-PCR was performed to determine alterations in α-internexin gene expression in response to cortical injury at 1, 7, 14 and 28 days following injury. α-internexin mRNA levels were significantly elevated at 14 days post-injury, but returned to control levels by 28 days post-injury.

$P < 0.05$

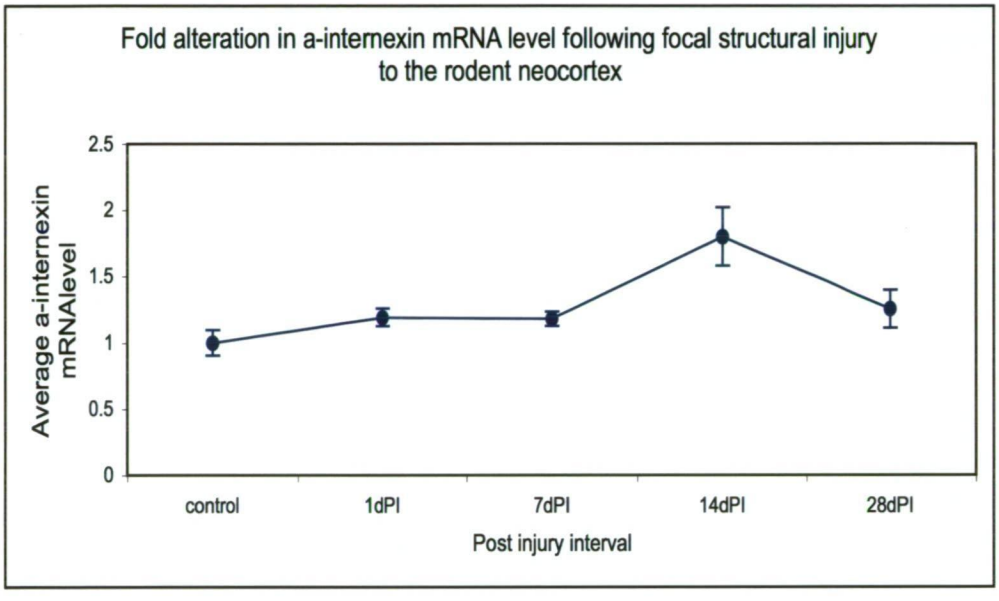
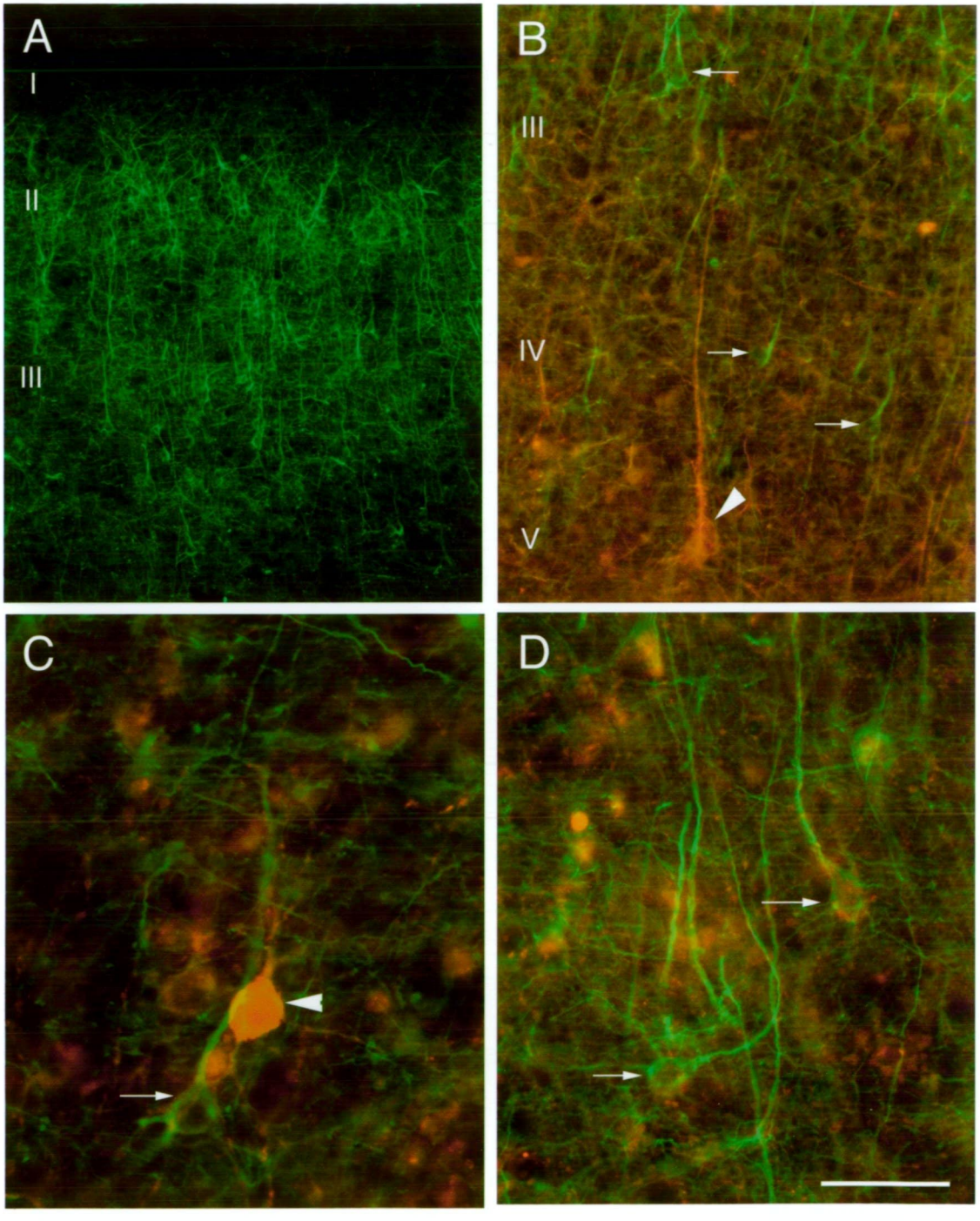

Fold alteration in a-internexin mRNA level following focal structural injury to the rodent neocortex
Average a-internexin mRNAlevel
2.5
2
1.5
1
0.5
0
control
1dPI
7dPI
14dPI
28dPI
Post injury interval

Figure 6.8 α-internexin was localised to a distinct subclass of pyramidal neurons in the human neocortex

Similar to the rat neocortex, α-internexin was localised to a population of pyramidal-like neurons in layer II/III of the human neocortex (A). Double labelling demonstrated that these cells labelled for α-internexin (arrows in B denote examples) independently of NF-M (arrowhead denotes example). Furthermore, α-internexin immunopositive cells (arrow in C) were a discrete class of pyramidal neurons and α-internexin was not localised to interneuron populations (arrowhead in C denotes an example of a parvalbumin-labelled cell). In addition to the distinct band of layer II/III α-internexin-labelled pyramidal neurons, α-internexin was also localised to an array of irregularly orientated cells within layer VI of the human cortex (arrows in D denote examples). Images were taken from the inferior temporal gyrus of a 65-year-old control case.

Scale bar: A = 140μm; B = 60μm; C = 30μm; D = 50μm

Images are courtesy of Dr Tracey Dickson.


A
I
II
III
B
III
IV
V
C
D

predominantly of large NF-M-labelled pyramidal neurons. Within layer VI of the human cortex, similar α-internexin immunopositive neurons to those described for layer II/III were observed. Occasionally these pyramidal-shaped cells exhibited irregular orientation (Figure 6.8D).

6.3.6 α-internexin alterations in end-stage AD cases

Previous investigations have demonstrated a close association between neurofilament triplet protein aberrations and ß-amyloid plaques in AD (see introduction). Double labelling for α-internexin relative to ß-amyloid and tau was performed to determine whether abnormalities in α-internexin distribution were associated with ß-amyloid plaques and tau DNs (Figure 6.9). Indeed, clusters of α-internexin immunoreactive DNs were frequently associated with ß-amyloid plaques (Figure 6.9A). Moreover, bulb-like α-internexin-labelled DNs were occasionally observed to have a tau-immunoreactive core (Figure 6.9B).

6.3.7 α-internexin alterations in preclinical AD cases

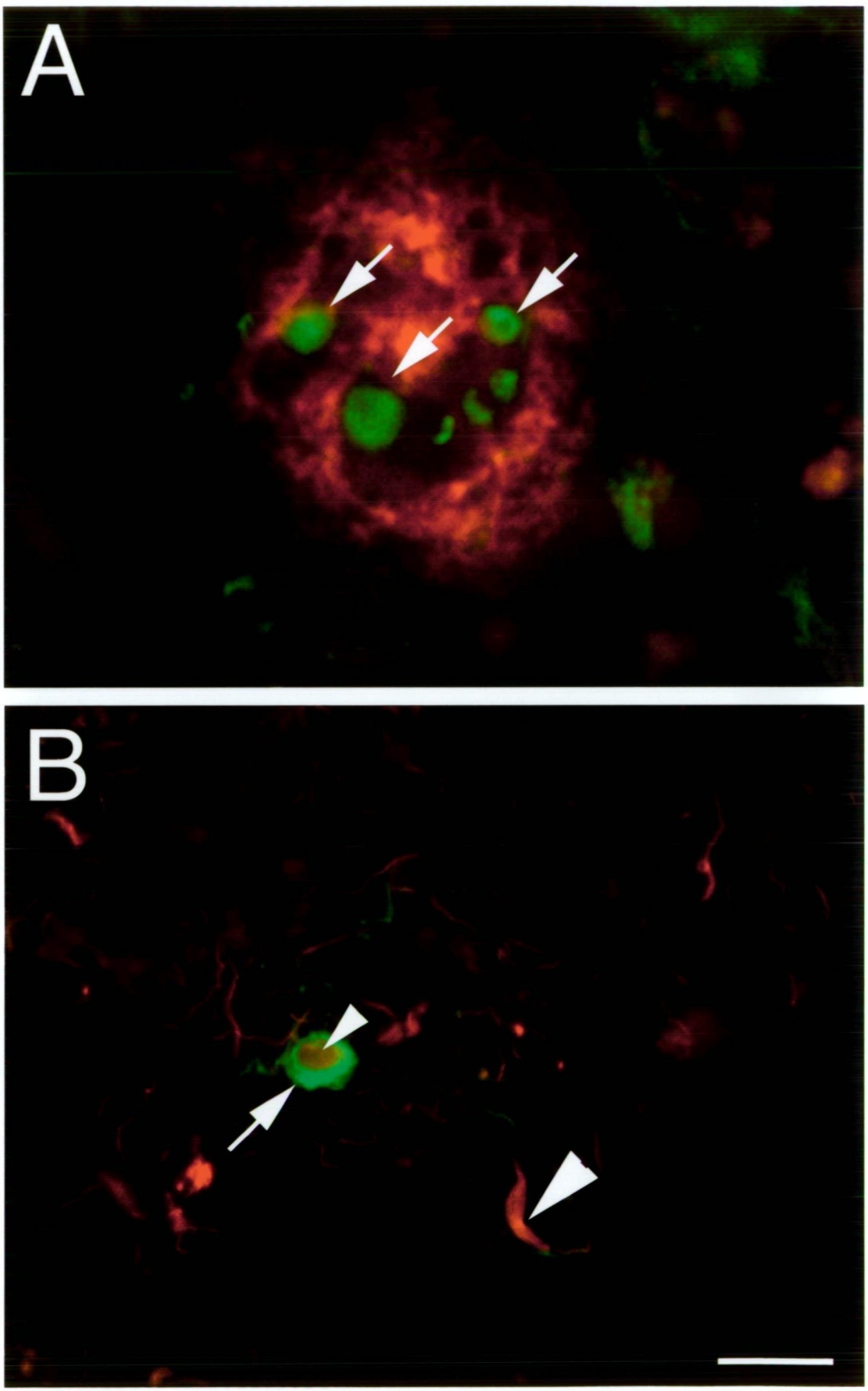
Immunolabelling of preclinical AD cases demonstrated that, in addition to the previously described neurofilament triplet immunoreactive dystrophic/abnormal neurites associated with ß-amyloid plaques in the neocortical grey matter, abnormal accumulations of α-internexin were also localised within ß-amyloid plaques. Consistent with NF-M, α-internexin was localised to a variety of dystrophic neurite subtypes, including bulb-like and ring-like dystrophic neurite morphological forms. Additionally, the ring-like DNs displayed morphology identical to the ring-like

Figure 6.9 α-internexin aberrations were associated with ß-amyloid plaques and tau dystrophic neurites in end stage AD

Double labelling for α-internexin (A, green) relative to ß-amyloid (A, red), demonstrated that clusters of abnormal α-internexin-labelled neurites (arrows) were localised to regions occupied by fibrillar ß-amyloid deposits located within the inferior temporal gyrus and superior frontal gyrus. Classical tau elongated and fusiform abnormal neurites (B, large arrowhead) were observed within α-internexin dystrophic neurite clusters. Occasionally, tau cores (B, small arrowhead) were present within bulb-like α-internexin-labelled dystrophic neurites (B, arrow). Images were taken from the inferior temporal gyrus from a 74-year-old female AD case.

Scale bar: A and B = 30μm

Images are courtesy of Dr Tracey Dickson.


A
B

accumulations of α-internexin observed in experimental models of physical injury (Figure 6.10A).

Plaque-associated, α-internexin-labelled ring-like structures showed variable patterns of co-localisation with NF-M. Of the ring-like structures examined, 48.5±5.9% (SE) were only labelled for α-internexin, whereas only 10±2.2% (SE) were exclusively labelled for NF-M. Co-localisation of NF-M and α-internexin occurred in 41.4 ±5.9% (SE) of rings. These patterns of localisation were independent of the laminar location of the plaque with which the ring-like structures were associated. Additionally, the majority of plaques contained accumulations of both NF-M- and α-internexin-labelled filaments.

6.3.8 Abnormal ring-like accumulations of α-internexin, but not NF-M, were present in end-stage AD cases

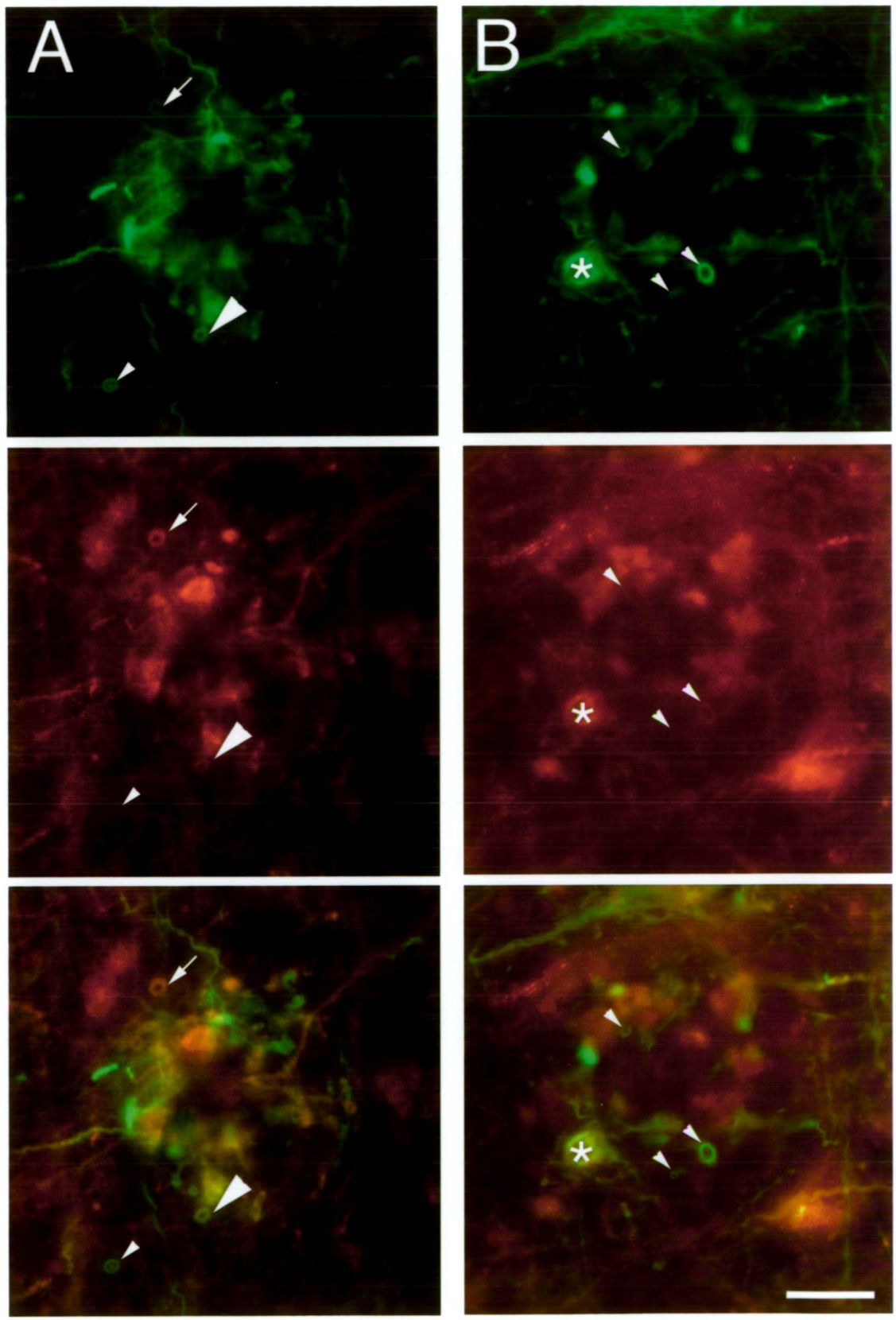
Although immunolabelling demonstrated the presence of both NF-M and α-internexin immunolabelling in DNs associated with ß-amyloid plaques in preclinical Alzheimer's disease cases, end-stage cases were defined by a distinct lack of NF-M immunoreactive ring-like DNs, with the continuing presence of α-internexin ring-like structures (Figure 6.10B). Identical to preclinical AD cases, these α-internexin-labelled ring-like structures were located within the plaque-associated clusters of DNs.

Figure 6.10 α-internexin was involved in the evolving cytoskeletal pathology associated with AD

Aberrant α-internexin immunoreactive ring-like structures were characteristic of preclinical (A) and end-stage (B) AD. Double immunofluorescence labelling for α-internexin (green) relative to NF-M (red) demonstrated distinct similarities and notable differences between preclinical and end-stage AD. In preclinical AD 3 distinct labelling patterns of ring-like structures were observed; α-internexin only, NF-M only and α-internexin-NF-M co-labelled ring-like structures (denoted by small arrowhead, arrow and large arrowhead in A). In end-stage AD, ring-like structures were exclusively labelled for α-internexin and were never observed to be immunoreactive for NF-M (small arrowheads in B). Asterisk in B denotes a spherical bulb-like dystrophic neurite co-labelled for α-internexin and NF-M. Images in panel A were taken from the inferior temporal gyrus of an 84-year-old male. Images in panel B were taken from the inferior temporal gyrus of a 72-year-old male.

Scale bar: A = 20μm; B = 30μm

Images are courtesy of Dr Tracey Dickson.


A
B

6.4 Discussion

Although substantial alterations in neurofilament triplet proteins have been reported to occur in response to a variety of experimental neuronal injury paradigms as well as the evolving pathology of certain neurodegenerative diseases (reviewed by Maxwell et al., 1997; Vickers et al., 2000; Lariviere and Julien, 2004), α-internexin by comparison has received relatively little attention with regard to injury-related alterations and involvement in the pathogenesis of AD. However, recent studies have indicated that aggregations of α-internexin form a major hallmark pathological alteration in NIFID (Cairns et al., 2004a, b, c; Momeni et al., 2005; Mosaheb et al., 2005).

In general, α-internexin has largely been regarded as a predominantly developmental protein. In this respect, α-internexin has been reported as the major intermediate filament component of the neuronal cytoskeleton in early brain development, prior to the bulk expression of the neurofilament triplet proteins (Pachter and Liem, 1985; Fliegner et al., 1990, 1994; Kaplan et al., 1990; Shaw, 1991; Giasson and Mushynski, 1997). Whereas in the mature CNS, neurofilament triplet proteins are the most abundantly expressed neuronal intermediate filament proteins (Shaw, 1991; Hall et al., 2000; Lariviere and Julien, 2004) and are restricted to specific neuronal subclasses, including the large pyramidal cells of the neocortex (Hayes and Lewis 1992; Hof and Nimchinsky, 1992; Hof et al., 2000; van der Gucht et al., 2001; Kirkcaldie et al., 2002).

A recent investigation has, however, reported substantial alterations in α-internexin in response to physical injury in both CNS- and PNS-derived neurons (McGraw et al., 2002), suggesting that the normal expression and role of α-internexin may not be restricted to developmental periods (Julien, 1999; Lariviere and Julien, 2004) and that α-internexin may indeed be involved in neurodegenerative and neuroregenerative events in injury and disease. This chapter, therefore, aimed to re-examine the normal cellular distribution of α-internexin during the development of cortical neurons as well as in response to neocortical injury and the evolving pathology of AD, with the view that ß-amyloid deposition structurally injures surrounding neocortical neurons (reviewed by Vickers et al., 2000; Woodhouse et al., 2005).

Overall, α-internexin was widely expressed in both the rodent and human neocortex, where it was specifically localised to a sub-set of pyramidal neurons. In response to physical damage, α-internexin underwent distinctive changes in expression and cellular distribution and these alterations were demonstrated in both *in vitro* and *in vivo* experimental neuronal injury models. Notably, these abnormalities were identical to alterations in the neurofilament triplet proteins in response to injury and included the formation of ring- and bulb-like structures and the elaboration sprout-like structures into injury sites.

Immunohistochemical analysis demonstrated morphologically and neurochemically identical alterations in α-internexin in human brain tissue from preclinical and end-stage AD cases. Moreover, the neuronal intermediate filament composition of DNs

associated with neuritic ß-amyloid plaques differed between preclinical and end-stage AD cases. Notably, α-internexin immunoreactive ring-like DNs, localised to ß-amyloid plaques, represent a previously undescribed pathological alteration in the evolving pathology of AD. This finding implicates the specific neuronal intermediate filament complement of a neuron in contributing to its relative vulnerability to AD pathology. Furthermore, the similarities between the reactive alterations in α-internexin in models of experimental neuronal injury and AD provide further evidence that ß-amyloid plaques may structurally injure neurons.

6.4.1 The cellular distribution of α-internexin differs from that of NF-M during neuronal development *in vitro*

Similar to the neurofilament triplet proteins, α-internexin demonstrated distinct cellular distribution in developing neocortical cells. However, contrary to reports of α-internexin preceding the expression of neurofilament triplet proteins (Fliegner et al.; 1990; Kaplan et al., 1990; Giasson and Mushynski, 1997), relatively young neurons often lacked α-internexin immunoreactivity, but contained NF-M. This suggests that α-internexin may not be performing a scaffolding function for future triplet protein construction in these cells and that, at least in cortical neurons, α-internexin may not have as large a role in neurite development as previously thought (Shea and Beermann, 1999). Similar to findings from the present study, Benson et al. (1996) reported the presence of α-internexin immunoreactivity in hippocampal neurons at all developmental stages, where it was distributed as long filaments and short fragments in axons and dendrites respectively. Importantly, studies by Benson

et al. (1996) and Suzuki et al. (1997) demonstrated that α-internexin was localised to dendritic spines and post-synaptic densities, implicating it in the formation and maintenance of these structures (Benson et al., 1996; Suzuki et al., 1997). Additionally, Levavasseur et al. (1999) demonstrated that α-internexin is not required for axonal growth during development. Collectively these studies demonstrate that α-internexin may have a variety of roles within developing neurons, including those not typically ascribed to neurofilament triplet proteins.

6.4.2 Injury-induced α-internexin alterations resemble those occurring in NF-M in both *in vitro* and *in vivo* models of experimental neuronal injury

Collectively, results from both the *in vitro* and *in vivo* injury paradigms demonstrated that α-internexin underwent a distinct stereotypy of reactive alterations following neuronal injury, which has been previously described to occur in neurofilament triplet proteins (Dickson et al., 2000; King et al., 2001). These changes included the early predominance of α-internexin-labelled ring-like structures as well as the localisation of α-internexin in swollen neurites surrounding the lesion site and later predominance of α-internexin-labelled bulb-like structures. These alterations resolved over time, reflecting the acute nature of the injury paradigms and indicating that these alterations may comprise a transitory phase in preparation for regeneration (Dickson et al., 2000; King et al., 2001).

Compared to the robust regenerative response of axons in the developing nervous system and PNS, axons in the mature mammalian brain are generally unable to mount a sustained attempt at regeneration *in vivo* (as discussed in Chapter 5). The

present investigation, therefore, aimed to determine the role of α-internexin in potential axonal regenerative attempts utilising *in vitro* and *in vivo* experimental models of neuronal injury capable of eliciting a neurite sprouting response (as demonstrated in Chapters 4 and 5). Immunohistochemical analysis demonstrated that both injury paradigms were capable of inducing the elaboration of numerous α-internexin–labelled sprouts into lesion sites. Moreover, α-internexin expression was significantly elevated following injury, returning to control levels by 28 post-lesion. McGraw et al. (2002) have also recently demonstrated similar alterations in α-internexin in axonal regenerative attempts in both PNS and CNS neurons. Collectively these findings highlight the involvement of α-internexin in neuronal regenerative attempts and emphasise that formation of the glial scar may perturb this response (as discussed in Chapter 4). The increase in α-internexin expression may indicate a final regenerative attempt by a sub-set of neurons prior to establishment of the glial scar. The expression of the neurofilament triplet proteins remained unaltered in response to injury (see Chapter 4), this may reflect the different contributions of neuronal intermediate filament proteins in neuronal regenerative attempts.

6.4.3 α-internexin-labelled DNs represent an additional hallmark pathology associated with neuritic ß-amyloid plaques in preclinical and end-stage AD

An array of cytoskeletal, synaptic, apoptotic and regenerative markers are localised to plaque-associated DNs and these alterations correlate with disease stage (Masliah et al., 1993; Wang and Munoz, 1995; Saunders et al., 1998; Dickson et al., 1999;

Arendt, 2001). Importantly, the presence of ß-amyloid plaques associated with neuronal intermediate filament-immunoreactive DNs and a lack of tau neurofibrillary pathology have been proposed to represent a preclinical, or very early, phase of AD in which only minor cognitive impairment is apparent and end-stage cortical pathology is absent (Braak and Braak, 1991; Morris et al., 1996). In this regard, plaque-associated alterations in neurofilament triplet proteins are proposed to represent the very first cytoskeletal aberration that may predict subsequent development of end-stage AD pathology, including extensive tau-immunoreactive neurofibrillary pathology (Masliah et al., 1993; Vickers et al., 1994, 1996; Su et al., 1996, 1998; Nakamura et al., 1997; Dickson et al., 1999).

An important feature of end-stage AD is the distinct absence of neurofilament triplet immunopositive ring-like DNs, (Vickers et al., 2000; Woodhouse et al., 2005). In this regard, Vickers et al. (1996) and Dickson et al. (1999) have described DNs as developing through 4 distinct phases from preclinical to end-stage AD: (I) the initial formation of neurofilament triplet rings; (II) continued neurofilament triplet accumulation to form bulb-like structures; (III) the appearance of tau cores within neurofilament triplet immunoreactive bulb-like DNs; (IV) DN maturation into predominantly tau/paired helical filament containing structures. Thus, phases I and II are characteristic of preclinical AD, whereas in end-stage AD, phase III and IV DNs have developed (Masliah et al., 1993; Vickers et al., 1994).

Findings from this chapter have demonstrated that α-internexin DNs were a characteristic feature of both preclinical and end-stage AD. In this regard, α-

internexin was localised to ring-like, and bulbous DNs and a proportion of these demonstrated tau cores in end stage cases. However, contrary to the neurofilament triplet proteins, α-internexin-labelled ring-like DNs were present in both preclinical and end-stages of AD, whereas NF-M ring-like DNs were exclusively restricted to the preclinical phase of the disease.

6.4.4 How are ß-amyloid deposition and DN formation linked?

Importantly, the mechanisms linking plaque formation with the earliest stages of neurofibrillary pathology remain to be fully elucidated. The presence of α-internexin ring-like DNs in end-stage AD implicates α-internexin-containing neurons as being vulnerable to the earliest stages of DN formation in this advanced stage of the disease. In this regard, the neuronal intermediate filament complement of neuronal populations may contribute to differential neuronal vulnerability to AD pathology.

The pervading view regarding neurodegeneration in AD is explained by the "amyloid cascade hypothesis" (Selkoe, 1994), which asserts that ß-amyloid deposition results in neuronal death by evoking neurotoxic, biochemical and metabolic pathways (Hashimoto and Masliah, 2003). Intriguingly, the specific neurofilament triplet and α-internexin DNs observed in preclinical and end-stage AD were morphologically and neurochemically identical to the reactive cytoskeletal ring- and bulb-like accumulations characteristic of axonal damage in both of the structural neuronal injury paradigms investigated (Dickson et al., 2000; King et al., 1997, 2001).

The neuronal response to axotomy follows a stereotypical sequence of events, including degeneration of the distal axonal segment and reactive alterations in the surviving proximal axon, which remains attached to the cell body, as well a subsequent retrograde, or cell body, reaction (Smith and Meaney, 2000). Neurofilament triplet proteins may accumulate into bulb-like structures in the severed proximal stump and into ring-like structures within distal axonal terminals (a location from which they are normally excluded) (Maxwell et al., 1997).

Thus, aberrant ring- and bulb-like DNs observed in preclinical and end-stage AD may represent a 'reactive' response of axons, which have undergone some form of physical injury. In this regard, a 'mass-effect' variant of the amyloid cascade hypothesis postulates that continued deposition of ß-amyloid generates space-consuming lesions within the brain, which progressively compress and constrict surrounding neurons and/or block axonal transport to ultimately cause axotomy-like injuries to surrounding neurons (Vickers et al., 1996; King et al., 1997; Dickson et al., 1999; Vickers et al., 2000; Dickson and Vickers, 2001; Woodhouse et al., 2005).

6.4.5 ß-amyloid plaques may physically disrupt surrounding neurons and their processes

The complement of neuronal changes occurring in AD pathogenesis may be explained by physical injury exerted by ß-amyloid plaques (Vickers et al., 1997, 2000). For instance, aberrations in dendritic structure and density are associated with ß-amyloid plaques in both human AD cases and transgenic animal models. Plaque associated dendritic changes include decreased density, abnormal geometry (Le et

al., 2001), dendritic spine loss and atrophy (Tsai et al., 2004) and loss of the dendritic protein MAP-2 within fibrillar plaques (Adlard and Vickers, 2002). Moreover, plaque-associated axonal alterations may include axonal varicosities, swelling, disconnection and loss (Benes et al., 1991; Tsai et al., 2004). Interestingly, these features are also common to traumatically injured axons (Maxwell et al., 1997). Furthermore, neurons surrounding plaques often demonstrate distorted morphologies, whereby they are bent around the plaque and this abnormality can be reversed by treatment with ß-amyloid antibody (Lombardo et al., 2003). Collectively, these studies provide evidence that ß-amyloid plaques may physically displace and/or injure neurons.

Moreover, physical injury in experimental models of neuronal trauma is typically transient, however, ß-amyloid plaques may develop over many years and thus generate chronic lesions that continually activate attempts by the affected neurons to regenerate (Vickers et al., 2000). In this regard, aberrant axonal sprouting and dystrophic boutons, immunoreactive for GAP-43, have also been associated with ß-amyloid plaques (Masliah et al., 1993; Phinney et al., 1999). It has been postulated that ß-amyloid plaque formation may induce a neurotrophic effect on surrounding neurites (Phinney et al., 1999). Conversely, chronic activation of aberrant regenerative attempts, including alterations in the neuronal cell body (the retrograde reaction), may ultimately culminate in the neurofibrillary pathology that contributes to neurodegeneration and neuronal loss (Vickers et al., 2000).

6.5 Conclusion

This study has shown that cortical neurons differ in their complement of neurofilament triplet and α-internexin intermediate filament proteins and has identified a distinct class of pyramidal neurons in both the rodent and human adult brain, which exclusively contain α-internexin. Furthermore this study highlights some of the distinct similarities and discrepancies that exist between these two protein classes during development, in response to injury and during the progressive cytoskeletal pathology associated with AD. Strikingly, both neurofilament triplet proteins and α-internexin were involved in the reactive and regenerative response of structurally injured neurons. Specifically, identical to the neurofilament triplet proteins, α-internexin abnormally accumulated into ring- and bulb-like formations and was localised to sprouting axons. The eventual resolution of triplet protein and α-internexin ring-and bulb-like accumulations in models of experimental neuronal injury suggests that this phase of the neuronal response to injury is like to be transient and associated with a subsequent phase of regeneration, indicated by the elaboration of axonal sprouts.

Identical reactive alterations in α-internexin were shown to be associated with neuritic plaques in both preclinical and end-stage AD cases. These findings provide support for the hypothesis that ß-amyloid deposition in AD causes structural injury and displacement of surrounding neurons and their neurites. The discovery of α-internexin ring-like DNs represents a novel hallmark pathology of AD. Furthermore, the persistence of early indicators of neuronal damage, namely α-internexin ring-like DNs, in end-stage AD highlights the differential vulnerability of neuronal

intermediate filament-containing pyramidal neurons in the different stages of AD and the ongoing ability of ß-amyloid plaques to induce structural injury throughout the disease process and into advanced stages of the disease.

General Summary and Discussion

7.1 Background

The inability of the adult brain to undergo full structural and functional restoration following physical injury is evidenced by the fact that acquired brain injury frequently results in prolonged or permanent mental and/or physical deficits. Intriguingly, however, episodes of mild to moderate TBI are often associated with some degree of functional recovery (Goldberger and Murray, 1988; Wilson, 1998; Medana and Esiri, 2003; Constantinidou et al., 2005; Ding et al., 2005; Povlishock and Katz, 2005). This response may be due to an initial subsidence of various inflammatory reactions and other responses such as diaschisis, where un-injured neurons undergo temporary loss of function (Duff, 2001). Post-injury recovery may also be attributed to long-term plasticity and compensation by undamaged brain regions, possibly including frank replacement of degenerated neurons (Kruger and Morrison, 2002; Emsley et al., 2005) and/or lost neuronal connections (Kristt, 1987; McHale et al., 1995; Dusart et al., 1999; Christman et al., 1997, Deller and Frotscher, 1997; McKinney et al., 1997).

An accumulating body of literature has demonstrated striking examples of adult brain plasticity, adaptability, repair and regeneration in response to CNS injury. Indeed, the adult CNS undergoes a transient increase in plasticity following injury, which is thought to recapitulate changes observed during development and PNS regeneration (Emery et al., 2003). The intrinsic repair mechanisms utilised by the adult brain, in addition to experimental repair strategies aimed at enhancing these responses, have demonstrated that the adult brain is much more adaptable than was

historically appreciated. However, although the endogenous adaptive response may be enhanced by various rehabilitative therapies (Wilson, 1998; Tillerson and Miller, 2002; Nudo, 2003), no surgical or pharmacological treatment currently exists to fully restore pre-injury neural integrity following trauma to the human brain (Smith and Meaney, 2000). Additionally, it is likely that no single therapy will adequately overcome all consequences of brain degeneration following injury and instead several combined and interactive approaches will probably be required.

The inability of the adult brain to undergo effective repair following injury revolves around several factors including the combined influences of an inhibitory CNS milieu, an intrinsic inability of mature neurons to initiate adaptive growth processes and a limited capacity for neurogenesis within the adult brain (Fawcett and Asher, 1999; Schwartz et al., 1999; Domeniconi and Filbin, 2005). Contemporary research is directed at understanding the molecular and cellular factors that inhibit neural regeneration as well as potential adaptive endogenous responses that may be extrinsically manipulated to facilitate effective repair mechanisms (Selzer, 2003). A large literature now exists supporting various experimental approaches aimed at either evoking neurogenesis or promoting axonal regeneration and synaptogenesis in the injured brain. In general, these methods either directly target damaged axons or endeavour to promote a neurogenic response. Alternatively, indirect strategies have been developed to block inhibitory factors associated with the post-injury sequela, such as inhibitory molecules in CNS myelin and the glial scar as well as toxic factors associated with glial activation, cell degeneration and blood-brain barrier breakdown, thus allowing endogenous neuronal responses to be revealed.

Importantly, although many strategies to promote neural repair have been successful in experimental settings, many of these therapies have fallen short in translating to cases of human head trauma. Largely, this lack of transplantation may be attributed to limitations in experimental models simulating all aspects of human head traumas as well as representing only a fraction of the vast variation in the combination of influences associated with the human population, such as age, gender, genetic back ground and environment (Statler et al., 2001). However, experimental models of neuronal/brain injury have proven invaluable in understanding the molecular and cellular mechanisms underlying neurodegeneration and regeneration following injury.

For endogenous reparative attempts to be effective, damaged neurons must survive the initial insult as well as confounding factors, such as glial activation and damage due to breeches in the blood brain barrier. Neurons must subsequently have the capacity to correctly navigate their processes to pre-injury targets. Additionally or alternatively, a traumatic event may induce the elaboration of new neurons from endogenous neural progenitor populations, which subsequently differentiate and integrate into the damaged neural circuitry. In the brain, these changes must occur appropriately within a background of confounding glial responses. Thus, the capacity for endogenous neurogenesis and axonal regeneration following TBI remain contentious and are widely accepted to be significantly influenced by extra-neuronal factors.

It is imperative to note that although many neuro-repair strategies are focussed upon

promoting axonal regeneration and neurogenesis, the intrinsic capacity the damaged brain possesses to evoke these responses remains incompletely understood. It will be crucial to determine the capacity for endogenous brain repair prior to developing therapies that can effectively target intrinsic and potentially latent responses. For instance, the inextricably complex nature of the adult CNS may have evolved poor regenerative capacity for good reason and promoting axonal growth or neurogenesis in an unregulated manner may have dire consequences. This thesis investigated the endogenous mechanisms underlying axonal regenerative attempts and brain repair following structural injury. The following discussion reviews the aims and major findings of this thesis and discusses the relevance of these findings in relation to current understanding of endogenous brain repair mechanisms and methods that are being developed to enhance this response.

7.2 Review of general thesis aims and main findings

This thesis primarily sought to investigate the endogenous capacity for repair following structural injury to the adult mammalian brain and the intrinsic potential for regeneration of axotomised mature central neurons. Collectively, findings from this thesis contribute to understanding the molecular and cellular events responsible for endogenous neuronal regenerative attempts and brain healing following structural injury and highlight important aspects of the neuro-glial response to injury that may be therapeutically targeted to enhance brain repair and neuronal regeneration. The major findings from this thesis are as follows:

7.2.1 The adult mammalian brain possesses the capacity to recruit proliferating and progenitor cells to sites of injury to bring about brain healing

Aim 1:

The initial aim of this thesis was to determine the fate of injury-induced proliferating and progenitor cells and determine whether structural brain injury evokes a neurogenic response capable of producing neurons that replace those lost by injury.

Finding 1:

Acute structural brain injury evoked extensive cell proliferation and induction of neural progenitor cells. Importantly, the primary germinal region of the adult brain, the SVZ, was involved in this process and was likely to elaborate a proportion of the cells, which congregated at the lesion margin and contributed to brain healing by

formation of a glial scar. Ultimately, proliferating and progenitor cells associated with the injury site were identified to be newly generated glial cells or reactive astrocytes, and not neurons. Thus an acute focal lesion to the rodent neocortex did not evoke a neurogenic response or neuronal replacement within damaged cortical tissue.

7.2.2 Co-ordinated and adaptive responses involving all major neural cell populations contribute to brain healing following structural injury

Aim 2

A second goal of this thesis was to investigate the molecular and cellular mechanisms directed at endogenous brain repair, therefore, determining the co-ordinated short- and long-term interactive responses between astrocytes, microglia/macrophages, oligodendrocytes/myelin, neurons and brain vasculature in contributing to brain healing.

Finding 2

Acute structural brain injury elicited an adaptive and co-ordinated neuro-glial response involving microglial activation, astrogliosis and regenerative neuronal alterations, including axonal sprouting and altered gene expression. Although astrogliotic changes may have ultimately hindered axonal regrowth within the injury site, these changes contributed to a brain healing response that resulted in the restoration of relatively normal neural cytoarchitecture within close proximity of the lesion site. Importantly, molecular changes occurring during neuronal regenerative,

or sprouting, events (including up-regulation of the developmentally important genes, GAP-34 and βIII-tubulin) were similar to the genetic alterations characterising early neurite development.

7.2.3 Adult central neurons possess a remarkable capacity to attempt regeneration following axotomy that, in some respects, reflects developmental axonal growth

Aim 3

Thirdly, the response of relatively mature axotomised neurons was investigated in an *in vitro* model of axonal injury to enable the intrinsic reaction of damaged axons to be studied, free of confounding glial factors such as myelination, astrogliosis and inflammation. Specifically, the cytoskeletal mechanisms underlying axonal re-growth following injury and during development were compared to determine whether axonal regenerative growth recapitulates initial neurite development.

Finding 3

Following axonal injury *in vitro*, relatively mature axotomised neocortical neurons demonstrated a remarkable intrinsic capacity for regeneration. This response was characterised by the rapid and sustained elaboration of axonal sprouts into lesion sites. Interestingly, injury-induced axonal sprouts demonstrated microtubule dynamics, morphological features and neurochemical composition, but not actin dynamics, which were similar to developing neurites. These findings indicate that poor axonal regeneration in the adult CNS may be more attributable to inhibitory

factors in the CNS environment than an intrinsic incapacity of injured neurons to re-initiate growth.

7.2.4 The neuronal intermediate filament protein, α-internexin, undergoes identical morphological and neurochemical alterations following physical injury relative to preclinical and end-stage AD

Aim 4

Much of the work covered in this thesis focussed on investigating the role of the neuronal cytoskeleton in injury-induced axonal degeneration and regeneration. Recent studies have drawn important parallels between the reactive cytoskeletal alterations that result from neuronal injury and the early cytoskeletal pathology of ß-amyloid plaque-associated DNs in preclinical and end-stage AD cases. For instance, bulb- and ring-like accumulations of neurofilaments following physical injury are morphologically and neurochemically identical to a sub-class of DNs in AD. Additionally, AD is associated with an aberrant ß-amyloid plaque-associated axonal sprouting response. Collectively, these findings implicate plaque-induced physical injury as a mechanism of neurodegeneration in AD. Thus, a final sub-aim of this thesis was to explore the evolution of DN formation utilising brain tissue from non-AD, preclinical and end-stage AD cases, relative to the reactive events that characterise the neuronal response to physical injury to further elucidate the similarities between plaque-associated DNs and physically injured axons Particular emphasis was placed on examining alterations in the neuronal intermediate filament protein α-internexin, which, unlike the neurofilament triplet proteins has received

relatively little attention in studies of neuronal injury and AD.

Finding 4:

The neuronal intermediate filament protein, α-internexin, undergoes similar alterations to neurofilament triplet proteins in the reactive and regenerative events that characterise the neuronal response to injury. In this regard, both α-internexin and neurofilament proteins abnormally accumulate into ring- and bulb-like formations within injured axons and are subsequently localised to injury-induced axonal sprouts. Intriguingly, morphologically and neurochemically identical DNs are characteristic of the evolving pathology of AD. These findings support the proposal that ß-amyloid deposition inflicts chronic structural injury on surrounding neurons and their processes, evoking an axotomy-like reactive response. Additionally, these findings identified a novel pathological hallmark in preclinical and end-stage AD, namely α-internexin-labelled ring-like DNs, implicating α-internexin containing cells as selectively, but differentially, vulnerable to AD pathology, relative to neurons containing neurofilament triplet proteins.

7.3 Discussion and implications of main thesis findings

A lack of appropriate micro-environmental cues in the adult brain may be implicated in its poor regenerative ability (Mitchell et al., 2004; Emsley et al., 2005). If these cues can be discovered and manipulated, perhaps latent regenerative and neurogenic responses may be revealed. It is now evident that the adult mammalian brain is capable of extensive functional and structural plasticity (Nudo, 2003). A variety of approaches are being investigated and developed to promote recovery from CNS injury. Overall, these strategies aim to replace lost neurons, support the survival of damaged neurons and/or enhance axonal regeneration (reviewed by Olson, 1997). The individual chapters in this thesis have discussed extensively the findings from each study. The remainder of this discussion will therefore examine the broader findings of this thesis, in terms of potential experimental manipulations currently being developed to enhance endogenous neural repair.

7.3.1 Manipulation of neural stem and progenitor cells as a therapeutic tool to promote brain repair following injury and in neurodegenerative diseases

7.3.1.1 Stem and progenitor cells in the adult brain confer neurogenic potential

The initial investigation in this thesis aimed to address a topical and contentious avenue of brain regeneration research, namely the ability of the injured adult mammalian brain to generate new neurons in the aftermath of a physical injury. Previous studies, utilising diverse brain lesion paradigms (as detailed in Chapter 3), have demonstrated significant alterations in cell proliferation and progenitor cell generation in response to brain lesion, including the recruitment of endogenous

migrating and/or progenitor cells to lesion sites, the differentiation of these cells into both neuronal and glial cells and the generation of new neurons that may contribute to neuronal replacement of degenerated neurons (Clarke et al., 1994; Duggal et al., 1997; Holmin et al., 1997; Li and Chopp, 1999; Sahin Kaya et al., 1999; Gu et al., 2000; Magavi et al., 2000; Dash et al., 2001; Jin et al., 2001; Kernie et al., 2001; Arvidsson et al., 2002; Chirumamilla et al., 2002; Parent et al., 2002a, b; Shi et al., 2002; Chen et al., 2003a, b; Nakamura et al., 2003; Tonchev et al., 2003; Douen et al., 2004; Goings et al., 2004; Romanko et al., 2004; Salman et al., 2004; Gotts and Chesselet, 2005; Ramaswamy et al., 2005; Tatsumi et al., 2005).

Rakic (2002) has noted many implications in the interpretation of neurogenic responses in experimental models. For example, many studies are performed in rodent models and may yield different results from studies performed in primates and, therefore, also humans. Additionally, Rakic (2002) highlights important aspects of the methods currently utilised to detect neurogenesis, such as BrdU incorporation and subsequent double labelling analysis. BrdU is incorporated into cells that are synthesising DNA and, thus, *post-hoc* labelling may detect proliferating cells as well as cells which are undergoing DNA repair and apoptosis (reviewed by Nowakowski and Hayes, 2001). However, BrdU detection of cells undergoing DNA repair is likely to be minimal (Cooper-Kuhn and Kuhn, 2002). Another noteworthy point involves studies that have concluded the occurrence of neurogenesis, when in fact the proliferating cells detected may actually be satellite glial cells, closely apposed to neurons (reviewed by Rakic, 2002). Recently developed methods for identifying neural precursor cells, including retroviruses combined with fluorescent markers and

transgenic mice specifically expressing fluorescent markers in a sub-set of precursor cells, in conjunction with sophisticated imaging techniques are now allowing this field to rapidly advance (reviewed by Song et al., 2005). Accumulating evidence indicating that endogenous neuro- and glio-genesis play roles in brain healing warrant further investigation, particularly if these processes can be appropriately manipulated (Plunchino et al., 2005).

It is now widely accepted that the adult brain is capable of producing new neurons. Adult-born neurons arise from stem or progenitor cells, which may be parenchymal-resident or retained within specific germinal zones (Gage, 2002; Emsley et al., 2005). In the SVZ, the exact origin of adult-born neurons is still debated, with evidence suggesting that ependymal cells, astrocytes, specialised adult radial glial and other precursor cells may all act as neural stem cells (Barres, 1999; Doetsch et al., 1999; Laywell et al., 2000; Seri et al., 2001; Doetsch, 2003; Morshead, 2004; Plunchino et al., 2005). Essentially, stem cells possess an unlimited capacity to self-renew and the ability to produce diverse mature cell types, whereas progenitor cells have a limited capacity for self-renewal and differentiation (McKay, 1997; Morshead, 2004). Additionally, the term 'precursor cell' may be used to describe a cell at an earlier developmental stage relative to another cell (McKay, 1997). Remarkably, discrete sub-sets of glia located throughout the brain may form an additional population of latent stem cells (Doetsch, 2003).

Specific regions of the adult mammalian brain, including the dentate gyrus of the hippocampus and SVZ of the lateral ventricles harbour stem and progenitor cells

capable of generating new neurons throughout life (Lois and Alvarez-Buylla, 1993; Eriksson et al., 1998; Johansson et al., 1999; Kukekov et al., 1999; Doetsch, 2003). Stem/progenitor cell proliferation, survival, migration, differentiation and integration are controlled by a vast array of extracellular molecular regulators, including proteins, neurotransmitters and hormones (reviewed by Hagg, 2005). Adult-born neurons have been shown to integrate into functional circuits (Carlén et al., 2002; van Praag et al., 2002). Although neural progenitor cells have been isolated from many regions of the adult brain (Reynolds and Weiss, 1992; Taupin and Gage, 2002; Lie et al., 2004; Emsley et al., 2005), whether the neocortex itself possesses the capacity for neurogenesis remains controversial (Kornack and Rakic, 2001; Gould and Gross 2002; Rakic, 2002; Koketsu et al., 2003). Accordingly, a large proportion of the literature, regarding neurogenesis in the normal adult brain and injury-induced neurogenic responses, indicates that new neurons are most likely to be derived from germinal regions such as the SVZ and subsequently migrate to lesion sites (Alvarez-Buylla et al., 2002; Ramaswamy et al., 2005), although some neurons may be generated from parenchymal resident progenitors (Magavi et al., 2000; Emsley et al., 2005).

Findings from this thesis demonstrated that proliferating cells included astrocytes, microglia/macrophages, neural progenitor cells and neuroblasts. Moreover, alterations in the distribution and expression of the neural progenitor cell marker, nestin, were suggestive of migration from the SVZ to the lesion site. However, double-labelling analysis, in addition to proliferation assays utilising BrdU incorporation, revealed that progenitor and proliferating cells did not undergo

neuronal differentiation in response to acute focal neocortical lesion. Moreover, DCX-labelled neuroblasts were not recruited from their normal migratory route (the rostral migratory stream) to the lesion site. Collectively, these findings demonstrated that this particular lesion paradigm largely results in a gliogenic, and not neurogenic response, in accordance with previous investigations (Holmin et al., 1997; Kuroda et al., 2002; Yoshiya et al., 2003; Douen et al., 2004; Salman et al., 2004). Other studies have, however, demonstrated neurogenesis in response to brain injury (Kernie et al., 2001; Chen et al., 2003b; Rice et al., 2003; Tonchev et al., 2003). The reasons for these conflicting findings were discussed extensively in Chapter 3, thus the remainder of this section of the discussion will focus upon the therapeutic manipulation of stem and progenitor cells in the treatment of brain injury.

7.3.1.2 Neural stem and progenitor cells may be manipulated to promote brain repair

An important feature common to most brain lesion paradigms is the generation of new cells. As discussed above, numerous studies have demonstrated that stem and progenitor cells exist within the adult mammalian brain, capable of generating astrocytes, oligodendrocytes and neurons under both normal and pathological conditions. These factors combined hold great therapeutic promise. For example, endogenous progenitor cells may be manipulated to undergo neuronal differentiation to replace neurons lost by injury or disease (Frisén et al., 1998; Kruger and Morrison, 2002; Lie et al., 2004; Mitchell et al., 2004; Schouten et al., 2004; Kulbatski et al., 2005; Taupin, 2005).

Aside from the manipulation of endogenous neural precursor cells, the transplantation of stem or progenitor cells from autologous, allogeneic or xenogenic sources, provides a second therapeutic avenue for neural repair following brain injury and in certain neurodegenerative diseases (Kulbatski et al., 2005; Taupin, 2005). Indeed, clinical trial have demonstrated that this may a viable method of cell replacement and recovery in Parkinson's disease (reviewed by Bjorklund, 2005; Snyder and Olanow, 2005; Sonntag et al., 2005) and studies are underway to adapt this technique to promote brain repair in Huntington's disease (reviewed by Dunnett and Rosser, 2004). The use of neural stem/progenitor cell transplantation also holds promise for the treatment of human brain trauma. Importantly, in autologous transplantation a patient's own stem cells may be harvested, propagated and manipulated *in vitro* and transplanted back into damaged or diseased regions, thus overcoming potential problems associated with host-donor reactions and ethical implications (Frisén et al., 1998). However, transplanted cells must survive, differentiate and integrate appropriately to be of beneficial value.

Manipulation of endogenous cells may not be limited to producing new neurons. The transplantation of precursor cells or manipulation of endogenous progenitors to differentiate into glial cells capable of enhancing neuronal regeneration may be beneficial. Accordingly, as discussed in Chapter 4, both astrocytes and microglia/macrophages may play important molecular and/or physical functions in supporting neuronal re-growth. Thus promoting, for example, the generation of microglia and macrophages following injury may indirectly result in greater neuronal regeneration through the release of neurotrophic factors. The poor regenerative

capacity of the adult brain has been partially attributed to its immuno-privileged nature, which under normal circumstances acts to protect the CNS, but in cases of injury may act maladaptively to limit the activity of immune cells capable of evoking extensive tissue repair and remodelling (Schwartz et al., 1999). Indeed, studies by Batchelor et al. (1999, 2000, 2002a, b) indicate that microglial and macrophages provide extensive trophic support to regenerating axons following striatal injury. Moreover, neural progenitor cells may be genetically manipulated to produce a range of supportive factors (Pincus et al., 1998). Philips et al. (2001) have shown that stem progenitor transplants producing nerve growth factor result in improved cognitive and motor function following brain injury. The use of transplanted stem cells may also be of therapeutic value in terms of myelin replacement following CNS injury, as well as in a range of demyelinating diseases (reviewed by Keirstead, 2005).

The literature to date has provided varied results regarding the capacity of transplanted stem and progenitor cells to differentiate, migrate and integrate into neuronal circuitry to bring about functional recovery from CNS lesion (for example Fricker et al., 1999; Englund et al., 2002; Hoane et al., 2004; Shear et al., 2004; Wennersten et al., 2004; Brunet et al., 2005). The transplantation of more differentiated neurons may be a more effective transplantation method (Frisén et al., 1998). However, unless transplanted cells are able to integrate and function appropriately they will be of little use in assisting recovery of function following brain injury. Rakic (2002) notes that the relative lack of neurogenesis in the adult brain may not foster an environment that is facilitative for transplant therapies.

It is crucial that the manipulation of endogenous and transplanted neural stem/progenitor cells to promote brain repair be carefully regulated and monitored. The reasons for this are several fold. For instance, such therapies have a variety of ethical implications regarding sources for cell transplantation. Many technical problems are also of paramount importance, including the selection of the ideal cell types to transplant or manipulate and how these cells can be optimised, maintained and tracked (Kulbatski et al., 2005). Additionally, the restriction of neurogenesis in the adult mammalian brain to discrete regions may be an adaptive mechanism to prevent the development of aberrant circuitry. Whereas ongoing neurogenesis in the hippocampus is implicated in memory and learning, seizure-induced neurogenesis has been implicated in the development of abnormally hyper-excitatory circuitry (Parent and Lowenstein, 2002; Parent, 2002).

Abrahams et al. (2004) review work describing *de novo* neurogenesis following stroke, bringing into question whether cell transplants are necessarily required, if endogenous neurogenic responses can be appropriately manipulated. Cell implantation also runs the risk of tumour development and the mechanisms underlying this response remain to be elucidated (Paul et al., 2002; Batchelor and Howells, 2003). Nevertheless, lesion-induced, potentially compensatory, neurogenesis is a viable avenue for future research and warrants further, yet careful, examination (Kozorovitskiy and Gould, 2003). The potential therapeutic effects of manipulating endogenous and transplanted neural precursor cells may be invaluable considering irreversible and extensive neuronal loss frequently accompanies brain injury (Hagg, 2005).

7.3.2 The intrinsic neuronal response to injury is more adaptive than historically appreciated

7.3.2.1 Certain populations of mature CNS neurons demonstrate remarkable capacity to attempt regeneration following injury

There now exists general agreement that the adult mammalian brain is able to mount a transient attempt at regeneration following injury. One of the major factors used to gauge regeneration is the degree spontaneous axonal regrowth following a traumatic incident. Axonal regrowth is identified as the elaboration of fine axonal sprouts into or around lesion sites (Foerster, 1982; Povlishock and Becker, 1985; Kristt, 1987; McHale et al., 1995; Salin et al., 1995; Christman et al., 1997; Deller and Frostcher, 1997; McKinney et al., 1997; Batchelor et al., 1999, 2002b; Dusart et al., 1999; Dickson et al., 2000; Moon et al., 2000; Pastor et al., 2000; King et al., 2001; Chung et al., 2002, 2003; Haas et al., 2004). Taken together, these studies establish that mature CNS neurons are able to re-initiate growth following injury and implicate the up-regulation of important growth related genes and cytoskeletal reorganisation in this process. Additionally, although glial reactions may be detrimental to axonal regrowth, accumulating evidence indicates that certain glial activities may in fact promote axonal regeneration (for example Prewitt et al., 1997; Batchelor et al., 1999, 2002a, b; Dusart et al., 1999; King et al., 2001).

Since the discovery that the adult CNS retains a remarkable degree of intrinsic regenerative capacity, a major avenue of contemporary neuronal repair research has been directed at facilitating axonal regrowth following injury through manipulation

of micro-environmental signals, glial modulation and cell/tissue transplant strategies. The ability of CNS neurons to undergo axonal regeneration depends on the combined influences of a neurons ability to re-express growth-related genes, the availability of substrate and trophic factors that facilitate regeneration and the presence of inhibitory molecules (Tuszynski and Gage, 1995). Intra-neuronal factors include the survival of the cell body, axonal sprouting from the proximal stump, axonal re-myelination and appropriate formation of contacts between newly generated axon terminal and their targets (Rhodes and Fawcett, 2004). Repair strategies are aimed at addressing one or several of these factors.

Findings from this thesis demonstrated that some of the aspects of attempted axonal regeneration recapitulate developmental patterns of growth, including morphological alterations, microtubule dynamics and cytoskeletal composition. However, regenerative growth cones were observed to be less complex than developmental growth cones and regenerative growth was less reliant on actin dynamics, as indicated by continued axonal sprouting in the presence of the actin depolymerising agent latrunculin A, which largely abolished developmental neurite growth. Additional discrepancies between developmental and regenerative growth have also been noted by other authors (reviewed by Selzer, 2003). These include an abundance of neurofilaments at the tips of regenerating axons, in addition to a lack of a discrete growth cone (McHale et al., 1995) and the formation of a dystrophic growth cones on regenerating axons (Tom et al., 2004).

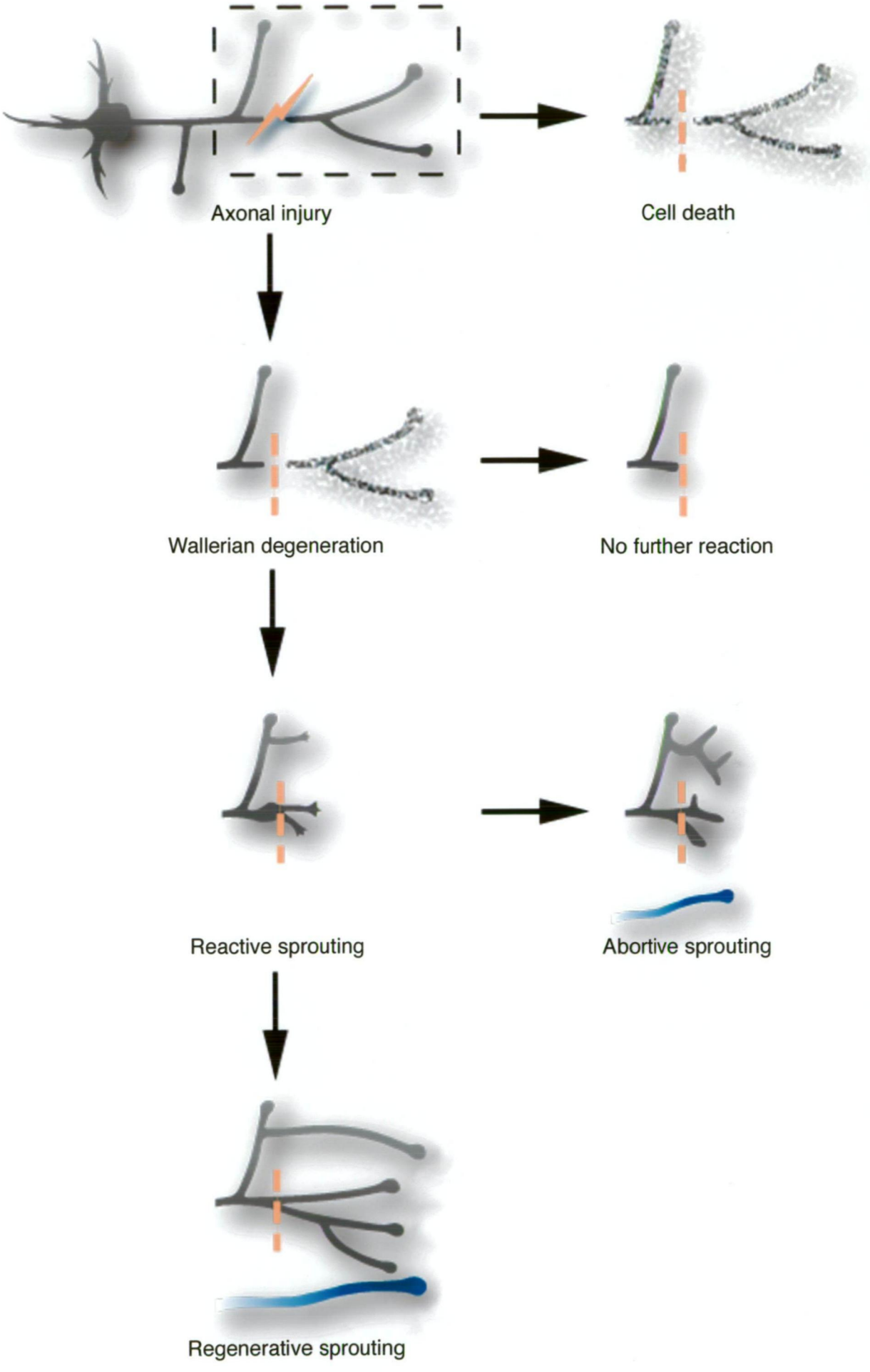
Axonal regrowth following injury may be either abortive or regenerative. It is useful

here to clarify the difference between these growth patterns. Essentially, axonal regeneration is the regrowth of a damaged axon to innervate its original target, whereas abortive growth defines a transient, but unsuccessful, attempt by an injured axon to regrow (Cotman et al., 1994; Kapfhammer, 1997). In this respect, neurons from the adult CNS are largely believed to undergo an abortive sprouting response following injury, whereas those in the PNS may effectively regenerate. Injury-induced axonal sprouting in the adult CNS is usually localised to the local neuropil and does not involve long distance re-growth (Rhodes and Fawcett, 2004). The terms 'abortive' and 'regenerative' may also be used more loosely to describe the continuum or variety of different responses that may occur following axonal injury, involving both frank axonal growth from the injured axon in addition to collateral axonal growth from undamaged regions of the injured axon or entirely un-injured axons from other neurons. Ultimately, axonal growth may result in reinnervation of the original target or compensatory regrowth to approximate targets (see Figure 7.1). The formation of new synapses following an injury is referred to as reactive synaptogenesis (Cotman et al., 1994) and may have adaptive or maladaptive consequences.

Collateral sprouting from undamaged axons is believed to be the major mechanism of functional regrowth in experimental models of CNS injury (Deller and Frostcher, 1997; Kapfhammer, 1997), however, some studies, including observations from this thesis, indicate that terminal sprouting may also occur (Povlishock and Becker, 1985; King et al., 2001). In many studies of brain lesion, including stroke (Stroemer et al., 1995; von Euler et al., 2002; Carmichael, 2003) and physical trauma (as cited

Figure 7.1. Diagrammatic representation of potential neuronal responses to axotomy

Axonal injury may evoke degenerative, reactive and/or regenerative outcomes. Attempted regeneration in the adult CNS manifests as an axonal sprouting response, generally restricted to the local neuropil. Post-injury sprouts are believed to arise from collateral sources, i.e. undamaged axons or undamaged regions of the injured axon. However, terminal sprouting may also occur, where sprouts are elaborated from the severed axonal tip. The occurrence of terminal sprouting in the CNS remains contentious. The diagram to the right shows how some of these processes may occur. Not all possibilities are illustrated and combinations of these processes may also occur. Axonal injury may result in cell death. Alternatively, the proximal axonal segment and cell body may survive following Wallerian degeneration of the distal segment. The proximal axonal segment may undergo no further change or exhibit a reactive axonal sprouting response. Reactive sprouting may proceed to abortive sprouting, localised to the local neuropil, or regenerative sprouting, whereby terminal sprouts and/or collateral sprouts re-innervate the original target cell(s). Notably, reactive sprouting may contribute to innervation of non-target cells and generate aberrant circuitry. This process has been implicated in the development of post-traumatic epilepsy following human brain trauma. Grey processes indicate terminal and collateral axonal sprouts elaborated from the axotomised neuron, the blue process represents collateral sprouting from an un-injured neuron.


Axonal injury
Cell death
Wallerian degeneration
No further reaction
Reactive sprouting
Abortive sprouting
Regenerative sprouting

above), it is not clear whether axonal sprouts derive from collateral sources or frank regeneration of damaged axons. Interestingly, studies from the PNS and nervous systems of lower vertebrates and invertebrates suggest that frank axonal regeneration is common in these systems (as discussed in Chapter 5) and therefore, potentially in the CNS of mammals. However, fundamental differences in growth cone formation may contribute to poorer regenerative potential in the mammalian CNS (Tom et al., 2004). Utilising a neonatal spinal cord injury model Bernstein-Goral et al. (1997) have shown that collaterally sprouting and regenerating axons may have different growth requirements. Nevertheless, axonal sprouting is likely to be implicated in the gradual recovery observed following human brain trauma. Intriguingly, certain populations of neurons may survive for several months prior to undergoing an axonal sprouting response, as indicated in the case of cerebellar Purkinje cells (Dusart et al., 1999).

Historically it has commonly been assumed that neurons from the mature mammalian CNS have very limited capacity for regeneration. In light of accumulating data regarding CNS regeneration, it is now evident that certain populations of mature central neurons possess a capacity for repair following injury, which may be largely limited by a non-facilitative environment (Olson, 1997). In fact, studies reviewed by Fawcett (1997) and Olsen (1997) indicate that most CNS neurons are capable of extensive regeneration when provided with a facilitative environment. These studies demonstrate that peripheral neurons are able to grow in CNS white matter and regenerating CNS axons grow effectively into peripheral nerve grafts. This raises the possibility that injured CNS tissue, rather than non-

injured CNS tissue is the main inhibitory factor in CNS regeneration (Rhodes and Fawcett, 2004). In this respect, many growth inhibitory molecules, generally categorised as myelin- or extracellular matrix-associated, are only associated with, or are up-regulated in, the astroglial scar (reviewed by Rhodes and Fawcett, 2004).

Although many studies have provided examples of axonal sprouting following injury, the mechanisms underlying this process are still not fully understood and the extent to which injured axons are able to undergo intrinsically driven attempts at regeneration remains to be elucidated. Findings from Chapters 4 and 5 of this thesis, in combination with the studies cited previously, demonstrate that neurons from the CNS are able to undergo an adaptive sprouting response following injury. Moreover, results from Chapter 5 of this thesis indicated that this response is likely to be an intrinsically driven process, at least for neocortical neurons, which were able to undergo a sustained axonal regrowth when grown *in vitro,* free of confounding glial effects.

7.3.2.2 Numerous experimental approaches are now being developed to facilitate axonal regeneration

Although no therapies currently exist to treat brain injury and resultant axonal damage, various strategies have been used to slow the progression of degeneration. These approaches include magnesium administration, as magnesium levels are often reduced following brain injury; administration of Dexanabinol, a non-competitive NMDA receptor antagonist; administration of hypertonic saline; mild post-injury hypothermia; decompressive craniectomy to combat raised intracranial pressure; and

CsA administration, which may prevent mitochondrial damage and abnormalities in calcium regulation that cause cytoskeletal and other damage (Sahuquillo and Poca, 2002; Bayir et al., 2003). Many studies regarding the capacity of CNS neurons to regenerate have focused on injury to bundles or tracts of neurons associated with a high degree of myelination, such as those in the spinal cord. In this respect, the lack of regeneration of mammalian CNS neurons has been primarily attributed to inhibitory factors within CNS myelin and the extracellular matrix as well as factors associated with formation of the glial scar. A large proportion of experimental approaches are therefore directed at circumventing these factors. Interestingly, axons are capable of parallel, but not non-parallel, growth on CNS white matter, however, these studies were performed by applying cultured cells to sections of brain tissue and thus axons may have contacted potentially facilitative substrates, to which they are not normally exposed to during regenerative attempts (Pettigrew and Crutcher, 1999). Additionally, work reviewed by Batchelor and Howells (2003) brings into question the extent of myelin-associated inhibitory activity during axonal regeneration.

Due to the relative accessibility of the spinal cord compared to the brain, models of spinal cord injury are frequently utilised to assess the outcome of various experimental strategies to promote axonal regeneration. It is important to note, however, inherent problems associated with the methods utilised to perform spinal cord injuries. It is crucial to differentiate between fibres that extend into or across lesion sites, which may represent frank axonal regeneration of axons that were spared from the injury (Selzer, 2003). Nevertheless a variety of approaches have

been employed to promote regeneration of CNS axons. These strategies can be broadly divided into transplantation or cellular approaches and molecular approaches (reviewed by Tuszynski and Gage, 1995; Olson, 1997; Fawcett and Geller, 1998; Fawcett and Asher, 1999; Batchelor and Howells, 2003; Selzer, 2003).

Peripheral nerve grafts or foetal tissue can be transplanted into the injured CNS to guide regrowing axons and to bridge lesions. Additionally, injection/transplantation of particular cell populations, including neural stem/progenitor/precursor cells, olfactory ensheathing cells, or macrophages, can be utilised to replace degenerated cells, guide axonal regrowth or produce trophic factors that enhance axonal growth, respectively. Molecular approaches target the underlying molecular mechanisms that inhibit axonal growth (Selzer, 2003). In this regard, a variety of strategies are under development, including the use of antibodies to block inhibitory molecules, the enzymatic digestion of inhibitory molecules, the addition of appropriate trophic factors and genetic manipulation of factors intrinsic to neurons, such as an up-regulation of survival- and regeneration-associated genes. Additionally, studies have also determined that robust regenerative attempts can be promoted in the absence of CNS glia, however, this is not necessarily paralleled by increased functional outcome (Moon et al., 2000). Collectively, these studies demonstrate that, when provided with a facilitative environment, central neurons are capable of substantial and sustained regenerative growth.

Interestingly, different forms of metallothionein may be crucial for regulating the neuronal response to injury. Metallothioneins are small zinc binding proteins found

predominantly within astrocytes and thought to confer neuroprotection to neurons from oxygen free radical and metal-induced neurotoxicity (reviewed by Chung and West, 2004). Recent studies also indicate that metallothioneins may have a pivotal role in the neuronal response to injury. Metallothionein III has been shown to inhibit axonal regeneration *in vitro* (Chung et al., 2002), whereas metallothionein IIA promotes axonal regeneration *in vitro* and brain healing *in vivo* (Chung et al., 2003). Interestingly, signals released from injured neurons appear to be crucial for stimulating metallothionein expression in astrocytes (Chung et al., 2004), reiterating the complex and dynamic interactions between neuronal and glial cells in the CNS response to injury.

7.3.3 Aberrant sprouting responses may be generated by structural brain injury and the evolving pathology of AD

7.3.3.1 Axonal injury may evoke maladaptive reactive and regenerative responses

Similar to therapies that aim to promote post-injury neurogenesis or cell replacement, strategies that enhance regrowth of damaged axons must also be tightly controlled. Thus, although evading factors that inhibit axonal regeneration is a major aim for restoring connectivity within well defined long distance axonal tracts, such as in the spinal cord, inhibition of aberrant regeneration might prove a useful approach in certain forms of acquired brain injury. Accordingly, agents that specifically hinder or promote fundamental stages of the stereotypical axonal response to injury may be utilised to combat potentially maladaptive axonal responses. In this regard, findings from this thesis have demonstrated that targeting certain components of the neuronal

cytoskeleton, to cause microtubule stabilisation or destabilisation may be of therapeutic value.

Microtubules are often lost following physical axonal injury, thus, stabilisation of the microtubule cytoskeleton, with agents such as taxol, may counteract microtubule dissolution, a notion supported by findings from Adlard et al. (2000). Moreover, findings from this study in addition to a studies by Letourneau (1984) and Vuorinen (1988, 1990) indicate that taxol induced microtubule interference impedes axonal regeneration and may therefore prevent maladaptive neurite growth. Perez-Espejo et al. (1996) demonstrated that taxol improves functional outcome following spinal cord injury. Additionally, Chung et al. (2002) have shown that metallothionein III application inhibits axonal regeneration *in vitro*, causing similar morphological alterations to those observed in taxol-induced microtubule stabilisation.

Incorporated in the process of axonal growth following injury is the possibility of aberrant re-innervation of targets or innervation of inappropriate targets. In this regard, studies in both *in vitro* (McKinney et al., 1997) and *in vivo* (Salin et al., 1995) experimental models suggest that post-injury sprouting may not always be functionally appropriate. These studies have provided evidence that aberrant hyper-excitable axonal connectivity may result from experimental brain lesion. This phenomenon has been implicated in the development of post-traumatic epilepsy following human head trauma (Larner, 1995; McKinney et al., 1997; Prince, 1997; Prince et al., 1997; Jacobs et al., 2000; Santhakumar et al., 2001).

Importantly, intrinsic mechanisms may be in place to actively prevent plasticity in certain regions of the adult CNS. Indeed, the existence of perineuronal nets (specialised accumulations of extracellular matrix molecules around the cell bodies and dendrites of certain populations of neurons, including cortical neurons) may act to inhibit aberrant neuronal growth and accidental circuitry formation (Rhodes and Fawcett, 2004). Thus, when endogenous reactive and regenerative mechanisms become pathological or when intrinsic regulation of regeneration is disrupted it may be necessary to therapeutically intervene. Taken together, these studies highlight the need for further investigation of true versus maladaptive regenerative attempts following neuronal/brain injury and indicate some agents which may be utilised therapeutically to target specific aspects of the neuronal response to injury to bring about appropriate axonal regrowth and inhibit detrimental alterations.

7.3.3.2 The evolving pathology of AD involves similar reactive and regenerative cytoskeletal alterations to those observed following structural neuronal injury

As discussed in Chapter 6 of this thesis, DNs associated with ß-amyloid plaques in preclinical and end-stages of AD are morphologically and neurochemically identical to the reactive alterations described in experimental models of structural neuronal/brain injury (Vickers et al., 1996, 2000; King et al., 1997, 2000a, b, 2001; Dickson et al., 1999, 2000; Woodhouse et al., 2005). This has lead to the proposition that ß-amyloid deposition causes structural axonal injury (reviewed by Vickers et al., 2000; Woodhouse et al., 2005). One abnormality frequently observed in association with ß-amyloid plaques is profuse, aberrant axonal sprouting (Masliah et al., 1991,

1992), indicating that, as a result of the chronically evolving nature of ß-amyloid depositions, neurons are promoted into a state of un-regulated growth/regeneration (Vickers et al., 2000). As discussed above, microtubule stabilisation effectively blocks axonal sprouting. Common methods may be therefore utilised to prevent maladaptive ß-amyloid plaque-associated axonal sprouting in AD.

AD is characterised by various abnormalities in protein folding and aggregation, including the accumulation of the microtubule binding and stabilising protein, tau, into fibrillar cytopathological inclusions and the extracellular deposition of ß-amyloid into ß-amyloid plaques. Utilising an *in vitro* model, Michaelis et al. (1998, 2005) demonstrated that taxol application was protective against ß-amyloid toxicity and may therefore hinder neuronal degeneration in AD. Furthermore, pathological alterations in tau are associated with impeded axonal transport and brain degeneration (Trojanowski et al., 2005). Microtubules are the main conduit for axonal transport and their function relies upon appropriate interactions with microtubule-associated proteins including tau (Tanaka and Sabry, 1995; Korey and Van Vactor, 2000). Thus, microtubule stabilising compounds may be of therapeutic use in preventing tau-associated pathological disruptions (Zhang et al., 2005).

Trojanowski et al. (2005) review a large body of literature indicating that protein misfolding, resulting in pathological loss- or gain-of-function, is common to many neurodegenerative diseases and is implicated in aberrant axonal transport. For example, tau recruitment into cytopathological inclusions, such as NFTs, DNs and neuropil threads, characteristic of AD, may result in microtubule destabilisation and

loss, warranting strategies that re-stabilise this cytoskeletal component (Trojanowski et al., 2005; Zhang et al., 2005). Microtubule-stabilising drugs may also have additional value in numerous disorders involving tauopathies (Trojanowski et al., 2005; Zhang et al., 2005).

Taxol and its derivatives have been approved for the treatment of various cancers by the Federal Drug Administration (FDA) and therefore hold particular therapeutic promise in the treatment of AD and other related neurodegenerative diseases (Trojanowski et al., 2005). Although the studies discussed above indicate that certain interventions may be therapeutically viable in terms of preventing maladaptive neuronal responses and neurodegeneration, it is important to note that the underlying mechanisms these treatments aim to target must be fully elucidated. In this respect, microtubules are ubiquitous and thus interfering with this cytoskeletal component may have widespread and unanticipated outcomes. For instance, previous studies have indicated that taxol may be implicated in peripheral neuropathy, although the mechanisms underlying this are not fully understood (reviewed by Mielke et al., 2005). Investigations have demonstrated additional implications of taxol administration in the PNS, where taxol-induced microtubule accumulation resulted in the detachment of Schwann cells from axons (Roytta et al., 1984, Roytta and Raine, 1985). Taxol has also been shown to induce apoptosis in cortical neurons (Figueroa-Masot et al., 2001). However, Trojanowski et al. (2005) suggest that taxol and its derivates may be utilised at relatively low doses in the treatment of neurodegenerative diseases, compared doses currently used in chemotherapy treatments, thus over coming neuropathological effects.

7.4 Concluding remarks

In the race to develop strategies to effectively promote neural repair following brain trauma, some of the basic mechanisms underlying the endogenous aspects of this response have been overlooked. It is imperative to fully elucidate the mechanisms of endogenous brain and neuronal repair so that aspects of this response can be specifically targeted and/or manipulated towards effective brain repair. Moreover, it will be crucial to determine the ultimate effect of glial cell activation during attempted neuronal regeneration. So far, a multitude of conflicting results have been generated regarding the extent of endogenous neuronal regeneration and the role of microglia, astrocytes and oligodendrocytes in neural repair. In this respect, the potential for endogenous neuronal regeneration following brain injury has generally been viewed as bleak and glial cell populations have largely been attributed a deleterious role in this process, including formation of the glial scar and the generation of neurotoxic and inhibitory factors.

This thesis specifically sought to determine the intrinsic potential for axonal regeneration following injury as well as the interactive, and potentially facilitative, role glial cells play in this process. Intriguingly, contrary to popular belief, mature central neurons were demonstrated to undergo a sustained regenerative attempt follow axotomy. This process was shown to rely heavily upon microtubule dynamics and, in many respects, recapitulated initial axonal development. This remarkable regenerative capacity was demonstrated in an *in vitro* model of axonal injury, highlighting the importance of *in vitro* systems in analysing intrinsic neuronal responses free of confounding glial reactions and as providing a platform for testing

potential physical, chemical and/or molecular therapies for promoting neuronal regeneration. Axonal sprouting was also demonstrated following *in vivo* acute focal brain injury and was associated with significant alterations in gene expression. This response was ultimately limited by formation of a glial scar, however, prior to this was associated with substantial cell proliferation, neural progenitor cell production and neuro-glial interactions, suggestive of an adaptive response directed toward brain healing.

Overall, the results from this thesis demonstrate that mature neocortical neurons possess a remarkable and previously unappreciated capacity for regeneration and that structural brain injury, common to many human head traumas, evokes a repertoire of adaptive and interactive responses resulting in the restoration of relatively normal cytoarchitecture. The findings from this thesis also draw interesting parallels between the early reactive cytoskeletal alterations characterising the neuronal response to injury and the evolving pathology of AD, providing further evidence that physical displacement and/or compression of neurites by ß-amyloid plaques may be an additional or alternative mechanism of neuronal degeneration in AD.

The endogenous mechanisms utilised by injured neurons must be discovered if these responses are to be harnessed and therapeutically manipulated. In this regard, it will be imperative to determine why experimentally successful approaches to neural regeneration do not always translate to effective therapies in cases of human head trauma. It is likely that this is due to species differences, difficulties in modelling all aspects of human head trauma and, importantly, a lack of understanding of the basic

mechanisms underlying neuronal degeneration and regeneration. A combination of approaches will likely be required to bring about effective recovery from brain trauma. Interestingly, complete regeneration or replacement of damaged neurons may not be required to restore a substantial proportion of function.

This thesis provides insight into the molecular and cellular mechanisms underlying endogenous neural repair and highlights important components of this response that may be targeted to enhance recovery from brain injury, for example driving neuronal differentiation of injury-induced proliferating and progenitor cells, promoting the facilitative effects of injury-activated glial cell populations and developing strategies to increase appropriate long distance axonal regrowth and synaptogenesis. Additionally, results from this thesis have provided evidence supporting the use of agents that act specifically on components of the cytoskeleton in preventing maladaptive sprouting responses following axonal trauma and in the pathogenesis of certain neurodegenerative diseases. Taken together, studies of neural regeneration indicate that the adult brain possesses remarkable capacity for repair and regeneration, which involves gross structural and functional adaptability in addition to plasticity on a minute scale involving synaptic, axonal and dendritic remodelling. In summary, these findings provide exciting new avenues for future research into neurodegeneration and regeneration involved in brain trauma and neurodegenerative diseases.

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Appendix

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