Therapeutic Intervention in Alzheimer's Disease

by

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(1) **Adlard, P.A.**, West, A.K. and Vickers, J.C. (1998) Increased density of metallothionein I/II immunopositive cortical glial cells in the early stages of Alzheimer's disease. *Neurobiology of Disease* 5(5), 349-356.

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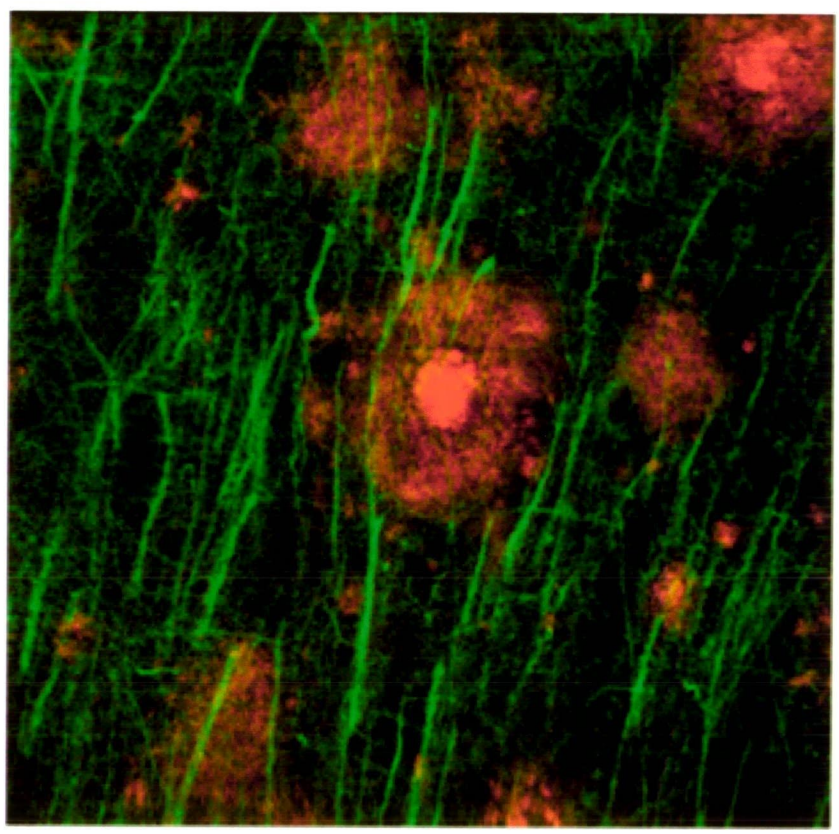
A preclinical Alzheimer's disease (AD) case double-labelled with ß-amyloid (red) and microtubule-associated protein-2 (MAP2) (green). MAP2-positive dendrites are shown deflecting around, and terminating within, the area occupied by the dense-cored plaque (see chapter 3 for details). Such structural deformation of neurites, by ß-amyloid plaques, may underlie the evolution of the neuropathology which precedes neurodegeneration in AD.



Abstract

Alzheimer's disease (AD) results from a series of dysfunctions which spread throughout the cortex in a precise spatiotemporal manner, and which subsequently give rise to a characteristic pattern of cognitive and non-cognitive symptoms. Whilst some of these symptoms can be controlled with specific drug regimes, there are still no treatments available to prevent the disease. Similarly, there are no drugs available which will reverse or even halt the progression of the pathological changes which occur in the AD brain.

It is hypothesised that ß-amyloid deposited within the brain as "plaques' causes slowly evolving physical damage to neurons, which then triggers a stereotypical neuronal response to trauma. This involves specific cytoskeletal alterations which give rise to the characteristic neuropathology of AD, and which can be experimentally modeled in both in vivo and in vitro models of neuronal injury. That the pathogenesis of AD crucially involves the cytoskeleton, and that targeting these changes may be an effective method of delaying or even preventing neurodegeneration in AD, was explored in this thesis.

A number of broad hypotheses were posed. Firstly, that there are significant cytoskeletal alterations in the AD brain which may be ameliorated by the use of cytoskeletal stabilising agents, and which may subsequently limit the evolution of the neuropathological changes characteristic of AD. Secondly, that metallothioneins may play a role in AD, and perhaps be able to prevent the aberrant neuronal sprouting associated with AD.

These hypotheses were addressed in a number of aims. The major conclusions from these

investigations were that morphologically distinct plaque types differentially affect the architecture of the brain in the early and late stages of AD, to result in significant cytoskeletal alterations. Similar observations were made following experimental cortical injury, where it was demonstrated that the administration of cytoskeletal stabilising drugs can both prevent and delay the onset of neuropathological changes. Finally, metallothioneins were shown to be upregulated in both the early stages of AD and following cortical injury, suggesting that they may have a role in the pathogenesis of AD.

This thesis has, therefore, demonstrated that it is possible to intervene in the sequence of events which ultimately leads to neurodegeneration in AD. Agents which target cytoskeletal alterations may represent alternatives to current therapeutic strategies.

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Abbreviations

Aß	ß-amyloid
ACh	Acetylcholine
AD	Alzheimer's disease
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
BDNF	Brain-derived neurotrophic factor
CERAD	Consortium to establish a registry for Alzheimer's disease
CNS	Central nervous system
DAI	Diffuse axonal injury
DIV	Days *in vitro*
DN	Dystrophic neurite
DNA	Deoxyribose nucleic acid
DS	Down's syndrome
ERT	Estrogen replacement therapy
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
GAP43	Growth associated protein 43
GFAP	Glial fibrillary acidic protein
GIF	Growth inhibitory factor
HBS	HEPES-buffered saline
IF	Intermediate filament

KSP	Lysine-serine-proline (amino acid repeat)
MAO-B	Type B monoamine oxidases
MAP	Microtubule associated protein
mRNA	Messenger ribonucleic acid
MS	Multiple Sclerosis
MT	Metallothionein
NF	Neurofilament
NFH	High molecular weight neurofilament subunit
NFL	Low molecular weight neurofilament subunit
NFM	Medium molecular weight neurofilament subunit
NFT	Neurofibrillary tangle
NGF	Nerve growth factor
NSAIDS	Non-steroidal anti-inflammatory drugs
NT	Neurotrophin
PAGE	Poly acrylamide gel electrophoresis
PBS	Phosphate buffered saline
PHF	Paired helical filament
PI	Post-injury
PMI	Post-mortem interval
PNS	Peripheral nervous system
TBI	Traumatic brain injury

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Literature review

1.0 Introduction

Alzheimer's disease (AD) is the commonest cause of dementia in people of all ages (Wilcock, 1996), affecting approximately 11% of the population greater than 65 years of age and up to 50% of people aged 85 years and older (Hof and Morrison, 1994). AD results from a series of dysfunctions which spread throughout the cortex in a precise spatiotemporal manner, and which subsequently give rise to a constantly changing, and deteriorating, pattern of cognitive and non-cognitive symptoms (Folstein and Bylsma, 1994; Delacourte, 1998). The disease itself may arise on many different backgrounds, and perhaps have many independent and dynamic processes at its cause (Delacourte, 1998).

1.1 Clinical course of Alzheimer's disease

The clinical course of AD can range from a few years to many decades, with an extremely rapid course of illness in less than three years very unusual, and greater than 25 years also uncommon. The majority of sufferers will experience a 7-10 year time frame of their illness (Berg and Morris, 1994). The clinical manifestations can also vary, with approximately 20% of probable AD patients showing an atypical presentation (Berg and Morris, 1994). The majority, however, will experience a gradual onset of disease with a slow, but progressive, decline in most higher cortical functions (Berg and Morris, 1994; Foster, 1994; Katzman and Kawas, 1994).

AD is insidious in onset, and sufferers are often unaware of their condition. Most will experience increased forgetfulness, and perhaps some decline in their ability to perform

everyday tasks.

Within a few years there are signs of impairment in other cognitive activities such as oral and written language, the ability to calculate and make judgments also declines, as does the ability to cope with new and complex tasks. There is also some change in visuospatial recognition. Aside from these issues, the patients are generally neurologically normal.

AD-sufferers may then begin to experience personality changes, such as apathy and restlessness. Mood changes, including major depression, often occur within the first 3-4 years of disease onset and are present in 15-20% of the AD population (Folstein and Bylsma, 1994). Hallucinations may also be a feature of AD, occurring in between 7-49% of individuals, but these are less common than delusions, which are frequent in this early to middle phase of the disease (Folstein and Bylsma, 1994). Other psychiatric manifestations are unusual.

In the middle to moderate stage of AD, people become more reliant on others, they are largely only able to recall long established memories and they will become lost in familiar surroundings. Previously impaired abilities such as judgment and calculation worsen and confusion is evident. Other abnormal behaviours, such as hostility and personality changes, will also often become exaggerated in this phase of the disease.

In the more advanced stages of the disease, memory loss becomes more significant, sufferers become nearly totally dependant on others, to the point where they need help dressing and washing, are often both fecally and urinary incontinent and many are rendered mute. Comorbidity is high in this phase of the disease, with patients often

experiencing other disorders such as depression and stroke.

In very advanced stages, patients become bedridden, unresponsive to verbal communication are often unable to swallow and may meet the criteria for a persistent vegetative state. Terminal illnesses may include pneumonia, sepsis and pulmonary embolism (Berg and Morris, 1994).

The symptoms of AD are, therefore, quite extensive and display a progressive course, with the gradual erosion of higher cortical functions. Many of these symptoms can be observed in the aging population, and so it has been postulated that AD is merely an exacerbation of normal aging. AD and normal aging, however, are both qualitatively and quantitatively different with regards to the neuronal pathology which occurs. Thus, AD is a unique disease entity which is not merely an exaggeration of the effects of normal aging (Foster, 1994).

1.2 The Alzheimer's disease brain

AD is a central nervous system (CNS) related disorder, and so the brain is the main locus of effect. There are two main pathological structures found within the AD brain, ß-amyloid plaques and neurofibrillary tangles (NFT). Both the ß-amyloid plaque and the NFT, whilst not individually unique to AD, have a characteristic distribution and density in this disease (Hof and Morrison, 1994), and are considered its hallmark pathological features. There are, however, a host of other alterations which occur in and contribute to the gradual devastation of, the AD brain and its various functions. These include both atrophy of a number of cortical regions as well as more microscopic changes, such as alterations in various cytoskeletal networks within nerve cells. These, together with the hallmark pathologies, contribute to the overall pathogenesis of AD.

1.2.1 Macroscopic

Macroscopically, the AD brain shows an abnormal enlargement of different cortical structures. The leptomeninges, for example, is often thickened, whilst the lateral ventricle may be massively increased in size. There is also atrophy of the cerebral cortex, with many gyri, particularly those in frontotemporal areas, affected in AD. The hippocampus may also become significantly involved and can reduce in size by half (Terry *et al.*, 1994).

1.2.2 Microscopic - amyloid

ß-amyloid, which comprises the plaques found in the AD brain, is derived from the proteolytic cleavage of a larger protein, the amyloid precursor protein (APP). APP is encoded by a single gene on chromosome 21 and occurs in three different protein isoforms. These molecules are expressed in most tissues and are integral membrane-spanning glycoproteins. The normal proteolysis of APP, by alpha-secretase, results in cleavage through the beta A4 region to give short soluble fragments and no ß-amyloid. However, the action of putative beta- and gamma-secretases is to cleave APP on either side of this region to give rise to a soluble full length ß-amyloid peptide. The position of the cleavage by gamma-secretase is crucial, and will give rise to either shorter (Aß1-40) or longer (Aß1-42/43) ß-amyloid peptides. Aß1-42/43 comprises the most abundant species of ß-amyloid found in neuritic plaques (Hardy, 1997).

The precise mechanism by which these proteins become insoluble and contribute to the evolution of different plaque types is yet to be determined.

1.2.2.1 Plaques

The deposition of small (7-10 nm) extracellular filaments, comprised of a ~4kDa insoluble form of the ß-amyloid protein within the brain to ~~from~~ form plaques is a central

event in the pathogenesis of AD, and one which occurs in all stages of the disease process (Glenner and Wong, 1984; Masters *et al.*, 1985). ß-amyloid deposits are heterogeneously distributed, affecting most cortical areas (Delacourte, 1998). There does seem to be some preference, though, for the parahippocampal gyrus and the superficial layers of the neocortex (Hof and Morrison, 1994), and, indeed, the deposition of ß-amyloid in particular cortical sites correlates well with cognitive decline (Cummings and Cotman, 1995; Cummings *et al.*, 1996).

The morphology of ß-amyloid plaques is heterogeneous, and as such it can form diffuse, amorphous deposits through to the more well defined, typically spherical plaque structures. There are several different types of plaques that have been described, including diffuse plaques, which contain sparse amounts of filamentous ß-amyloid and are not normally associated with dystrophic or abnormal neurites (Terry *et al.*, 1994). In contrast, neuritic plaques contain masses of dense bundles of ß-amyloid fibrils and are associated with dystrophic neurites (DNs) (Terry *et al.*, 1994; Dickson, 1997). Ultrastructurally, these DNs are quite varied, with some containing paired helical filaments (PHF) and other laminated bodies, whilst others contain neurofilaments (NFs), laminated bodies, synaptic vesicles, mitochondria, lysosomes and a host of other proteins (Terry *et al.*, 1994; Dickson, 1997).

1.2.2.2 Amyloid angiopathy

Amyloid angiopathy, where ß-amyloid infiltrates the walls of blood vessels, particularly the leptomeningeal and cortical vessels within the brain, is common in AD. Whilst some ß-amyloid plaques appear to be associated with blood vessels, there is very little correlation between amyloid angiopathy and the density or frequency of plaques (Lippa *et al.*, 1993; Terry *et al.*, 1994).

1.2.3 Microscopic- neurofibrillary pathology

Neurofibrillary pathology, which primarily consists of NFTs, DNs and neuropil threads, comprises a significant amount of the pathological alterations that occur within the AD brain. These changes are crucial to neurodegeneration and the subsequent development of dementia.

1.2.3.1 Neurofibrillary Tangles (NFT)

NFTs are one of the major features of AD, however, they are not unique to AD and can be found in other conditions such as Parkinson's disease and dementia pugilistica (Roberts *et al.*, 1990; Tokuda *et al.*, 1991; Terry *et al.*, 1994; Mendez, 1995). Their presence, however, is generally related to neuronal dysfunction (Geerts, 1998), and correlates well with the existence of dementia (Bierer *et al.*, 1995; Berg *et al.*, 1998). Ultrastructurally, NFT are identified as accumulations of abnormal components of the cytoskeleton, and are primarily composed of PHF. It has been suggested that PHF are comprised of aberrantly phosphorylated forms of the microtubule associated protein, tau (Wischik *et al.*, 1988; Goedert *et al.*, 1993; Hof and Morrison, 1994; Terry *et al.*, 1994; Delacourte, 1998; Geerts, 1998), and that the latter may account for up to 80% of its structure (Geerts, 1998). However, it is also suggested that, at least in the early stages of NFT formation, that the tau comprising the PHF is not abnormally phosphorylated (Wischik *et al.*, 1995). This discrepancy may be due to post-translational modifications, which initially produce PHF-like changes in tau (Goedert *et al.*, 1996) and then subsequently promote its phosphorylation (Hasegawa *et al.*, 1997).

NFT are normally found in the cerebral cortex and in subcortical regions that project to the cerebral cortex, such as the amygdala, basal forebrain and locus ceruleus (Hof and Morrison, 1994). Within the neocortex, there is an association between NFT and

pyramidal nerve cells, specifically, those in layer II, III and IV comprise the most affected cells in AD (Pearson *et al.*, 1985; Lewis *et al.*, 1987; Hof and Morrison, 1990; Hof *et al.*, 1995; Mann, 1996). These are primarily large, NF rich neurons that send out long cortical projections (Hof and Morrison, 1994), with small and medium sized neurons rarely affected (Hof and Morrison, 1994; Terry *et al.*, 1994). Indeed, NF antibodies have been shown to label NFT (Lee *et al.*, 1988; Zhang *et al.*, 1989; Vickers *et al.*, 1992, 1994; Nakamura *et al.*, 1997), and NF have been suggested to be essential to the development of neurofibrillary changes in degenerating neurons in AD (Vickers *et al.*, 2000). The formation of NFT and the subsequent complete degeneration of affected neurons, therefore, is not a non-specific process, but rather, involves particular neuronal types and cortical regions preferentially to others (Morrison *et al.*, 1987; Hof *et al.*, 1990; Hof and Morrison, 1990; Hof *et al.*, 1995).

1.2.3.2 Neuron Loss

Similarly, neuronal loss, which is a consistent finding in AD, occurs in specific regions of the brain. There is, for example, a severe loss of neurons in the nucleus basalis of Meynert, which then impacts upon cholinergic activity in the cortex and hippocampus and subsequently affects memory. Similarly, the loss of neurons in the locus ceruleus affects noradrenergic activity throughout the cortex (Terry *et al.*, 1994). The loss of neurons and synaptic connections, therefore, is undoubtedly a feature which contributes to the symptoms observed in the disease.

1.2.3.3 Neuropil threads

Neuropil threads are short fibres that contain PHF and are characterised by labelling for tau and other antibodies. The majority of neuropil threads arise from degenerating dendrites, but some may be axonal in origin (Hof and Morrison, 1994; Terry *et al.*,

1994). These structures normally appear early in the disease process, and occur more frequently in the hippocampus and entorhinal cortex (Hof and Morrison, 1994).

1.2.3.4 Hirano Bodies

Hirano bodies can also be a prominent feature in the pyramidal layer of the hippocampus in the AD brain. These structures are not unique to AD, and are found adjacent to, or within, pyramidal cells. They are composed of 7-10μm thick filaments that label with antibodies to actin and other proteins such as tropomyosin (Terry *et al.*, 1994).

1.2.3.5 Dystrophic neurites

Dystrophic, or abnormal, neuronal processes are a consistent finding in AD and are found in particular association with ß-amyloid plaques (Hof and Morrison, 1994), although they are also found in the non-AD brain (Vickers *et al.*, 1996). These degenerating neurites most likely represent altered, probably abnormally sprouting axons (Masliah *et al.*, 1993a; Praprotnik *et al.*, 1996; Vickers *et al.*, 1996).

As mentioned earlier, DNs display a variety of neurochemical profiles, and this may reflect their occurrence in different stages of the disease process (Dickson *et al.*, 1999). Early forms of DNs, for example, are associated with profound neurofilamentous changes in the absence of tau abnormalities (Vickers *et al.*, 1996), with tau pathology occurring later in the development of DNs (Benzing *et al.*, 1993; Su *et al.*, 1996). DNs can, therefore, be distinguished as either tau- or NF- abundant forms (Dickson *et al.*, 1988; Masliah *et al.*, 1993b; Vickers *et al.*, 1994; Su *et al.*, 1996). In addition to the localisation of NFs (Arai *et al.*, 1990; Cras *et al.*, 1991; Schmidt *et al.*, 1991; Schmidt *et al.*, 1994; Su *et al.*, 1996, Nakamura *et al.*, 1997; Su *et al.*, 1998) and tau (Nakamura *et*

al., 1997; Su *et al.*, 1998) to DNs, there are also accumulations of APP (Perry *et al.*, 1988; Ghiso *et al.*, 1989; Ishii *et al.*, 1989; Shoji *et al.*, 1990; Cole *et al.*, 1991; Cras *et al.*, 1991; Joachim *et al.*, 1991; Saunders *et al.*, 1998), synaptophysin and chromagranin (Masliah *et al.*, 1989; Brion *et al.*, 1991) and the growth associated protein, GAP43 (Masliah *et al.*, 1991) found within plaque-associated DNs.

1.2.4 Cytoskeletal changes in Alzheimer's disease

There are profound cytoskeletal changes in AD. Microtubules, for example, are reported to be lost in affected neurons in AD (Gray, 1986; Gray *et al.*, 1987; Paula-Barbosa *et al.*, 1987). This may be due to an alteration in one of the microtubule associated proteins, such as tau. Conversely, the destabilisation and loss of microtubules may lead to the subsequent changes in tau, which are characteristic of AD. It has also been postulated that the paucity of microtubules in AD may be due to their displacement by the formation of PHF within dendrites (Geddes *et al.*, 1994; Ashford *et al.*, 1998). Similarly, recent investigations have suggested that the deposition of ß-amyloid in the neocortex may affect microtubular structure and organisation by causing gradual damage to dendrites which pass through plaques, resulting in morphological changes which eventually lead to the dissociation of tau from microtubules, and a subsequent destabilisation of the cytoskeleton (Knowles *et al.*, 1998, 1999). The various mechanistic issues aside, it is clear that dendrites, and the microtubules and microtubule associated proteins within them, are significantly altered throughout the course of AD.

The NF system within the cell is also dramatically altered in affected neurons in AD. The normal NF network within the various neuronal domains such as the cell body and axon are replaced by abnormal neurofibrillary structures in AD (Metuzals *et al.*, 1988; Vickers *et al.*, 1992, 1994). Abnormally phosphorylated NF subunits, for example, are seen to accumulate in areas from which they are normally excluded, such as neuronal

cell bodies (Vickers *et al.*, 1992, 1994; Nakamura *et al.*, 1997). The early stages of DN formation are characterised by accumulations of NFs (Vickers *et al.*, 1996; King *et al.*, 1997), and there is also a down regulation of NF expression in AD (Kittur *et al.*, 1994).

1.3 Risk Factors

A number of factors have been associated with a higher risk of dementia, however, the majority of these may only have a modest or inconsequential effect on the development of AD. Similarly, there are several factors associated with a decrease in the incidence of AD. For example, it has been suggested that education is protective, with higher incidences of AD reported in manual labourers (Katzman and Kawas, 1994; Dartigues *et al.*, 1998). Conversely, being single and having low weight and height have been associated with an increased prevalence of AD (Dartigues *et al.*, 1998).

There are, however, a number of accepted risk factors for the development of AD. These include age, the existence of a family history of dementia, Down's syndrome and apolipoprotein E (ApoE) genotype (Katzman and Kawas, 1994; Jorm, 1997). A history of head trauma is also gaining wider acceptance as a causative factor in the development of AD (Vickers, 1997).

1.3.1 Age

AD is an age-related disorder, and so the greatest risk factor for the development of AD is old age. AD affects approximately 11% of the population older than 65 years (Hof and Morrison, 1994), and its prevalence is reported to double every five years up to the age of 95 (Jorm, 1997).

1.3.2 Family history and genetic mutations

Approximately 25% of AD patients have another relative with dementia (Bird, 1994). In families where only a single case is documented, the risk to other family members is no greater than the basal level of occurrence (Bird, 1994). However, if it is a parent or sibling with the disease, then the risk of developing AD is increased more than three fold (Jorm, 1997). Lautenschlager and colleagues (1996) report that the risk of developing AD for first degree relatives is 5% at age 70, 16% at age 80 and up to 33% at 90, and for children whose both parents had the disease, the risk is 54% by age 80. In cases of early onset AD, the risk of other relatives developing AD is also greater than in families of late onset AD.

Familial AD, then, is classed as a family in which there is greater than one person with the disease. Whilst this genetic component sets these AD patients apart from others, the clinical range and type of symptoms are not any different between familial or sporadic AD cases (Bird, 1994).

There are a number of genetic alterations that can contribute to AD, some of which have been postulated to be responsible for both familial and sporadic AD. These include mutations in a number of different genes.

1.3.2.1 Amyloid precursor protein (chromosome 21)

APP mutations, of which more than five have been described that give rise directly to AD, account for approximately 5% of all familial AD cases (Chartier-Harlin *et al.*, 1991; Goate *et al.*, 1991; Murrel *et al.*, 1991; Hendricks *et al.*, 1992; Mullan *et al.*, 1992; Hardy, 1997; Van Broeckhoven, 1998). Mutations in this gene can alter its metabolism such that there is an increase in either the total amount of ß-amyloid

produced (Citron *et al.*, 1992; Cai *et al.*, 1993; Citron *et al.*, 1994) or the amount of the proteolytic fragment Aß1-42/43 generated (Suzuki *et al.*, 1994; Tamaoka *et al.*, 1994; Delacourte, 1998). This peptide has a greater propensity for fibrillogenesis than the shorter Aß1-40 (Yankner, 1996; Van Broeckhoven, 1998), and so this ratio shift may be crucial to ß-amyloid deposition.

1.3.2.2 Presenilin 1 (chromosome 14) and Presenilin 2 (chromosome 1)

The presenilin 1 and 2 genes encode proteins of 463 and 448 amino acids, respectively. These are transmembrane proteins which are localised to the endoplasmic reticulum and Golgi complex (Kovacs *et al.*, 1996) and which share a high degree of homology (~67%), suggesting a shared functional role. Their precise cellular function, however, remains unknown. Recent reports, though, suggest that the presenilins may regulate APP processing (Karran *et al.*, 1998; Haass and Strooper, 1999). It has been hypothesised that they may directly interact with APP to traffic it to a specific cellular site of, and perhaps assist in its presentation for, gamma-secretase cleavage (Hardy and Israel, 1999; Verdile *et al.*, 2000). Alternatively, the presenilins themselves may be, or be cofactors for, gamma-secretase (Steiner *et al.*, 1999; Wolfe *et al.*, 1999; Li *et al.*, 2000). Further to this, there have been greater than fourty mutations described in these genes, which, along with mutations in the APP gene itself, give rise to altered APP metabolism and processing. The result is an increase in Aß1-42/43 and a subsequent increase in ß-amyloid deposition (Citron *et al.*, 1992; Cai *et al.*, 1993; Citron *et al.*, 1994; Suzuki *et al.*, 1994; Tamaoka *et al.*, 1994; Scheuner *et al.*, 1996; Hardy, 1997; Citron *et al.*, 1998; Delacourte, 1998; Mehta *et al.*, 1998; De Jonghe *et al.*, 1999). The presenilin genes may, therefore, be crucially involved in ß-amyloid deposition.

1.3.2.3 Apolipoprotein E (chromosome 19)

The ApoE gene, located on chromosome 19, occurs in three forms, which are designated ε2, ε3 and ε4, and which differ by only a few amino acids at codons 112 and 158. Their occurrence in the population varies markedly, with the ε2 allelic variant found in approximately 7% of people, ε3 in 78% and ε4 in 15% (Mahley, 1988; Van Broeckhoven, 1998). Biologically, ApoE is considered to be a chaperone molecule, aiding in the transport of cholesterol and other hydrophobic molecules (Van Broeckhoven, 1998), and it is postulated to play a role in neuronal repair (Rubinsztein, 1995).

Whilst Apo ε3 is the most common allele in the general population, the ε4 variant is the most common in the AD population, and is considered a risk factor for the development of AD. Inheritance of an ε4 allele is believed to account for between 20-50% of AD cases (Corder *et al.*, 1993; Van Broeckhoven, 1998), and is reported to increase the risk of AD by eight fold (Masters and Beyreuther, 1998). People that are homozygous for Apo ε4 also show a greater risk for the development of AD than those with only 1 or no ε4 variants, and this is believed to give rise to an earlier age of onset (Corder *et al.*, 1993; Van Broeckhoven, 1998). Individuals who carry an ε4 allele, however, can live to old ages without developing AD (Henderson *et al.*, 1995), and so ApoE genotype is not an absolute determinant for the development of AD.

The inheritance of an ε4 allele is associated with increased levels and accelerated deposition of ß-amyloid (Rebeck *et al.*, 1993; Corder *et al.*, 1994; Van Broeckhoven, 1998), perhaps due to its increased affinity for ß-amyloid as compared to the other variants (Van Broeckhoven, 1998). In AD, then, ApoE may promote the development of insoluble plaques (Ma *et al.*, 1994).

Interestingly, inheritance of an ε2 allele is believed to be protective against the development of AD (Corder *et al.*, 1994).

1.3.3 Down's syndrome

Individuals with Down's syndrome (DS) typically develop AD-like changes in their brains before the age of 40 (Wisniewski *et al.*, 1985), with the appearance of neocortical plaques in the second to third decade of life and then subsequent neurofibrillary changes and eventual degeneration (Wisniewski *et al.*, 1985; Mann *et al.*, 1986; Mann and Esiri, 1989; Giaccone *et al.*, 1989; Motte and Williams, 1989). This disease is unique, in that people with DS possess two copies of the APP gene on chromosome 21. This not only gives rise to neuropathological changes similar to AD, but also results in the earlier appearance of this pathology than observed in AD.

1.3.4 Head trauma

While the association between head trauma and AD has remained controversial, it has been reported that in between 2 and 20% of AD cases there is a prior history of head injury (Mortimer *et al.*, 1991; Rasmusson *et al.*, 1995). It is also suggested that the effect of head injury on the incidence of AD can be significantly altered by the presence of an Apo ε4 allele (Mayeux *et al.*, 1995).

1.4 The cause of Alzheimer's disease ?

It has been postulated that ß-amyloid plays a crucial role in the early stages of the disease process, and is involved in the neurodegeneration that leads to dementia in AD (Selkoe, 1991, 1994). It is also suggested that ß-amyloid does not have a central role in the disease due to its presence in the apparently non-demented elderly (Neve and Robakis, 1998). So, is ß-amyloid critical to the development of AD-like

neuropathological changes ? Studies which demonstrate that individuals with DS develop neurofibrillary pathology subsequent to the early deposition of ß-amyloid in the cortex, as well as the localisation of DNs to a subset of plaques in AD, suggest that ß-amyloid is a key component in neurodegeneration. Transgenic animals which carry a mutation in the APP gene also demonstrate similar DNs in association with neocortical plaques (Masliah *et al.*, 1996; Irizarry *et al.*, 1997; Sturchler-Pierrat *et al.*, 1997).

The pervading hypothesis is that ß-amyloid is toxic to nerve cells, although numerous *in vivo* and *in vitro* studies have both supported and refuted this hypothesis (Yankner, 1996; Neve and Robakis, 1998). Indeed, specific ß-amyloid peptides can cause nerve cell death both *in vitro* (Pike *et al.*, 1991; Takadera *et al.*, 1993; Lambert *et al.*, 1998) and *in vivo* (Kowall *et al.*, 1992; Weldon *et al.*, 1998). It has, however, been demonstrated that ß-amyloid has very little biological effect *in vivo* (Games *et al.*, 1992; Stephenson and Clemens, 1992). It has also been suggested that the physiological level of ß-amyloid required to cause nerve cell death is unlikely to be achieved within the AD brain (Neve and Robakis, 1998), and the fact that ß-amyloid does not result in cell death in the immediate vicinity of the deposit argues against a diffusable chemical-mediated mechanism (Sampson *et al.*, 1997). The abnormal localisation of DNs to plaques must, therefore, be a result of a mode of action unrelated to the chemical toxicity of ß-amyloid. Examination of the early stages of AD may provide clues to the mechanism of ß-amyloid-induced neurodegeneration.

1.5 Preclinical Alzheimer's disease cases

The existence of the so-called 'preclinical', or transition stage, AD cases has remained controversial. However, they are defined as those cases in which there is the appearance of some diffuse neocortical plaques, but which lack significant neuronal degeneration and neurofibrillary pathology (Crystal *et al.*, 1988; Morris *et al.*, 1991, 1996; Coria *et*

al., 1993; Vickers *et al.*, 1996). Individuals with preclinical AD, therefore, do not show overt signs of dementia (Terry *et al.*, 1994), but are likely to go on and develop the full clinical symptoms of AD (Morris *et al.*, 1996; Vickers *et al.*, 1996; Troncoso *et al.*, 1998). It has been postulated that a large percentage of the older population are in this preclinical phase of the disease (Davies *et al.*, 1988).

Neuropathologically, preclinical AD cases demonstrate accumulations of NFs in abnormal neurites associated with plaques (Vickers *et al.*, 1996). This initial pathology precedes PHF-like changes in tau, and morphologically, the DNs appear as either bulb- or ring-like structures. The DNs remain NF positive further into the course of the disease (Dickson *et al.*, 1988; Cras *et al.*, 1991; Masliah *et al.*, 1993b; Vickers *et al.*, 1994; Su *et al.*, 1996; Dickson *et al.*, 1999). This suggests then, that one of the very early changes associated with the deposition of ß-amyloid in the cortex is a disruption to the cytoskeletal elements present within the processes of nerve cells. Neuronal cytoskeletal proteins may, therefore, be critical in the pathogenesis of AD (Matsuyama and Jarvick, 1989; Vickers, 1997; Vickers *et al.*, 2000).

1.6 The Cytoskeleton

The axonal cytoskeleton consists of a number of different components, namely, microfilaments, microtubules and intermediate filaments (Okabe *et al.*, 1993; Lin and Szaro, 1995), between which there is an elaborate system of structural connections (Hirokawa, 1991).

1.6.1 Microfilaments

These are short 4- to 8- nm filaments which are involved in a number of cellular functions, including synaptic transmission, receptor anchoring, endocytosis, filament

linking and contact inhibition (Bamburg and Bernstein, 1991; Nixon, 1991). An example of a microfilament is actin.

Actin, a major constituent of striated muscle, is also an important component of non-muscle cells (Bamburg and Bernstein, 1991). In neuronal cells actin is the main cytoskeletal component of the postsynaptic cytoplasm, whilst actin and microtubules comprise the presynaptic terminals. The arrangement of actin filaments within these domains is believed to be closely related to synaptic transmission (Hirokawa, 1991). Actin also participates in a host of other cellular events (Bamburg and Bernstein, 1991).

1.6.2 Microtubules

Microtubules are an essential component of the neuronal cytoskeleton, without which it could not exist (Lasek *et al.*, 1985). Linear microtubule polymers are formed by the polymerisation of α- and ß-tubulin under the influence of a variety of factors, such as microtubule-associated proteins (MAPs) (Burgoyne, 1991; Huizing *et al.*, 1995), and they normally appear in groups, forming small bundles within the axon (Hirokawa, 1991).

Microtubules are involved in a number of important intracellular functions, including mitosis; fast axonal transport of membranous organelles; slow axonal transport and axonal growth; maintenance of cell shape and the modulation of interactions with cell surface receptors (Lasek *et al.*, 1985; Burgoyne, 1991; Huizing *et al.*, 1995; Lin and Szaro, 1995). Microtubules act to stabilise the cytoskeleton (Lasek *et al.*, 1985), and can be destabilised by a variety of mechanisms. Post-translational modifications to various MAP species can induce the disassembly of preformed microtubules, whilst similar modifications can occur to the greater than 20 different tubulin isoforms, the

components from which microtubules are formed, to regulate the kinetics of microtubule assembly/disassembly (Burns, 1991; Cambray-Deakin, 1991). There are other proteins, also, such as microtubule-inhibitory-protein, which, under the correct environmental conditions, can cause the partial disassembly of microtubules (Burns, 1991).

1.6.3 Microtubule associated proteins

The major MAPs isolated from the mammalian brain include MAP1A, 1B, 1C, MAP2 and tau, all of which demonstrate a specific pattern of localisation which may vary as the brain develops (Burgoyne, 1991; Hirokawa, 1991). These proteins are associated with microtubules and have a number of functions including the assembly and stabilisation of microtubules and the regulation of interactions between microtubules and other cytoskeletal elements and organelles (Burgoyne, 1991). Indeed, once the microtubule has formed, the MAPs have a marked effect on its function and behaviour (Burns, 1991).

1.6.3.1 MAP1

MAP1 is found to a greater extent in the axon than any other neuronal compartment, and is composed of three antigenically distinct isoforms, 1A, 1B, 1C (320-350 KDa) (Burgoyne, 1991; Hirokawa, 1991). MAP1A and 1B are the major constituents of the sidearms that connect microtubules to each other (Burgoyne, 1991; Hirokawa, 1991). MAP1C, or brain dynein, has, however, been proposed to be an axonal transport motor molecule (Hirokawa, 1991). It is believed that MAP1C is anterogradely transported, in an inactive form, by another motor molecule (perhaps kinesin) along the axon, prior to being activated and performing its function as a retrograde transport motor (Hirokawa, 1991). MAP1A is neuronal specific within the CNS, whilst MAP1B is found in both

glia and neurons. In axons, MAP1B is in a phosphorylated form, whilst, in dendrites, it is unphosphorylated (Burgoyne, 1991).

1.6.3.2 MAP2

Within the mature CNS, MAP2 is confined to the dendritic compartment and is a component of the cross bridges which link microtubules to each other and to NF in the dendritic compartment (Burgoyne, 1991; Cambray-Deakin, 1991; Hirokawa, 1991). It also stimulates the assembly of microtubules and stabilises assembled microtubules (Burgoyne, 1991). There are three different isoforms of MAP2 (2A, 2B and 2C). MAP2A and 2B are of high molecular weight (~275 KDa) and are predominant in the mature brain. MAP2C (~70 KDa), however, is abundant in the developing brain, where it is expressed in axons (Burgoyne, 1991).

Post-translational modifications of MAP2, such as phosphorylation, can significantly disrupt microtubule organisation. Phosphorylation of MAP2 by cAMP-dependant protein kinase, for example, decreases the affinity of MAP2 for microtubules, causes the disassembly of preformed microtubules and interferes with the interaction between microtubules and other cytoskeletal elements (Burgoyne, 1991). The phosphorylation of MAP2 can, therefore, regulate its function. There are also other regulatory mechanisms. The binding of MAP2 to NF, for example, is inhibited in a calcium dependent manner by calmodulin (Burgoyne, 1991).

1.6.3.3 MAP3, 4 and 5

MAP3, which is localised to the axon and appears to stimulate microtubule assembly, is found in glia and NF-rich neurons (Burgoyne, 1991; Hirokawa, 1991). MAP4 is only present in non-neuronal cells (Burgoyne, 1991). MAP5 is found in high levels in the

immature brain, but declines during development. It is found in both glia and neurons (Burgoyne, 1991), and has been shown to be homologous to an isoform of MAP1, MAP1B (Burgoyne, 1991; Matus, 1991).

1.6.3.4 Tau

The tau family consist of 55-62 KDa proteins which are formed by the alternative splicing of a single gene to give six different isoforms (Goedert *et al.*, 1989). Tau selectively stabilises the axon, and is, therefore, primarily localised to the axonal compartment (Kanai and Hirokawa, 1995). It has been suggested that tau is not essential to the formation of axons, with tau-deficient mice demonstrating a histologically normal CNS, and hippocampal neurons cultured from these animals successfully elongating axonal structures (Harada *et al.*, 1994). Some small calibre axons in tau-deficient mice, however, had a significantly altered organisation of microtubules, which had also become destabilised (Harada *et al.*, 1994). Larger calibre axons demonstrated increases in MAP1A, perhaps in compensation for the lack of tau (Harada *et al.*, 1994). That other MAPS could compensate for the loss of tau is problematic in deciphering its biological significance. It is of interest, then, that a recent investigation utilised a cell culture technique which caused an acute loss of tau without any associated compensatory changes in other MAPs (Liu *et al.*, 1999). This study demonstrated that tau is required for normal neurite elongation.

Tau is found in close association with microtubules, and forms projections from the microtubule surface (Hirokawa, 1991). Tau has a proposed role in the growth and maintenance of nerve cell processes, in crosslinking adjacent microtubules to form microtubule-bundles and in promoting the polymerisation of tubulin (Hirokawa, 1991; Zemlan *et al.*, 1999).

Tau can be extensively phosphorylated at up to eleven different sites which are preferentially associated with serine or threonine amino acids. Such a modification increases both the length and rigidity of tau, and this change in conformation results in its dissociation from microtubules (Hirokawa, 1991; Geerts, 1998). This may, therefore, disrupt the microtubule network. Other post-translational modifications include glycosylation and glycation.

1.6.4 Intermediate Filaments

Intermediate filaments (IFs) have been divided into six different classes based upon their amino acid composition and similarity. Class I and II consist of acidic and basic keratins respectively; class III includes vimentin, desmin, glial fibrillary acidic protein (GFAP) and peripherin; class IV includes the NF triplet proteins and alpha-internexin; class V includes the nuclear laminins and nestin comprises the novel sixth class of IF (Oblinger *et al.*, 1989; Steinert and Liem, 1990; Shaw, 1991).

Intermediate filaments consist of three domains. A ~40 KDa rod shaped domain, which is primarily α-helical in nature, is conserved across all the members of this class. This is proposed to be the area responsible for filament assembly and other functions shared between the different IF proteins. This domain is flanked on either side by hypervariable regions which comprise the amino and carboxy terminals of the proteins. It is these hypervariable domains which are responsible for the functional variations between the members of this class of filament (Lasek *et al.*, 1985). A characteristic feature of the IF is that, depending upon the requirements of the cell, their molecular composition can vary, and this is often related to the stage of maturation of the cell (Lin and Szaro, 1995).

All the different classes of intermediate filaments are preferentially expressed in a particular cell type. For example, the cytokeratins are found in epithelia, NF triplet proteins in neurons, GFAP in glia, desmin in myogenic cells, laminins in the nuclear envelope and vimentin in early embryonic cells and mesenchymal cells (Lasek *et al.*, 1985; Julien and Grosveld, 1991). The majority of mammalian neurons express the type IV class of IF, the NF triplet proteins, which in turn are primarily expressed in neurons with large myelinated axons (Oblinger *et al.*, 1989). Throughout development, however, the IF network undergoes significant compositional changes, depending on the neuron's changing demands for plasticity and stability, before a stable mature system is achieved. Similarly, CNS trauma results in an alteration to the expression of the different IFs (Oblinger *et al.*, 1989; Nixon and Shea, 1992).

The IFs initially expressed in the developing CNS include nestin, vimentin, peripherin and alpha-internexin. Nestin, or neuroepithelial stem cell protein, functions in neuroblast migration and is expressed in radial glial cells, the progenitors of both neurons and glia (Lendahl *et al.*, 1990). Vimentin mediates cell division and alterations in cellular shape, and may assist in initial neurite outgrowth (Nixon & Shea, 1992). Peripherin helps to maintain plasticity during outgrowth and regeneration, but is primarily localised to the peripheral nervous system (Portier *et al.*, 1984). Alpha-internexin, which also helps maintain plasticity during outgrowth and regeneration, is hypothesised to be the first type IV class of IF that is expressed in post-mitotic CNS cells of a neuronal fate. It is most abundant during development, but declines to a stable level, with the onset of the NF triplet, which remains throughout adulthood and it persists as the major IF in small calibre axons (Patcher & Liem, 1985; Nixon and Shea, 1992; Benson *et al.*, 1996).

The NF triplet proteins, which eventually replace the majority of these other IFs, establishes neuronal phenotype and stabilise neuronal circuitry (Nixon and Shea, 1992). There is an association between this class of IF protein and AD (Dahl *et al.*, 1982; Perry *et al.*, 1985; Cork *et al.*, 1986; Haugh *et al.*, 1986; Miller *et al.*, 1986, Dickson *et al.*, 1988; Masliah *et al.*, 1993b; Vickers *et al.*, 1994; Su *et al.*, 1996), and so a more detailed outline of NFs is given below.

1.6.5 Neurofilaments

The NF triplet proteins belong to the type IV family of IFs, which all form 10nm filaments (Shaw, 1991). The NF triplet forms a series of long unbranched filaments that run longitudinally and in parallel with each other. They are linked to each other via a series of bridges (4-6 nm diameter) which run perpendicular to the NF, and are connected to microtubules by fibrils of a similar diameter, but which are 20-50 nm long (Hirokawa, 1991).

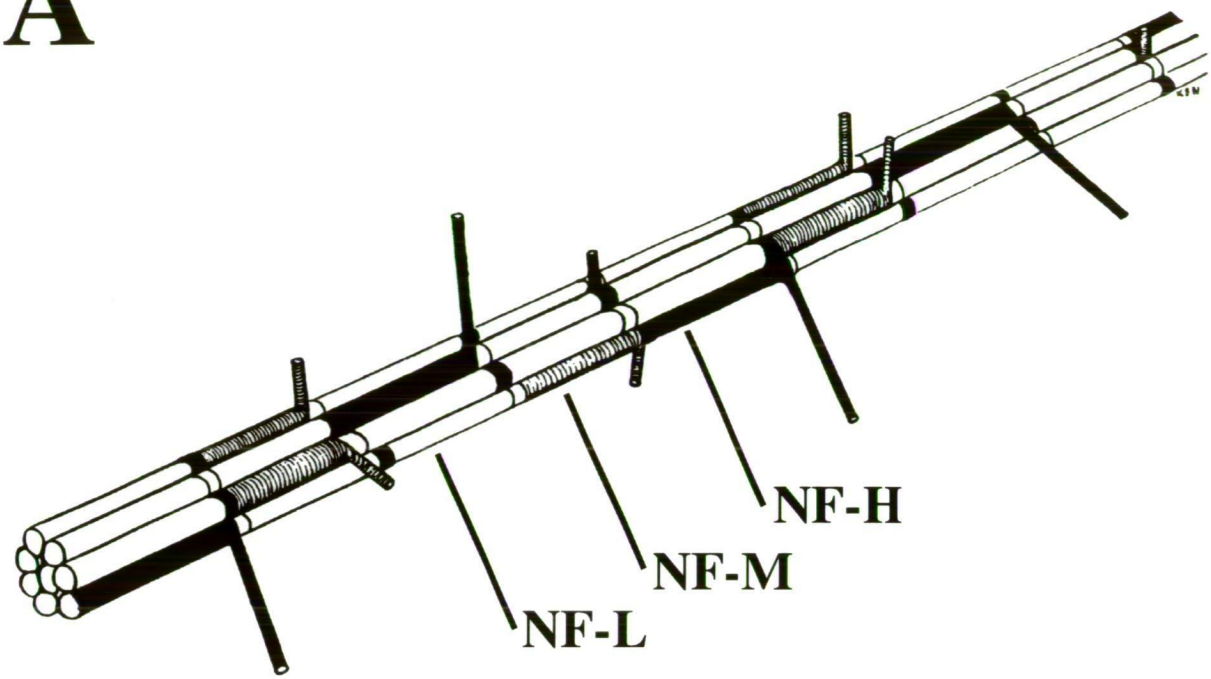
1.6.5.1 Neurofilament structure

The NF triplet is composed of three different subunits, NFL (68 KDa), NFM (150 KDa) and NFH (200 KDa), which copolymerise to form the NF triplet (Figure 1.1A). NFL, along with NFM and NFH, forms the central core, whilst NFM and NFH form components of the sidearms which project from the filament. These projections are of varied length, based upon the differences in carboxy-terminal domains of NFM and NFH (Hirokawa, 1991).

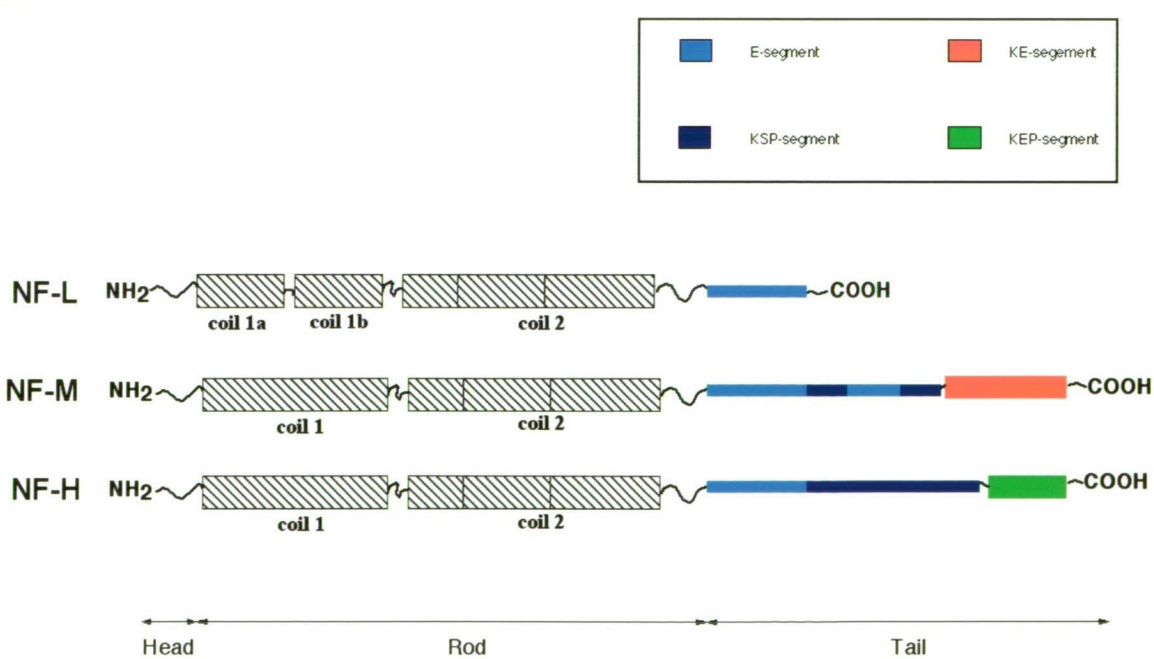
The NF triplet proteins are composed of several regions, including amino-terminal head, alpha-helical rod (conserved among all intermediate filaments) and carboxy-terminal tail domains (Shaw, 1991) (Figure 1.1B). The head domain is very basic and is

Figure 1.1. (A) shows a stylised representation of the neurofilament (NF) triplet protein present in mature axons (modified from Nixon, 1993), showing the low (NFL), medium (NFM) and high (NFH) molecular weight NF subunits. (B) is a diagrammatic representation of the amino acid sequences of the three NF triplet subunits. The vertical lines within coil 2 indicate areas where the heptad pattern is broken due to the insertion of an extra amino acid. 'E' segments correspond to areas within the sequence which are rich in glutamic acid, whilst KSP segments, which consist of lysine-serine-proline repeats, are found in variable numbers in different sections of each of the NF subunits. At the end of NFM is the 'KE' domain, which has a high concentration of lysine and glutamic acid. The carboxyterminus of NFH is referred to as the 'KEP' segment and is rich in lysine, glutamic acid and proline. (Modified from Shaw, 1991)

A


NF-H
NF-M
NF-L

B


E-segment
KE-segement
KSP-segment
KEP-segment
NF-L
NH2
coil 1a
coil 1b
coil 2
COOH
NF-M
NH2
coil 1
coil 2
COOH
NF-H
NH2
coil 1
coil 2
COOH
Head
Rod
Tail

composed of predominantly ß-sheet and ß-turn structures which range in size from ~4 to ~9 KDa (Shaw, 1991).

The central rod domain, of the tripartite structure, is a ~37 KDa alpha-helical structure which is much less varied than both the head and tail domains among the intermediate family of proteins (Shaw, 1991). The amino acids which comprise this region form a heptad repeat structure which produces a helical strip of hydrophobic amino acids along the side of each alpha-helix. This strip can then intercalate with hydrophobic amino acids on another alpha helix to form a stable dimer. The result is an elongated, rigid structure called a coiled-coil. There are two breaks in these rod domains (in all the intermediate filaments except NFM/H, which don't posses the break at coil 1A), each containing between 10 and 22 amino acids, in the heptad repeat which subsequently define three regions in the coiled coil (Coil 1A, 1B and 2) (Shaw, 1991). A tetramer of two coiled-coils in antiparallel probably form a protofilament of 2-3 nm, and then, subsequently, 8 protofilaments form one 10nm filament, with the flanking head and tail domains conferring functional specificities (Julien and Grosveld, 1991).

The next region, the carboxy-terminal, is where the NFs demonstrate significant differences ~~to~~ from the remaining intermediate filaments. The initial sequence of the NF tail is homologous to the entire carboxy-terminal tails of the other intermediate filaments. The remaining regions are intermediate filament type IV specific, and commence with a segment, ~6KDa in size, which is rich in glutamic acid. The sequence of NFL finishes here and mutations in this region on NFL have been shown to result in the segregation of NF from microtubules (Toyoshima *et al.*, 1998).

The next section is perhaps one of the more important, as this is where there are

multiple repeats of the amino acid sequence lysine-serine-proline (KSP). This is the major site of phosphorylation in the NF sequence. The amino acids surrounding these repeats form the basis of NFM/H specific KSP motifs. After this are regions which, in NFM and NFH, are rich in lysine and glutamic acid and lysine, glutamic acid and proline respectively (Shaw, 1991).

The very large carboxy terminals of NFM and NFH, which form the structural sidearms present on NF, may explain why these two subunits cannot individually form 10nm filaments, but require the presence of the NFL backbone. This is in contrast to NFL, which can self assemble to form 10nm filaments (Julien and Grosveld, 1991; Shaw, 1991), although it still requires the expression of one or both of the other two subunits to form a complete filamentous structure (Hirokawa and Takeda, 1998). The absence of the typical IF break in coil I of NFM/H may also explain their inability to self-assemble into 10nm filaments, as the elongated coil I structure is extremely rigid (Nixon and Shea, 1992).

1.6.5.2 NF Expression and localisation

NFM and NFH are normally dephosphorylated in the perikaryon, but then extensively phosphorylated once they are translocated into the axon, where they are transported to the axon terminal at a rate of between 0.25 and 3 mm/day depending on the organism and cell type (Julien and Grosveld, 1991). It is postulated that NFs are stationary within the axon for between 85-99% of the time spent in transport, but then undergo extremely rapid periods of movement (Wang *et al.*, 2000). The phosphorylation state of the NF governs the relative affinity of the proteins for the transport mechanisms, as the highly phosphorylated forms of NF are associated with stationary NF within the axon (Nixon and Shea, 1992). Indeed, phosphorylation in general can affect a number of different

elements which comprise the neuronal cytoskeleton, and as such, it contributes to the formation of a stable filament network within the cell (Nixon and Shea, 1992). The phosphorylation of the amino-terminal of NFL, for example, will result in the disassembly of the NFL polymer and inhibit NFL polymerisation, thereby preventing the formation of the NF triplet (Strong, 1999). Similarly, phosphorylation of the carboxy-terminals of NFM and NFH facilitates NF crosslinking, stabilises the axonal cytoskeleton and protects NF from proteolysis.

The turnover within the axon is small, and proteases degrade the NFs at the axon terminal (Julien and Grosveld, 1991). Turnover is believed to occur at discrete sites on the filaments by the incorporation of new subunits by an unknown mechanism (Okabe *et al.*, 1993).

Developmentally, the expression of NFs is associated with the appearance of post-mitotic neurons. NFL and NFM are coexpressed prior to the formation of synaptic connections, and then NFH is expressed and is associated with a decrease in cytoskeletal transport (Julien and Grosveld, 1991). The appearance of NFs, associated with an increase in axonal calibre, together with myelination signify the maturation stage of development (Oblinger *et al.*, 1989).

1.6.5.3 Neurofilament function

The functions of NFs are hypothesised to be related to the maintenance of axonal calibre and cell shape, with a correlation existing between NF spacing and phosphorylation (narrow spacing of non-phosphorylated dendritic NFs, and wide in phosphorylated axonal NFs) (Shaw, 1991). Phosphorylation of the KSP repeats present in the carboxy tail domain of NFM/H increases the net negative charge causing an

increase in lateral extension of the terminal sidearms and, therefore, increasing the spacing and/ or increasing the crossbridging to other cytoskeletal elements (Hirokawa and Takeda, 1998; Julien, 1999). NFs may, therefore, directly contribute to the function of the cell by controlling axonal diameter, thereby determining the velocity of action potentials within the neuron (Lasek *et al.*, 1985; Xu *et al.*, 1993). Indeed NF-subunit knockouts have demonstrated an, often significant, effect on the cytoskeleton, including a decrease in axonal calibre, a decrease in IF number and an increase in the density of microtubules (Julien, 1999). Whilst it was originally reported that NFH was the most important subunit governing the regulation of axonal diameter by NFs, it has recently emerged that NFM is the most important subunit for the assembly and structure of NFs and for the radial outgrowth of large myelinated axons during development (Hirokawa and Takeda, 1998; Julien, 1999).

There are axons, however, which contain NFs, but whose diameter is not reliant on NF content. Presumably, then, NFs must have other roles, such as the strengthening of the cytoskeleton (Lin and Szaro, 1995). Indeed, once NFs have been formed, they are very hard to disassemble, thereby making them inherent structural supports for the cytoskeleton (Lasek *et al.*, 1985). NFs may also play a role during development and regeneration, with the emergence of different NF isoforms occurring at, and being associated with, different stages of growth (Lin and Szaro, 1995). It is also possible that, because NFs are degraded at axon terminals and then transported back to the perikaryon, that the NF breakdown products may serve as an indicator of the functional integrity of the cell. This may represent a positive feedback mechanism which can stimulate certain events following neuronal injury (Shaw, 1991). Parts of the NF molecule may, therefore, act either directly or indirectly as transcriptional or translational regulators (Shaw, 1991).

It should also be noted that not all nerve cells require NFs to function, which may be due to functional overlap with other cytoskeletal proteins such as microtubules. There is, for example, a naturally occurring recessive variant of the Japanese (quivering) quail which lacks NFL (Mizutani *et al.*, 1992). With the exception of a trembling phenotype, perhaps related to a decrease in stability of the cytoskeleton due to the absence of NFs, the animals function normally. They do, however, have fewer and thinner axons than normal, supporting the notion that NFs have a function in maintaining axonal calibre (Lin and Szaro, 1995). There are, similarly, other classes of neurons, such as particular populations of neurons found within the coeliac ganglion in the guinea pig, which don't contain NF (Vickers *et al.*, 1990). NFs, therefore, are not intrinsic components of the neuronal cytoskeleton of all neurons (Vickers *et al.*, 1990, 1991).

1.7 The importance of the cytoskeleton

Cytoskeletal proteins are, therefore, crucial cellular organelles whose normal maintenance and operation are required to maintain neuronal function and integrity. If these elements are altered or misprocessed, the result could be deleterious to the cell, with impairments in many cellular processes. In this respect it is clear that a number of cytoskeletal proteins are affected in AD, as detailed earlier. However, the exact mechanism by which this damage occurs, and the relationship that ß-amyloid has to it are yet to be fully elucidated. Recent work suggests that there may be similarities between the neuropathological changes which occur in the early stages of AD, and those which occur following traumatic brain injury (TBI), one of the reported risk factors for the development of AD. An understanding of the mechanisms underlying the neuronal change associated with TBI, together with information provided by examining preclinical AD cases, may give further clues as to the processes which leads to cellular change following ß-amyloid deposition in the cortex of AD sufferers.

1.8 Human Brain Injury

TBI in humans involves an immediate and direct disruption to the brain (Dunn-Meynell and Levin, 1997). Both focal and diffuse injuries result from TBI. Focal injuries are associated with cerebral contusions and haematoma, typically resulting from blows to the head (Smith *et al.*, 1997), whilst diffuse injuries, which are common after car accidents and falls from any height, are dependent upon the rapid movement and deformation of the brain (Adams *et al.*, 1984, 1989; Grady *et al.*, 1993; Abou-Hamden *et al.*, 1997; Smith *et al.*, 1997). These diffuse injuries are considered the most important pathology in severely brain injured people (Smith *et al.*, 1997), however, both types of injury are normally encountered following brain trauma (Graham *et al.*, 1993). It is these diffuse injuries following TBI which often lead to a prolonged, progressive neurodegenerative cascade involving diffuse axonal injury (DAI), gliosis and cell death (Erb and Povlishock, 1988; Gorman *et al.*, 1989; McIntosh *et al.*, 1989; Dunn-Meynell and Levin, 1997; Smith *et al.*, 1999). DAI remains the primary cause of death and neuronal dysfunction following TBI (Chen *et al.*, 1999). TBI is also one of the leading causes of death and disability in humans (Sosin *et al.*, 1995), particularly in the 15-24 year age group, where it accounts for ~550/100,000 deaths (Zemlan *et al.*, 1999).

DAI is associated with the unrestricted rotational acceleration and deceleration of the head (Gennarelli *et al.*, 1982). Due to the mass of the human brain and the inertial loading from the injury, there are shear, tensile and compressive strains on the brain which result in tissue deformation (Smith *et al.*, 1997). Clinically, DAI has been associated with both prolonged cognitive impairment (Brooks, 1972) and cognitive decline in aging (Corkin *et al.*, 1989; Stuss *et al.*, 1989). DAI is also associated, in between 2 and 20% of cases, with the development of AD (Mortimer *et al.*, 1991; Mayeux *et al.*, 1995; Rasmusson *et al.*, 1995).

The characteristic neuropathological finding in DAI is perturbations to various cytoskeletal elements, and the presence of axonal swellings at the ends of injured axons, which are immunoreactive for a number of proteins, but particularly NF (Povlishock, 1986; Povlishock, 1992; Yaghmai and Povlishock, 1992; Gultekin and Smith, 1994). In all grades of DAI, there is this widespread appearance of swollen axons and the formation of terminal axonal bulbs (Smith *et al.*, 1997), and similarly, axonal injury is reported to be associated with fatal head injury in nearly 100% of cases (Gentleman *et al.*, 1995). In the study by Gentleman and colleagues (1995), it was reported that the amount of axonal damage increased proportionately with survival time in the first 24 hours, but thereafter plateaued and declined over the next two months to the point that it was difficult to identify the typical axonal bulbs, whilst other features, such as Wallerian degeneration, had become the primary neuropathological finding.

Zemlan and colleagues (1999) have demonstrated that there is tau present in the CSF of head injured patients, and that it correlates with the clinical condition of a patient. They also hypothesise that it is a good predictor of not only the severity of head injury, but also perhaps patient outcome after discharge.

In single incidents of severe brain trauma there has been the demonstration of the occurrence of ß-amyloid deposition in the form of diffuse plaques within days of the injury (Roberts *et al.*, 1991, 1994; Graham *et al.*, 1995). In contrast, however, it has been reported (Geddes *et al.*, 1999), in a study examining five patients who experienced a variety of forms of mild chronic head injury, that NFT develop in the absence of ß-amyloid deposition. Other studies have also shown an increase in ß-amyloid in the CSF (Raby *et al.*, 1998), and a demonstrable increase in immunoreactivity for APP following brain trauma (Roberts *et al.*, 1994).

1.9 Animal models of human brain injury

Human TBI has been experimentally modelled in a number of different paradigms. In all these models, widespread axonal and cytoskeletal damage results, which has been principally identified utilising NF antibodies to highlight axonal damage and evolving axonal disconnection (Dixon *et al.*, 1991; Meller *et al.*, 1993; Povlishock, 1993; Foda and Marmarou, 1994; Kanayama *et al.*, 1996; Dunn-Meynell and Levin, 1997; Chen *et al.*, 1999). Whilst axonal damage is a consistent feature of all grades of injury, immediate primary axotomy following TBI, which has been shown to occur within the first twenty minutes post-injury (PI), only represents a small proportion of the most severely affected axons (Maxwell *et al.*, 1993; Maxwell *et al.*, 1997). The majority of neurons are subjected to a form of non-disruptive axonal injury which initiates a focal evolving process that progresses to the disruption of axoplasmic transport, local axonal swelling and the subsequent disconnection from the distal segment, or secondary axotomy (Pettus *et al.*, 1994; Pettus and Povlishock, 1996; Maxwell *et al.*, 1997; Povlishock *et al.*, 1997; Okonkwo *et al.*, 1998).

More specifically, there are initial intra-axonal changes, such as the misalignment and compaction of NFs, perhaps due to a change in phosphorylation state (Christman *et al.*, 1994; Okonkwo *et al.*, 1998); an increase in proteolysis of, for example, spectrin and NFs, with an altered phosphorylation state increasing the susceptibility of NFs to proteolysis (Goldstein *et al.*, 1987; Schlaepfer, 1987; Pant, 1988; Nixon and Sihag, 1991); microtubular loss and mitochondrial swelling (Meller, 1987). Focal alterations in axolemmal permeability ensue, and lead to impairments in axoplasmic transport with the subsequent accumulation of organelles, mitochondria, other vesicular structures and cytoskeletal components such as NFs (Blumke *et al.*, 1966; Lanners and Grafstein, 1980; McHale *et al.*, 1995). Specifically, NFs accumulate in the synaptic terminal, a

domain where they are normally not present, and this is often around a central mitochondrion which subsequently gives the morphological appearance of a ring-like structure (Gray and Hamlyn, 1962; Guillery, 1965; Meller *et al.*, 1993, 1994; King *et al.*, 1997). These distal portions gradually undergo Wallerian degeneration. There is also an accumulation of NFs in the proximal part of the axon and perikarya such that, following disconnection from the distal segment, a NF positive axonal swelling is evident (Torvick, 1976; Lanners and Grafstein, 1980; Meller, 1987; Schlaepfer, 1987; Christman *et al.*, 1994; Pettus *et al.*, 1994; Silveira *et al.*, 1994; McHale *et al.*, 1995; Pettus and Povlishock, 1996; Povlishock and Pettus, 1996; Povlishock *et al.*, 1997; Okonkwo *et al.*, 1998).

Other NF changes include the abnormal localisation of specific epitopes to different neuronal domains. Within the cell body, NFs are normally dephosphorylated (Sternberger and Sternberger, 1983), however, following injury phosphorylated NF epitopes appear in the cell body (Mansour *et al.*, 1989; Martin *et al.*, 1990; Yamada and Hazama, 1993). Similarly, NFs in the axonal domain are normally phosphorylated (Sternberger and Sternberger, 1983), but following injury there is the abnormal localisation of dephosphorylated epitopes to this neuronal domain (Meller *et al.*, 1993, 1994; Ross *et al.*, 1994; King *et al.*, 1997). Injury also results in a decrease in NF expression (Mikucki and Oblinger, 1991), whilst other cytoskeletal proteins such as tubulin and actin are reported to be increased following neuronal injury (Hoffman and Cleveland, 1988).

The abnormal accumulations of NFs within axons have been shown to develop as early as three hours PI (Dunn-Meynell and Levin, 1997), however, cytoskeletal change, which is a feature of axonal injury (Kanayama *et al.*, 1997), has been reported to occur

as early as the first minutes following injury with the destruction of dendrites (Gallyas and Zoltay, 1992; Gallyas *et al.*, 1992). This may be due to the significant decrease in MAP2, a major MAP which provides structural support to neuronal processes (Matus, 1994), that has been shown to occur in the first ten minutes PI (Hicks *et al.*, 1995), as well as at other times up to seven days PI (Taft *et al.*, 1992; Hicks *et al.*, 1995; Lewen *et al.*, 1996). It has also been shown that MAP2 abnormally accumulates in the neuronal perikarya following severe TBI (Kanayama *et al.*, 1997).

It should be noted, however, that the response to injury is not uniform when comparing different neuronal populations. In the case of stretch injury to the optic nerve, for example, it has been demonstrated that small and large calibre axons are affected differently. The small axons undergo NF compaction, lasting up to four hours PI, NF number increases and there is no significant change in other cytoskeletal elements such as MAP2 (Jafari *et al.*, 1997). Larger axons, however, experience two different forms of pathology. Where periaxonal spaces occur, there is a focal compaction of NFs for at least six hours PI, but no change in their number, and a decrease in the number of microtubules (Pettus and Povlishock, 1996; Jafari *et al.*, 1997). Where intramyelin spaces occur there is a significant decrease in the number of both NFs and microtubules and an increase in the spacing between both NFs and microtubules (Jafari *et al.*, 1997).

There are many factors hypothesised to underpin the axotomy which eventually occurs following TBI. One recent hypothesis was that mitochondrial failure may be a precipitating event in the progression to axotomy (Okonkwo and Povlishock, 1999; Okonkwo *et al.*, 1999). Okonkwo and colleagues (1999) suggested that the mitochondrial permeability transition pore may abnormally open and subsequently interfere with the production of high energy phosphates by the mitochondrion. These

molecules are essential to the function of the axolemmal membrane pumps which help maintain a homeostatic environment within the axon. This change, therefore, may lead to membrane deterioration and calcium influx which may participate in eventual axotomy (Okonkwo and Povlishock, 1999; Okonkwo *et al.*, 1999). This is supported by studies utilising cyclosporin A to inhibit this opening of the transition pore in the mitochondria. This was shown to slow the calcium-induced cytoskeletal change resulting from TBI and to decrease the number of disconnected and dysfunctional axons (Buki *et al.*, 1999). So, whilst mitochondria may serve a primary role in the progression of events following TBI, other factors may also have an equally important function. Calcium for example, has been shown to increase in the axon following mitochondrial failure (Okonkwo and Povlishock, 1999; Okonkwo *et al.*, 1999), as well as following membrane depolarisation after TBI (Folkerts *et al.*, 1998). Indeed, calcium has generally been shown to accumulate in cortical tissue following TBI (Shapira *et al.*, 1989; Fineman *et al.*, 1993). Calcium impacts upon many cellular functions, and a lapse in its normally rigorously controlled levels may result in the abnormal activation of kinases, which can subsequently impact upon many cellular functions including, for example, the assembly of MAP2, as well as an activation of calpain activity. Calpains have been shown to be upregulated as early as 15 minutes PI, and to remain changed for up to six hours (McCracken *et al.*, 1999) following TBI. This upregulation of calpain activity is also concomitant with cytoskeletal collapse in neurons (Posmantur *et al.*, 1994; Saatman *et al.*, 1996), and is attributed to the overactivation of calpains (Kampfl *et al.*, 1996). There are, undoubtedly, numerous facets to the evolution of neuropathology following brain injury. The influx of calcium, the overactivation of calpains and so on are all important aspects which contribute to the spectrum of changes which occur following TBI.

Animal models of TBI, therefore, demonstrate significant neuropathological similarities with human head trauma. They have also shown that neurons respond to injury in a stereotyped fashion, with a complex series of morphological, neurochemical and gene expression changes which are principally directed at an attempt to resprout or regenerate.

1.10 A link between Alzheimer's disease and head injury ?

It is clear from the studies of both human head trauma and animal models of TBI that the neuropathological sequelae which occur following physical injury to the brain closely resembles the neuropathological change which is found in AD. Interestingly, a number of studies have also examined the accumulation of ß-amyloid in the cortex of animals following experimentally induced head injury. Utilising a model of rotational acceleration of the head, Smith and colleagues (1999) demonstrated the occurrence of diffuse axonal pathology, concomitant with an accumulation, and colocalisation, of ß-amyloid and tau in damaged axons which demonstrated accumulations of APP and NF. This was observed between three and ten days PI. Other studies, however, have not confirmed the observation of ß-amyloid accumulation following experimentally induced cortical injury (Pierce *et al.*, 1996). The accumulation of phosphorylated tau in the neuronal perikarya has been shown, however, in a model of repeated mild cortical impact (Kanayama *et al.*, 1996).

To briefly recap, in AD there is an accumulation of NF in the perikarya (Vickers *et al.*, 1992, 1994; Nakamura *et al.*, 1997); the formation of NF positive bulb- and ring-like structures, which are early forms in the progression to DN formation, associated with plaques (Vickers *et al.*, 1996; King *et al.*, 1997); a down regulation of NF expression (Kittur *et al.*, 1994); phosphorylated NF epitopes are abnormally localised to nerve cell

bodies (Masliah *et al.*, 1993b; Vickers *et al.*, 1994); there are vesicular and mitochondrial changes and accumulations (Gonatas *et al.*, 1967; Dickson, 1997); a loss of microtubular structures in damaged axons (Gray *et al.*, 1987; Paula-Barbosa *et al.*, 1987) and the early forms of DN undergo a stereotypy of changes directed at attempts to regenerate. That such a similarity exists between the neuropathological sequelae of physical injury to the cortex and AD, suggests that ß-amyloid deposition within the cortex functions to physically damage the cortical tissue. The hypothesis, therefore, is that ß-amyloid is not harmful to neurons via a chemical toxin, but rather, its deposition within the brain causes physical damage to neurons, which triggers them to enter into a programmed response to that trauma (Vickers, 1997; Vickers *et al.*, 2000). This then results in a number of neuronal and cytoskeletal abnormalities which subsequently give rise to the characteristic neuropathology of AD. Whereas, in head trauma, there is an immediate deformation of cortical cells, ß-amyloid deposition is a slowly evolving process which ultimately leads to the formation of an insoluble plaque. The continued presence of this structure, then, is likely to continually damage neurons and result in the repeated stimulation of the neuronal response to trauma.

That the pathogenesis of AD crucially involves the cytoskeleton suggests that targeting these changes may be an effective method of delaying or even preventing neurodegeneration in AD (Matsuyama and Jarvick, 1989; Vickers, 1997; Vickers *et al.*, 2000).

1.11 Therapeutic intervention in Alzheimer's disease

There are currently no treatments available to prevent AD. Similarly, there are no drugs yet available which will successfully reverse, halt or slow the progression of pathological changes which occur in the AD brain.

The difficulty in treatment of AD arises from its multifactorial nature. The heterogeneity of the disease is such that it can arise on vastly different genetic backgrounds and past histories and often develops in the absence of any apparent cause. In terms of the course of the illness, it is by no means a 'single-effect' disease. AD affects multiple neurotransmitter systems (Hardy *et al.*, 1985); multiple brain regions and neuronal types. Although there appears to be some selectivity with the latter. A classical antagonist/ agonist approach to drug therapy for AD is, therefore, not suitable (Shvaloff, 1996).

Despite decades of research spent trying to find a 'cure', AD sufferers are still limited to the symptomatic treatment of their illness. This has two major foci. Firstly, the alleviation of behavioural problems, such as restlessness, agitation and mood swings, associated with AD. This remains an important step in improving the quality of life of AD patients, as the lifetime risk of a dementia patient experiencing such behavioural changes is close to 90% (Tariot *et al.*, 1997). Secondly, and perhaps more importantly, the amelioration of the cognitive decline experienced, by the augmentation of neurotransmitter levels in the AD brain. Whilst numerous neurotransmitter systems, such as the GABAergic system, are affected in AD, the one of primary interest and apparent greatest benefit from treatment, is the cholinergic system (Brodaty and Sachdev, 1997). The goal remains, however, to achieve a more fundamental approach to prevent disease, including strategies based on an increased understanding of, for example, the disruption of the neuronal cytoskeleton from NFT formation.

1.11.1 Cholinergic Agents and other neurotransmitter therapy

The use of cholinergic agents in the treatment of AD is based upon the cholinergic hypothesis (Bartus *et al.*, 1982). This hypothesis states that the learning and memory

discrepancies which occur in both the aged and the AD population may be attributable to a decline in the activity of one of the main neurotransmitter systems associated with memory, the cholinergic system. Specifically, the nucleus basalis of Meynert, a basal forebrain site where cortically projecting cholinergic neurons are located, is a centrally affected region in AD, and demonstrates a loss of cholinergic cells and a decrease in cholinergic function (Whitehouse *et al.*, 1981, 1982; Shvaloff, 1996). This region of the brain produces choline acetyltransferase- a molecule which links choline from the synaptic space, via high affinity choline uptake, to acetyl molecules to form the neurotransmitter, acetylcholine (ACh). It is also a biochemical marker for cholinergic neurons, and was utilised to demonstrate a loss of such neurons in the AD brain (Davies and Maloney, 1976; Perry *et al.*, 1977). The deterioration of this cholinergic system is associated with the early decline in cognitive function in AD (Perry *et al.*, 1978, 1981 and 1992; Palmer *et al.*, 1987; Reinkainen *et al.*, 1990; Dekosky *et al.*, 1992; Lehericy *et al.*, 1993; Brodaty and Sachdev, 1997; Tariot *et al.*, 1997; Rogers *et al.*, 1998), and so preventing the decline in ACh became a target for the treatment of AD.

The four main mechanisms that can be utilised to enhance ACh activity are precursor loading (with choline or lecithin) to augment ACh synthesis, increasing ACh release (with linoprine or ondansetron), inhibition of cholinesterase activity (physostigmine) to delay the intrasynaptic degradation of ACh and agonism of nicotinic and muscarinic receptors (arecholine). The latter has relevance to AD, as post synaptic muscarinic cholinergic receptors are relatively intact in AD, and presynaptic muscarinic receptors (which are decreased in AD) regulate acetylcholine release (Shvaloff, 1996; Tariot *et al.*, 1997). Muscarinic agonists then, such as xanomeline and talsaclidine, have been demonstrated to slow cognitive decline and have positive behavioural effects (Bymaster *et al.*, 1997; Ensinger *et al.*, 1997).

The most effective method of preserving the decreasing amounts of ACh within the AD brain to date, however, has been the use of acetylcholinesterase inhibitors (Wilcock, 1996; Brodaty and Sachdev, 1997). These agents target the molecules which, within the synaptic cleft, selectively metabolise ACh (Shvaloff, 1996). The other main enzyme utilised within the brain for degrading ACh is butyrlcholinesterase (Shvaloff, 1996), but drugs which antagonise its action have had little development.

The first cholinesterase inhibitor to be utilised, and the first drug to be approved for use in the treatment of AD by the Food and Drug Administration (FDA) in the United States, was tacrine hydrochloride (Cognex®) in 1995. This is a centrally active, reversible, non-specific cholinesterase inhibitor. It gave modest improvement in cognitive function, with the largest responses in those on higher doses. In patients able to tolerate treatment, between 30-51% showed a significant improvement on global clinical scales and indices of daily living, as compared to between 16 and 25% of the placebo group (Wagstaff and McTavish, 1994). Those which continued with treatment, however, only represented approximately 50% of the initial patients, the remaining unable to take the drug due to its toxicity. Gastrointestinal problems (Brodaty and Sachdev, 1997), as well as a host of other issues related to the use of cholinergic agents, including nausea, vomiting, hepatotoxicity and bradycardia (Wagstaff and McTavish, 1994; Tariot *et al.*, 1997) were experienced. The improvement in cognitive function was equated to the patients reverting to their cognitive status of at least six months prior to the commencement of treatment. There were also positive behavioural modifications as a result of treatment, including less anxiety, apathy, hallucinations and aberrant motor behaviours (Raskind *et al.*, 1997). The average duration of benefit was approximately three months (Brodaty and Sachdev, 1997; Tariot *et al.*, 1997), but the benefits, in some cases, can be extended out to 12 months (Giacobini, 1998; Parys, 1998). It is unlikely

that patients will receive benefit past this, due to tolerance to the drug and/or progression of the disease state (Giacobini, 1998). A review of the therapeutic efficacy and pharmacokinetic properties of tacrine can be found in Wagstaff and McTavish (1994) and Eagger and colleagues (1994).

Newer cholinesterase inhibitors have been developed which are at least as effective in the treatment of AD as tacrine, such as donepezil hydrochloride (Aricept®), which was approved by the FDA in 1996. This is a highly selective piperidine based molecule which reversibly inhibits cholinesterase activity, and which shows greater specificity for the CNS than tacrine (Rho and Lipson, 1997; Rogers *et al.*, 1998). Again, these drugs give statistically significant improvements in global clinical scales and indices of daily living, however, this benefit can be obtained as early as the first three weeks of treatment for demonstrated improvements on cognitive tests, and within the first 6 weeks for improvements in global function (Rogers *et al.*, 1998). It has been reported that the number of patients showing cognitive decline is reduced from 20% in the control group to 11% in those taking the drug (Rogers *et al.*, 1996). The benefits of these newer agents are that they are tolerated better and have fewer cholinergic side effects, the dosing regime is easier and achieves therapeutic levels quicker (Rogers *et al.*, 1996, 1998; Rho and Lipson, 1997).

Despite the positive benefits of these cholinesterase inhibitors, no drug has yet been able to return cognitive function to normal control levels, and they do nothing to prevent the disease progression (Wilcock, 1996; Brodaty and Sachdev, 1997; Giacobini, 1998). Despite this, the improvements that are gained in cognitive function and relief from various behavioural problems give a real improvement in the quality of life for AD patients in the short term (Giacobini, 1998).

These two agents, tacrine and donepezil, were the first two drugs to be approved for use in the treatment of AD, however, there are numerous other drugs which are in different phases of clinical trials which may also be later approved for use in AD, such as huperzine A (Cheng *et al.*, 1996; Da-Yuan *et al.*, 1996) and galanthamine (Rainer, 1997). Recently (April, 2000), another cholinesterase inhibitor, rivastigmine (Exelon™) (Sim, 1999), was approved by the FDA for use in the treatment of AD.

1.11.2 Other neurotransmitter therapy

Both aging and AD are associated with defects in monoaminergic transmission, as evidenced by a decrease in monoamine metabolite concentration in the brain and CSF. This is due to the increased activity of type B monoamine oxidases (MAO-B). MAO-Bs are mitochondrial enzymes which are responsible for the oxidative deamination of dopamine and other monoamines (Piccinin *et al.*, 1990). The inhibition of MAO-Bs with drugs such as L-deprenyl, therefore, has shown to have significant effects on memory and attention, which may be due in part to an improved function of the monoaminergic system, a decrease in oxidative stress or an increase in catecholamine levels and other chemicals such as dopamine and phenethlamine (Piccinin *et al.*, 1990; Wilcock, 1996; Brodaty and Sachdev, 1997; Tariot *et al.*, 1997).

Other neurotransmitters which are affected in AD have also been targeted. Attempts to boost serotonin levels, to normalise the catecholaminergic system, as well as to regulate NMDA receptor transmission and glutaminergic and GABAergic function, have all been trialled through the use of various precursors, agonists and antagonists, but to little or no positive behavioural or cognitive effect (Shvaloff, 1996; Wilcock, 1996; Brodaty and Sachdev, 1997; Tariot *et al.*, 1997).

1.11.3 Non-steroidal anti-inflammatory drugs

Retrospective analyses of patients with rheumatoid arthritis, who have a predilection to the use of non-steroidal anti-inflammatory drugs (NSAIDS), have demonstrated that there is an inverse relationship between the use of NSAIDS and the prevalence of AD, as compared to control patients (Jenkinson *et al.*, 1988; Broe *et al.*, 1990; McGeer *et al.*, 1990; Tariot *et al.*, 1997). NSAIDS such as indomethacin, therefore, appear to either delay the development of AD or slow the progression of both cognitive and behavioural symptoms (Rogers *et al.*, 1993; Rich *et al.*, 1995; Shvaloff, 1996; Wilcock, 1996).

There have been numerous studies on the use of NSAIDS, and their role in slowing the symptoms of AD, however, the majority of studies have been small and, therefore, lacked significance. McGeer and colleagues (1996) analysed 17 different epidemiological studies, however, and demonstrated that anti-inflammatory drugs may indeed be useful in protection against AD. Similarly, large population based studies have shown a possible protective effect of NSAIDS on the risk of the development of AD (Anderson *et al.*, 1995). In an interesting study by Breitner and colleagues (1994), twin pairs presenting with a disparity in the onset of AD by at least three years were examined. The patient with delayed onset, or in fact no AD symptoms, was more likely to have had prior treatment with either steroids or NSAIDS. Whilst the majority of these studies point to a role for NSAIDS in AD treatment, other studies, such as that by Henderson and colleagues (1997), have not been able to attribute any positive effect on the incidence of AD, or the slowing of symptoms, to the use of either aspirin or NSAIDS, even after more than three and a half years of treatment.

Whilst AD is unlikely to be initiated by inflammatory processes, there is little doubt that this plays a part in the disease process and presentation of symptoms. The AD brain

contains several markers of an inflammatory process, and is associated with an activation of the complement cascade and reactive astrocytes and/or activated microglia are found in association with both ß-amyloid plaques and neurofibrillary tangles (McGeer and Rogers, 1992; Breitner, 1996; McGeer *et al.*, 1996). NSAIDS are believed to function by inhibiting cell death, either by preventing astrocytic reuptake of glutamate and thereby interfering in glutamatergic transmission or by the suppression of cyclooxygenases, which catalyse the synthesis of prostaglandins and subsequently interfere in postsynaptic signal transduction (Breitner, 1996).

1.11.4 Hormone replacement therapy

Estrogen replacement therapy (ERT) is reported to decrease the risk of developing AD in postmenopausal women (Mortel and Meyer, 1995; Henderson, 1996; Tang *et al.*, 1996; Keller *et al.*, 1997; McBee *et al.*, 1997; Paganini-Hill, 1997; Paganini-Hill and Henderson, 1997; Sohrabji and Miranda, 1997; van Duijn, 1997). It has also been shown to result in an improvement in cognition and other behavioural indices (Fillit *et al.*, 1986; Kampen and Sherwin, 1994). However, there have also been a number of recent studies, which suggest that the short term (4 months) use of ERT does not have any positive effect on women with mild to moderate AD (Henderson *et al.*, 2000). Similarly, it has been demonstrated that the longer term (1 year) use of ERT does not slow the progression of AD, or result in a positive change in different functional and cognitive indices in women with mild to moderate AD (Mulnard *et al.*, 2000).

The use of estrogen has also been investigated in cell culture experimental models, where it has been shown to decrease both Aß(25-35)-induced toxicity and lipid peroxidation (Gridley *et al.*, 1997); block the toxicity of Aß(1-42) and increase neurite extension (Mook-Jung *et al.*, 1997); attenuate neuronal loss due to oxidative and excitotoxic stress (Regan and Guo, 1997) and to differentially and significantly regulate

cortical nerve cell outgrowth (Brinton *et al.*, 1997) and in particular, increase the density of spines on hippocampal neurons (Brinton, 1993). This latter point, which is postulated to directly affect memory, has also been demonstrated *in vivo* (Gould *et al.*, 1990; Woolley *et al.*, 1990; Woolley and McEwen, 1992, 1994). Whilst most studies have emphasised the action of estrogen on prevention of toxic-related nerve cell death and effects of cortical outgrowth, it may also impact upon the metabolism of APP and interact with ApoE (Brodaty and Sachdev, 1997).

Interestingly, the effects of estrogen are not confined to postmenopausal women. Phillips and Sherwin (1992) have demonstrated that 17 ß-estradiol enhances memory in not only women who have had surgically-induced menopause, but also in young neurologically normal adult women. It remains unlikely, however, that men would ever receive any benefit from ERT, as they have a constant source of estrogen until late in life from the intracerebral aromatisation of oestrone to oestrogen (Brodaty and Sachdev, 1997). This may, perhaps, explain the decreased prevalence of AD in the male population. The use of ERT may, therefore, represent a future therapy for AD.

1.11.5 Growth factor therapy

Another popular hypothesis is that the use of growth factors will be efficacious in the treatment of AD. Growth factors, in particular neurotrophins, are proteins which act on specific populations of neurons to support their growth, differentiation, survival and which may potentially have a role in repair of the CNS (Shvaloff, 1996). The neurotrophins, which all share a conserved domain and have a variable domain responsible for different receptor specificities, consist of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and NT-4/5. Other groups also exhibit neurotrophin-like activity, such as lymphokines, insulin-like growth

factors, fibroblast and epidermal growth factors (Wilcock, 1996; Bossi, 1998).

Studies have been undertaken in both animals and man, and the evidence in animal trials is fairly unanimous. The administration of NGF has been reported to have many effects. It is neuroprotective of basal forebrain cholinergic neurons, which are central to the cholinergic deficit in AD (Shvaloff, 1996). It will also promote neuronal survival, irrespective of the cause of the damage (Tariot *et al.*, 1997), prevent neuronal death, induce and accelerate recovery from injury and, with the appropriate conditions, both result in significant compensatory changes in cortical synaptic connections and improve behavioural symptoms associated with neurological deficit (Garofalo *et al.*, 1992; Tuszynski and Gage, 1995; Novikov *et al.*, 1997; Bossi, 1998). Different neurotrophins will also act on different populations of neurons. NGF, for example, will support the survival of septal cholinergic neurons whereas BDNF will support the survival of dopaminergic neurons in the substantia nigra (Bossi, 1998).

Trials of NGF administration in AD patients have, therefore, been undertaken (Olsen *et al.*, 1992; Seiger *et al.*, 1993; Eriksdotter *et al.*, 1998). These studies have shown little positive consistent effect, with side effects and drug penetration both being limiting factors.

The use of growth factors in the treatment of AD, however, may be harmful. It has been reported that there is massive somatodendritic sprouting of cortical neurons in AD (Ihara, 1988; McKee *et al.*, 1989). This may be due to a chronic stimulation of the neuronal reaction to physical trauma induced by the presence of ß-amyloid plaques within the brain. These sprouting attempts are futile and may eventually lead to cell death. The use of growth factors, therefore, may accelerate this response and cause a

faster degeneration of nerve cells and loss of cortical connections. This may subsequently result in a quicker cognitive decline.

1.11.6 Cytoskeletal strategies

The abnormal phosphorylation of tau proteins in AD, particularly in the cortex, results in a significant disruption to the neuronal cytoskeleton, which in turn results in impaired intraneuronal transport and may contribute to the formation of NFT. A potential target for therapy therefore, is an agent which acts to prevent this abnormal phosphorylation of tau, which may then result in a preservation of neuronal transport, including the retrograde transport of neurotrophic factors to the perikaryon and neurotransmitters to their site of release (Shvaloff, 1996; Wilcock, 1996; Tariot *et al.*, 1997). Another potential target is the cytoskeleton itself. Stabilisation of cytoskeletal networks against collapse, with drugs such as sabeluzole, may represent another therapeutic opportunity (Uberti *et al.*, 1997; Delacourte, 1998).

1.11.7 Anti-amyloid strategies

The recent literature has highlighted a promising avenue of therapy, anti-amyloid strategies. There have been a number of hypotheses put forward to prevent the deposition of ß-amyloid within the brain. These have included the use of antagonists against the enzymes which abnormally cleave APP into more amyloidogenic fragments (Shvaloff, 1996; Wilcock, 1996), and agents which break the structure of ß-amyloid, anti-ß-sheet peptides (Delacourte, 1998; Sigurdsson *et al.*, 1998). An example of a ß-sheet breaker peptide is Aß5. This peptide has been tested in both *in vitro* and *in vivo* paradigms. *In vitro* experiments have demonstrated that it reduces the neurotoxicity of Aß 1-42. It has shown similar promise *in vivo*, where it has reduced the size of ß-amyloid deposits by 49%, as well as removed histochemical positivity for Aß and

thioflavine-S (Sigurdsson *et al.*, 1998).

In another more recent study by Schenk and colleagues (1999), it has been demonstrated that the immunization of a transgenic mouse line, which expresses mutated human APP and subsequently develops the neuropathological hallmarks of AD, with ß-amyloid, can almost completely prevent the development of ß-amyloid plaques, dystrophic neurites and astrogliosis. This result was reliant on the immunisation of animals prior to the formation of the pathological structures. However, even in older animals where the plaques had already formed, immunisation resulted in a reduction in pathology.

This, therefore, remains perhaps one of the better, more fundamental approaches to the treatment of AD. It remains to be seen, however, whether or not this approach will work in humans, as it may prove difficult to immunise against native APP in man.

1.11.8. Other strategies

There have been innumerable agents postulated to be of benefit in the treatment of AD, with information based on historical anecdotes, animal studies and human trials. Agents have included nootropic drugs; neuropeptides; calcium channel blockers; chelating agents; antioxidants and ancient remedies such as ginkgo biloba (Brodaty and Sachdev, 1997). These drugs, however, will require thorough evaluation in modern clinical trials before they may become widely available for the treatment of AD.

Aims

Currently, the neurodegenerative cascade which leads to dementia in AD cannot be prevented. The only drugs which have been approved for the treatment of AD restore some cognitive abilities for a brief period of time, but do nothing to halt or even slow the progression of the disease. There is, therefore, a need for agents which will attack the underlying cause of the disease, and perhaps prevent its onset. In this respect, the central aim of this thesis has been concerned with establishing alternative routes of therapy for AD than those currently available. The approach is based on the hypothesis that there are very early cytoskeletal changes which occur within the AD brain as a result of plaque deposition, and that targeting these changes may be an effective method of preventing the neuropathology of AD.

Specific aim 1

To determine if ß-amyloid plaques are mediators of structural damage to the cytoarchitecture of the AD brain, and whether similar changes occur following experimentally induced cortical injury.

As outlined in the literature review, the deposition of ß-amyloid plaques in the AD brain is hypothesised to result in structural deformation to surrounding neurites. This theory will be analysed in this thesis by the examination of the dendritic changes that occur both in and around plaques in the early and late stages of AD. Similarly, the dendritic alterations which occur following a defined cortical lesion will be examined to determine whether the neuropathological changes which occur in, and are characteristic of, AD may be the result of a stimulation of the neuronal response to physical injury by the deposition of ß-amyloid plaques within the brain.

Specific aim 2

To assess the efficacy of potential therapeutic agents in preventing the cytoskeletal changes which are characteristic of AD.

Utilising an *in vivo* animal model of the early neuronal pathology of AD, agents which target and stabilise microtubules will be assessed for their efficacy in preventing the cytoskeletal changes which are characteristic of both cortical injury and AD. Similarly, the ability of microtubule-stabilising agents to prevent the evolution of the neuropathological structures which precede neurodegeneration will be assessed. The relative potential of such an approach in the treatment of AD will, therefore, be determined.

Specific aim 3

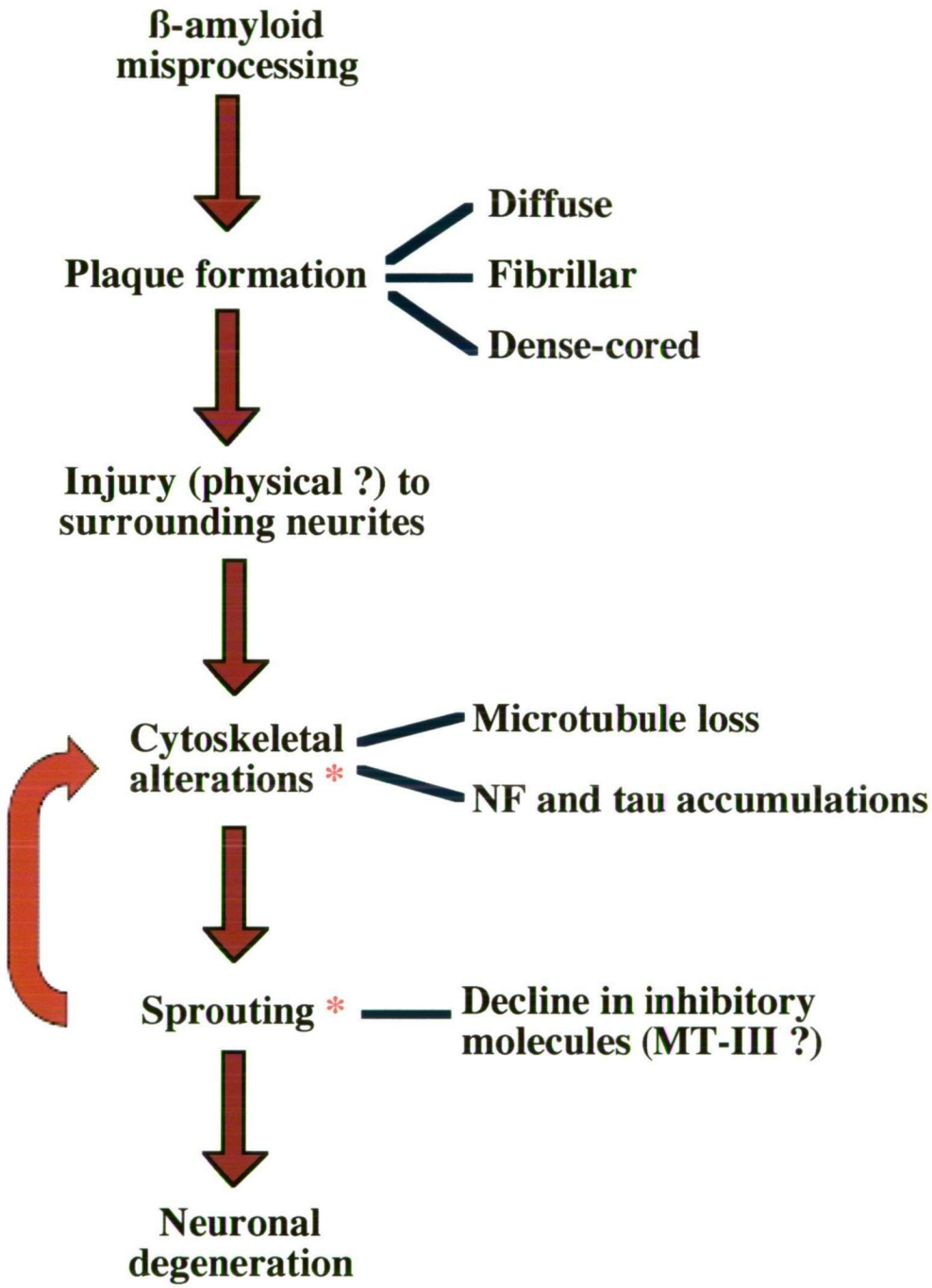
To examine the role of metallothioneins in AD and following cortical injury, and to determine their potential for the treatment of AD

It has previously been postulated that particular metallothionein (MT) isoforms may have a crucial role in the sprouting of damaged neurons in the AD brain. Examination of the expression of MT isoforms in the early and late stages of AD, as well as following cortical injury will be undertaken to establish whether this family of proteins may have a role in AD. Similarly, the *in vivo* animal model will be utilised to assess the relative effect of the exogenous administration of specific MT proteins on the neuronal response to physical injury. The potential use of these agents in the treatment of AD will, therefore, be determined.

In summary, then, this study will seek to further define the pathogenic mechanisms which may underlie neurodegeneration in AD, and to subsequently assess the effectiveness of targeting specific processes in the prevention of the evolution of the

neuropathology which is characteristic of AD. This thesis will be presented in two parts, the first three experimental chapters are concerned with specific aims one and two, whilst the remaining three experimental chapters deal with specific aim three. The main targets for therapeutic intervention in this thesis are summarised in Figure 1.2.

Figure 1.2. A simplified mechanism outlining the cascade of changes leading to neurodegeneration in AD. The specific aspects targeted in this thesis are shown (*).


ß-amyloid misprocessing
Plaque formation
Diffuse
Fibrillar
Dense-cored
Injury (physical ?) to surrounding neurites
Cytoskeletal alterations *
Microtubule loss
NF and tau accumulations
Sprouting *
Decline in inhibitory molecules (MT-III ?)
Neuronal degeneration

Materials and methods

2.0 Human material - Introduction and case definition

Investigations utilising human brain material were carried out in this thesis. Material was obtained from normal aged individuals, people diagnosed as suffering from Alzheimer's disease (AD) and also from people considered to be in a preclinical stage of AD. The source of this tissue and the fixation details are summarised in Table 2.1 (non-AD), 2.2 (preclinical AD) and 2.3 (AD). AD cases were diagnosed according to CERAD (Consortium to Establish a Register for Alzheimer's Disease) criteria (Mirra *et al.*, 1991), whilst preclinical cases were defined as cases in which there was the presence of ß-amyloid plaques throughout the neocortex, but no extensive neuronal pathology as indicated by thioflavine S staining or immunolabelling with PHF-tau or ubiquitin antibodies (Vickers *et al.*, 1996). These cases also showed no overt signs of dementia. They were, therefore, not diagnosed as AD according to CERAD criteria. Non-AD cases had no known neuropathological condition.

Brain tissue was cryopreserved and blocks from the superior frontal gyrus removed and sectioned at 40-50 μm on a freezing microtome (Leica CM1325). Tissue sections were then placed into storage medium until use (appendix, 11.1) Variations in fixation protocols and post mortem intervals to fixation did not result in different immunohistochemical profiles between cases.

Table 2.1. Summary of non-Alzheimer's disease cases utilised throughout this thesis.

(PMI = post mortem interval)

Code	Age (yr)	Sex	PMI (hrs)	Cause of death	Fixation details	Source
N-14	72	F	7	Cardiac failure	1	NH&MRC Brain Bank, Adelaide
15-98	83	F	8.5	Bowel disease	2	University of Sydney, Sydney
15-28	47	M	24	Heart disease	2	University of Sydney, Sydney
15-49	51	M	24	Pulmonary Embolus	2	University of Sydney, Sydney
15-69	58	M	28	Heart disease	2	University of Sydney, Sydney
15-87	65	M	15	Heart failure	2	University of Sydney, Sydney

1. Perfusion-fixed with 2% picric acid/ 4% paraformaldehyde
2. Blocks of cerebral cortex, immersion-fixed in 4% paraformaldehyde

Table 2.2. Summary of preclinical Alzheimer's disease cases used throughout this thesis.

(PMI = post mortem interval)

Code	Age (y)	Sex	PMI (hrs)	Cause of death	Fixation details	Source
PM-6060	70	F	4.4	Cardiac failure	2	University of Tasmania, Hobart
15-90	61	M	19	Heart disease	2	University of Sydney, Sydney
15-17	62	M	24	Cardiac failure	2	University of Sydney, Sydney
15-35	71	M	32.5	Cardiac arrest	2	University of Sydney, Sydney
N-17	78	F	18	Cardiac failure	1	NH&MRC Brain Bank, Adelaide
96-30	90	M	2.16	Resp. failure	2	Sun Health Research Institute, USA
97-09	81	F	3	Cardiac arrest	2	Sun Health Research Institute, USA
97-51	84	M	3	Cardiac arrest	2	Sun Health Research Institute, USA
98-37	78	M	2.25	Pneumonia	2	Sun Health Research Institute, USA
99-22	91	M	3	Cardiac failure	2	Sun Health Research Institute, USA

3. Perfusion-fixed with 2% picric acid/ 4% paraformaldehyde

4. Blocks of cerebral cortex, immersion-fixed in 4% paraformaldehyde

Table 2.3. Summary of Alzheimer's disease cases utilised throughout this thesis.

(PMI = post mortem interval)

Code	Age (y)	Sex	PMI (hrs)	Cause of death	Fixation details	Source
AD-1	65	M	3	AD	1	NH&MRC Brain Bank, Adelaide
AD-2	73	M	6.5	AD	1	NH&MRC Brain Bank, Adelaide
91-16	72	F	4	AD	2	Sun Health Research Institute, USA
91-11	66	M	2.8	AD	2	Sun Health Research Institute, USA
91-10	84	F	3	AD	2	Sun Health Research Institute, USA
99-07	92	F	2.25	Pneumonia	2	Sun Health Research Institute, USA
99-10	74	F	2	Pneumonia	2	Sun Health Research Institute, USA
99-13	74	M	2.75	Resp. failure	2	Sun Health Research Institute, USA
99-15	83	M	2.83	AD	2	Sun Health Research Institute, USA

1. Perfusion-fixed with 2% picric acid/ 4% paraformaldehyde
2. Blocks of cerebral cortex, immersion-fixed in 4% paraformaldehyde

2.1 Animal model - Introduction

AD has been experimentally replicated in a number of different models. Our laboratory has demonstrated that the *in vivo* focal cortical injury model utilised throughout this thesis accurately replicates the early neuropathological change associated with plaque formation in preclinical AD cases (King *et al.*, 1997). This model has the advantage of producing a defined lesion without confounding factors, and it also allows for the direct introduction of minute quantities of any agent into the damaged cortex, a method which has been exploited in this thesis.

All procedures were approved by the Ethics Committee (Animal Experimentation) of the University of Tasmania and are consistent with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.1.1 Technique

Adult Hooded Wistar rats (250-300g) were anaesthetised with sodium pentobarbitol ('Nembutal', 60 mg/Kg, intraperitoneal), the scalp was shaved and the head immobilised in a stereotaxic frame (Stoelting). The dorsal surface of the skull was wiped with alcohol and a midline incision made to expose the skull. Cortical landmarks were identified and, using the coordinates of Paxinos and Watson (1986), the Par1 region of the right somatosensory cortex was located. A hole was then created in the skull using a Dremel Multipro drill with an attached bit. The dura mater was removed with fine forceps and a 25 gauge blunt needle, with attached 2μl syringe (Hamilton), slowly inserted into the cortex to a depth of 1.5 mm. The needle, which penetrated all layers of the grey matter, but not the underlying white matter, was left in place for a total of ten minutes before being slowly removed. In animals

which received intracortical drug injections, the syringe was preloaded with 1μL of the given agent and then, once inserted into the cortex, delivered at a rate of 0.2μL/minute. The needle then remained in the cortex for the remainder of the ten minutes. Gelfoam (Upjohn) was utilised to fill the burr hole in the skull, and antibiotic powder (Cicatrin, Wellcome) sprinkled over the surface. The skin was closed with autoclips (9mm, Becton Dickinson) and an intramuscular injection of gentamicin (0.02 ml, David Bull Laboratories) given. A mild analgesic was supplied in the drinking water (Soluble 'Aspro', Roche). Animals were kept warm during the entire procedure and in the post-operative period. Once fully recovered, animals were closely monitored to ensure there were no behavioural abnormalities. In all experiments involving the injection of drugs into the cortex, appropriate controls of the vehicle solution were also performed.

At varying intervals post-surgery, animals were reanaesthetised with sodium pentobarbitol (60 mg/Kg, intraperitoneal) and perfusion fixed as detailed in the following section. Once animals were anaesthetised, they were immobilised on a perfusion table which allowed for the collection of waste material. The external surface of the skin was wiped with 70% alcohol and a midline incision made from the clavicle to the sternum. The skin was then laid back and the zyphoid process exposed. This structure was then clamped and incisions made down each side of the chest cavity. The diaphragm and rib cage were then cut through to open the thoracic cavity and allow access to the heart. The pericardial sac was removed and a 24 gauge cannula inserted through the left ventricle and into the aorta. The cannula was clamped in place with a haemostat and the right atrium pierced to allow exsanguination. Phosphate-buffered saline (PBS) (0.01M, appendix, 11.0) was then flushed through for a total of two minutes (or until the waste material had sufficiently cleared of red

blood cells) to remove blood from the circulation and allow better fixation and to enhance later immunohistochemical investigations. The perfusate was then changed to a 4% paraformaldehyde fixative solution (appendix, 11.2), and this was circulated for seven minutes. At the completion of perfusion, the skull cavity was opened with bone clippers and the brain carefully removed and placed in paraformaldehyde fixative for a further six hours of post-fixing.

The brain was blocked down to an area surrounding the injury site and glued to the stage of a 752M Vibroslice (Campden Instruments). The brain remained immersed in PBS (0.01M), and coronal sections were cut at 50μM. Sections were serially collected and were stored in PBS-azide until use (appendix, 11.2).

2.2 Immunohistochemistry

Analyses throughout this thesis have principally been conducted utilising the visualisation of specific antibody reactivity with fluorescence immunohistochemical techniques. Both single and double labelling methods have been utilised (an extensive outline of these techniques can be found in Vickers, 1999).

Briefly, material was initially washed three times in 0.01M PBS before incubation in primary antibodies. For investigations utilising human material, sections were initially pretreated with 90% formic acid for twenty minutes as well as 'quenched', which involved treating the material in 0.25% $KMnO_4$ for 20 minutes, washing in PBS, then placing in 1% $K_2S_2O_5$ and 1% oxalic acid until they turned white. Human material was then similarly

incubated in primary antibody, initially for several hours on an orbital shaker at room temperature, followed by overnight incubation at 4°C. Antibodies were diluted to the appropriate concentration in diluent (appendix, 11.0) to aid in their penetration into tissue. A list of the primary antibodies utilised, and their working concentration is shown in table 2.4 (monoclonal antibodies) and 2.5 (polyclonal antibodies). Primary antibodies will bind to a specific antigenic site, and this can be subsequently visualised by the use of secondary antibodies. Because the primary antibodies can be raised in different hosts, it is possible to combine multiple primary antibodies so that different antigenic sites can be targeted in the one tissue section. In this thesis, both single (one primary antibody, raised in either a mouse or rabbit) and double (two primary antibodies, one raised in mouse and one in rabbit) labelling techniques were utilised. Thioflavine-S staining was also used in conjunction with single labelling fluorescence investigations. Briefly, this involved incubating the human material in thioflavine-S (Polysciences) (0.0125% in a solution of 40% ethanol and 60% 0.01M PBS) for three minutes, followed by differentiation (in a solution of 50% ethanol and 50% 0.01M PBS) and washes in 0.01M PBS, prior to the overnight incubation in primary antibody.

Material was then given three ten minute washes in 0.01 M PBS before incubation in a secondary antibody. Secondary antibodies will attach to immunoglobulins of the primary antibody host species (either mouse or rabbit). They are utilised because they are conjugated to either a fluorophore or to biotin. In the former case, the fluorophore will emit fluorescent light when exposed to a specific wavelength of light, thereby allowing visualisation of antibody immunoreactivity. In the latter case, an avidin-conjugated

Table 2.4. Summary of mouse monoclonal primary antibodies utilised throughout this thesis.

Code	Reactivity	Dilution	Source
SMI32	dephosphorylated NF-M & NF-H	1:2000	Sternberger Monoclonals Inc.
SMI312	phosphorylated NF-M & NF-H*	1:5000	Sternberger Monoclonals Inc.
GAP43	Growth-associated protein-43	1:1000	Boehringer Mannheim
MAP2	Microtubule-associated protein 2	1:1000	Boehringer Mannheim
MT-I/II	Metallothionein isoforms I and II	1:500	DAKO

* A cocktail of monoclonal antibodies

Table 2.5. Summary of rabbit polyclonal primary antibodies utilised throughout this thesis.

Code	Reactivity	Dilution	Source
GFAP	Astrocytes	1:2000	DAKO
S-100 α+ß	Reactive protoplasmic and fibrous astrocytes	1:2000	DAKO
Tau	Tau	1:2000	DAKO
ß-amyloid	ß-amyloid 1-42(43)	1:500	Zymed
ß-amyloid (pan)	all ß-amyloid peptides	1:500	QCB
Ferritin	Reactive microglia	1:10000	DAKO

fluorophore can be added which will attach to the biotinylated secondary antibody. This, again, will emit fluorescent light when exposed to a specific wavelength of light. Double labelling studies exploit the technique of using two primary antibodies, raised in different hosts, and two secondary antibodies, conjugated to different complexes, which subsequently allows the simultaneous visualisation of two antibody species in the one tissue section by use of a fluorescent microscope with the capacity to excite different wavelengths of light. Other antibody detection systems also allow for the use of two fluorophore-conjugated secondary antibodies, eliminating the need to utilise a biotinylated secondary step in double labelling experiments.

Once the tissue has been thoroughly washed, either one or two secondary antibodies are added, and a list of those utilised is shown in Table 2.6. When injured animal tissue sections were utilised, the secondary antibodies used were 'rat adsorbed' to minimise binding to endogenous immunoglobulins. The sections were again incubated on the orbital shaker for two hours. To preserve the intensity of labelling, once a fluorescent label had been added to material, it was kept in the dark as much as possible. Sections were then washed three times in 0.01M PBS, and material which was single labelled using a fluorescein-conjugated secondary antibody was then mounted on glass slides with the aqueous mounting medium, Permafluor (Immunotech).

Sections, in which a biotinylated secondary was utilised, were then incubated in the avidin-conjugated fluorophore, streptavidin Texas Red, for two hours. Sections were then washed three times in 0.01M PBS prior to mounting on glass slides with Permafluor. In all

Table 2.6. Summary of secondary antibodies utilised throughout this thesis.

Lot	Reactivity	Raised in	Conjugate	Dilution	Source
F1206	Mouse IgG (H+L)	Horse	FITC	1:200	Vector
J0814	Mouse IgG (H+L)	Horse (rat adsorbed)	FITC	1:200	Vector
J0311	Rabbit IgG (H+L)	Goat	Biotin	1:200	Vector*

FITC - fluorescein isothiocyanate

* visualised with streptavidin Texas Red avidin D (Amersham 1:200, Lot J0310)

experiments, control sections were processed concurrently, but without the addition of primary antibody.

2.3 Cell Culture- Introduction

The development of a cell culture model of cortical injury/ AD was undertaken to provide an alternative to the *in vivo* cortical injury paradigm. A cell culture system would allow for the quicker screening of a much larger number of potential therapeutic agents, and, therefore, result in a minimisation of animal experimentation.

All procedures were approved by the Ethics Committee (Animal Experimentation) of the University of Tasmania and are consistent with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.3.1 Technique

Primary cortical cell cultures were established according to standard procedures (Banker & Goslin, 1991). Timed pregnant (gestational day 18) Hooded Wistar rats were anaesthetised by CO_2 asphyxiation. The abdomen was wiped with 70% alcohol and the skin then cut open and laid back. The abdomen wall was then cut through and the two horns of the uterus removed and placed in a sterile petri dish. This dish was briefly placed on ice to cold anaesthetise the foetuses (pups). The petri dish was then transferred to a laminar flow hood, where the remaining steps of the procedure were performed.

Foetuses were individually removed from the uterus and decapitated. The skull was then carefully unroofed using sterile forceps, and the meninges removed to expose the brain.

The cerebral cortex was then removed from both hemispheres of the brain. The cortical tissue was immediately placed into a sterile tube containing 4.5 ml 10 mM HEPES buffered saline (HBS) (appendix, 11.3). Once the required tissue was removed, 0.5 ml of 2.5% trypsin (from beef pancreas, BDH Chemicals) in PBS (0.01M) was added to enzymatically dissociate the tissue. This was incubated at 37°C for 20 minutes. The tissue was then washed, by removal of the HBS and the addition of a further 5 ml of HBS, followed by vigorous shaking. The solution was allowed to stand for five minutes, and then the procedure was repeated a further two times. The tissue was further dissociated by triturating through a series of decreasing calibre pipettes. The number of viable cells was then assessed utilising the trypan blue exclusion technique.

The required density of cells (4.5×10^5) was then plated into an 'initial' plating media (appendix, 11.3) on previously prepared glass coverslips. Prior to use, coverslips (Fisher Scientific) had been 'pitted' by acid treatment (two hours in 69% nitric acid) followed by extensive washes in MilliQ® water , heat sterilised (160°C overnight) and incubated overnight in poly-L-lysine (L-2, 6-Diaminohexanoic acid) (Sigma) (1 mg/ml in borate buffer (appendix, 11.3)). The poly-L-lysine was then removed and replaced with 1 mL of the 'initial' plating media (appendix, 11.3) in preparation for seeding with cells.

Once plated, cells were placed in an incubator (Flow Laboratories CO_2 incubator 220) at 37°C in a 5% CO_2 humidified atmosphere. Following one day in 'initial' plating media (1 day *in vitro*, DIV), the media was completely removed and replaced by a 'subsequent' plating media (appendix, 11.3). One third of the media on the cells was then exchanged for fresh 'subsequent' plating media every three days. The primary component of all media

used was Neurobasal™ (Gibco BRL, Life Technologies), which is selective for neuronal cells (Brewer *et al.*, 1993; Brewer, 1995, 1997).

2.3.2 Axonal transection

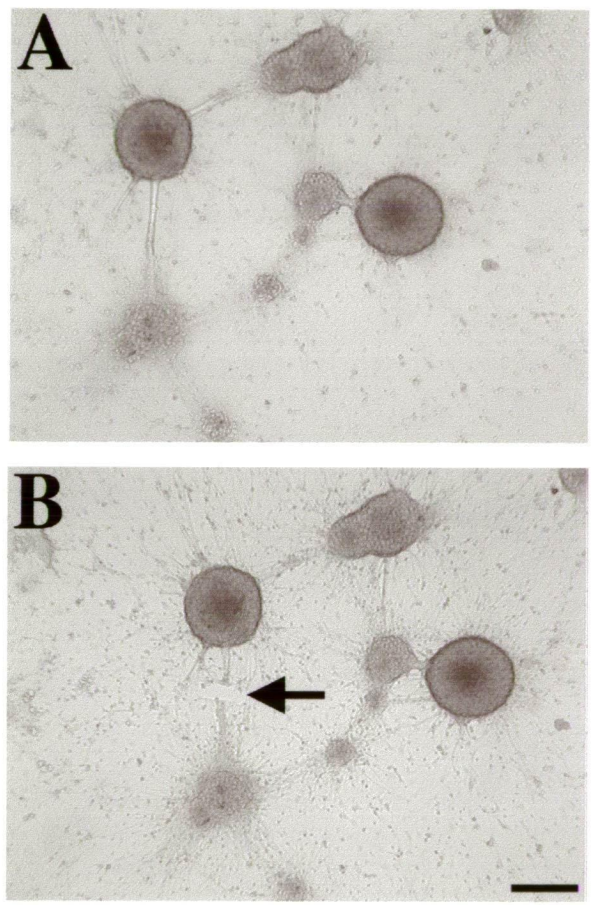
One day prior to the transection of cells (DIV 20), the coverslips were removed from the twelve wells trays and placed in individual petri dishes (35mm, Iwaki) with 1 ml of new 'subsequent' media and 1 ml of old media. The larger petri dishes allowed easier access to the coverslip for the transection of neurite bundles. The cells were reacclimatised for 24 hours prior to transection. Individual petri dishes were removed from the incubator and appropriate neurite bundles selected for transection under the inverted microscope (Leitz Fluovert). Utilising a size 15 scalpel blade attached to a scalpel handle, a number of cuts were made on each coverslip (example, Figure 2.1), with a montage of digital images captured both before and after each cut so as to allow easy relocation of the cut-site for immunohistochemical analysis. Analyses were performed at a variety of time points post-injury.

2.3.3 Fixation and labelling of coverslips

Prior to immunohistochemical analysis, cells were paraformaldehyde fixed on the coverslip. All the media was removed from the coverslip, and replaced by 37°C phosphate buffered paraformaldehyde/sucrose fixative (appendix, 11.3). This was then incubated for 30 minutes, removed and the coverslip washed with 0.01M PBS in preparation for labelling. The protocol for visualisation of antibody immunoreactivity in cell monolayers differed slightly from the standard method, as outlined below.

Figure 2.1. Unlabelled neuronal cultures at 21 days *in vitro*. (A) is before and (B) after localised physical injury. The arrow indicates the point of transection.

Scale Bar = 150 μm.


A
B

Following three 10 minute washes with 0.01M PBS, the coverslips were immersed in diluent for five minutes to permeabilize the cells. This was then removed and replaced with a blocking solution of 5% normal goat or horse sera (Vector) in PBS (0.01 M) and incubated on an orbital shaker for one hour. Coverslips were then incubated overnight in primary antibodies (diluted in 1% normal goat or horse sera) at 4°C. The following day the sera was removed and coverslips washed three times with PBS (0.01M) and then incubated in the appropriate rat adsorbed secondary antibody diluted in PBS (0.01M). Following further PBS washes, coverslips were left upside down to dry and then mounted on glass slides with Permafluor mounting medium.

2.4 Fluorescence microscopy and image analysis

A Leitz Dialux 22EB epifluorescence microscope was used to visualise fluorescence labelled material. This microscope is equipped with filters to allow the specific visualisation of different excitation wavelengths of light, specifically, those that allow the visualisation of FITC (fluorescein isothiocyanate) and Texas Red. With human material, it was also possible to utilise UV light to observe material that had been stained with thioflavine-S. This microscope was attached to a digital CCD camera (Ikegami ICD-4CE, Tsushinki Co) which in turn was attached to a Power Macintosh 7600 computer (with internal video capture card) running the NIH Image Analysis program (version 1.61). This allowed for the digital capturing of images from the microscope and subsequent analysis and preparation in Adobe Photoshop (version 5.5). Figures were printed with an Epson Stylus 800 Colour printer on photo quality paper (Epson).

Specific methods of quantitation are outlined in each chapter.

2.5 Confocal Microscopy

Confocal microscopy was performed using an Optiscan F900e krypton/argon scanning laser system attached to an Olympus microscope (BX60) and a Hewlett Packard Pentium II computer.

2.6 Protein Investigations- SDS-PAGE sample preparation

Samples were prepared by adding 16.5µl of sample to 7.5µl of 4X NuPage LDS sample buffer. The sample was then vortexed, and stored at 4°C until use. Just prior to use, 6µl of NuPage 10X Reducing Agent was added, and the sample heated for 10 minutes at 70°C, followed by thorough mixing.

2.6.1 SDS-PAGE running conditions

A Novex ready made 10% NuPage Bis-Tris gel was used in an electrophoretic apparatus (Mini-Protean II Cell). The gel was run at a constant 200V for 35 minutes, with 1XMES running buffer (Novex). Reducing conditions were required, so the upper running chamber contained 500µl of NuPage Running Buffer Antioxidant.

2.6.2 Western blotting

The Western Blot gel membrane sandwich was made up as per the instructions contained in the Novex Western Blot Unit user's manual. The gel was run at 20 V (approx 70 mA) overnight at 4°C. The following day, the gel was run at 40 V for an hour to ensure complete transfer. The Blot module was then carefully disassembled and the membrane gently removed and thoroughly washed. This involved firstly rinsing twice in PBS-T

(appendix, 11.4) buffer, followed by a wash in PBS-T for 5 minutes. The membrane was then placed in a heat-sealed, plastic bag with 10mls of 5% blocking reagent (appendix, 11.4) for 1 hour at room temperature with shaking. This was followed by another thorough washing process, involving four rinses in PBS-T at room temperature with shaking. The primary antibody, in 10 mls PBS-T, was then added to the membrane in a heat-sealed bag. This was then incubated overnight at 4°C. The following day, the membrane was again thoroughly washed, involving four rinses in PBS-T at room temperature with shaking. The secondary anti-body, anti-mouse Ig horseradish peroxidase, was diluted at a concentration of 1:1000 in 2.5% blocking solution (appendix, 11.4) and 10mls added to the membrane in a heat sealed bag, and incubated with shaking at room temperature for 1 hour. This was followed by washing of the membrane, involving four rinses in PBS-T at room temperature with shaking. Excess PBS-T was drained off the membrane and the detection solution prepared by mixing equal volumes of detection solution 1 and detection solution 2 (Lum-light Western Blotting Substrate kit, Roche Diagnostics). The membrane was then incubated, protein side up, in the detection solution for 1 minute with gentle agitation at room temperature. Excess solution was drained from the membrane and it was then wrapped in Glad Wrap, protein side up, to form an envelope with no air pockets. The membrane was then placed protein side up on an x-ray cassette and exposed to Hyperfilm ECL (Amersham Pharmacia Biotech), at varying exposure times.

Morphologically distinct plaque-types differentially affect dendritic structure and organisation in the early and late stages of Alzheimer's disease

3.0 Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease and is the commonest cause of dementia in people of all ages (Wilcock, 1996), affecting approximately 11% of the population greater than 65 years of age and up to 50% of people aged 85 years and older (Hof and Morrison, 1994). While the precise aetiology of the disease remains unknown, it is believed that the deposition of small (7-10 nm) extracellular filaments, comprised of a ~4kDa insoluble form of the ß-amyloid protein, within the brain to form plaques is a central event in the pathogenesis of AD, and one which occurs in all stages of the disease process (Glenner and Wong, 1984; Masters *et al.*, 1985). The precise mechanism by which ß-amyloid interacts with, and causes damage to, the brain also remains to be clearly defined. The formation of plaques in the neocortex, however, is known to be associated with a number of neuropathological changes which may be involved in the pathogenesis of AD, such as the development of dystrophic neurites (Benes *et al.*, 1991; Terry *et al.*, 1994; Dickson, 1997; Dickson *et al.*, 1999).

The quantitative examination of the influence of plaques on normal axon morphology has demonstrated that axons which pass through plaques are often swollen and have a disrupted organisation (Probst *et al.*, 1983; Benes *et al.*, 1991), with fewer axons found in association with the plaque relative to areas at a distance from the plaque (Benes *et al.*, 1991). Whilst it has been proposed that plaques disrupt the normal axonal cytoskeleton (Onorato *et al.*,

1989; Benes *et al.*, 1991; Vickers *et al.*, 1996; Dickson *et al.*, 1999), there has been a paucity of studies examining the effect of plaques on dendritic morphology. It has, however, been reported that dendrites may become affected in AD with the formation of neuropil threads early in the disease process. These are short fibers that contain paired helical filaments (PHF), and which predominantly arise from degenerating dendrites (Gray *et al.*, 1987; Hof and Morrison, 1994; Terry *et al.* 1994). The formation of neuropil threads involves a series of dendritic changes which not only include the accumulation of PHF-tau (Masliah *et al.*, 1992), but also dramatic structural alterations, such as swelling, localised 'transection' and eventual degeneration (McKee *et al.*, 1989; Braak *et al.*, 1994; Braak and Braak, 1997). Apical dendrites have also been shown to possess a significantly decreased number of spines in diseases such as Parkinson's disease, Creutzfeldt-Jacob disease and AD (Catala *et al.*, 1988).

Whilst it has been demonstrated that dendrites often have an altered, and abnormal, morphology within plaques (Probst *et al.*, 1983; Knowles *et al.*, 1998, 1999), the effect of various plaque types on dendrites has not been extensively investigated. To further define the effect of plaque formation on dendrites within the brain, then, we have examined microtubule-associated-protein 2 (MAP2), a marker for microtubules which is specifically localised to dendrites (Hirokawa *et al.*, 1996), within a number of morphologically distinct plaque-types in both the early and late stages of AD.

3.1 Materials and methods

Tissue source and processing

Forty μM sections of the superior frontal gyrus of each of 5 AD cases (case codes; mean age ± standard error: 99-07, 99-10, 99-13, 99-15, AD-2; 79.2 ± 3.7 years), 5 preclinical-AD cases (96-30; 97-09; 97-51; 98-37; 99-22; 84.8 ± 2.5 years) and 4 cases without any AD pathological changes (15-87; 15-69; 15-49; 15-28; 55.3 ± 3.9 years) were examined. Full case details are outlined in chapter 2. Prior to use, human material was pretreated with formic acid and autofluorescence quenched (chapter 2).

Standard immunohistochemical techniques for double labelling were utilised for visualisation of antibody immunoreactivity (chapter 2). In all fluorescence-double-labelling investigations, a monoclonal antibody which recognises MAP2 was utilised in combination with a rabbit polyclonal antibody against ß-amyloid (Zymed). In all immunofluorescence experiments, antibodies were visualised with a horse anti-mouse IgG conjugated to fluorescein isothiocyanate and a goat anti-rabbit IgG conjugated to biotin followed by avidin Texas Red.

It has been demonstrated that MAPs are not stable during post-mortem intervals in the rat (Irving *et al.*, 1997), however, there were no differences observed in the immunolabelling profile for either MAP2 or ß-amyloid across varying post-mortem to fixation intervals (PMI) or different fixation protocols. There was also no significant difference in PMI between preclinical AD cases (mean PMI ± standard error, 2.68 ± 0.19 hrs) and AD cases (3.27 ± 0.82 hrs)

Microscopy

For quantitative purposes, a Leitz Dialux 22 EB fluorescence microscope attached to a digital CCD camera (Ikegami ICD-4CE) and Macintosh computer (with internal video capture card), was utilised. The image analysis program, NIH Image (version 1.61), was utilised to capture individual fields of view. Based on previous investigations in this laboratory, three plaque types (diffuse, fibrillar and dense-core) were identified in each case examined. Ten plaques of each type, randomly chosen from throughout all layers of the grey matter, were then individually captured along with the corresponding field of view showing the labelling for MAP2 in that section. Adobe Photoshop (version 5.5) was then utilised to create a multi-layer, double-labelled, image of each plaque. The plaque area could then be accurately circumscribed and the area in the MAP2 field taken up by the plaque could be digitally isolated. Four further images (the same size as the plaque, and also in the MAP2 field) were taken from each side of the plaque. Utilising NIH Image software (version 1.61), the total area of MAP2 labelling both within, and surrounding, the plaque area was determined. This procedure was repeated for each individual plaque (ten of each of the different plaque types, thirty plaques per case), across all cases (five AD and five preclinical cases). Statistical analyses of all results (unpaired t-test, ANOVA) were carried out using StatView 4.5 for Macintosh.

Laser confocal scanning microscopy, using an Optiscan F900e krypton/argon system attached to an Olympus BX50 epifluorescence microscope, was also utilised to qualitatively assess microtubular changes associated with both preclinical and clinical AD.

3.2 Results

MAP2 and ß-amyloid immunohistochemistry

In all cases, MAP2 labelling was localised to cell bodies and dendrites, including long bundles of apical dendrites throughout the cortex, which extended from deeper layers to the external surface of the brain (Figure 3.1). Labelling remained consistent within case groups, however, qualitatively, there appeared to be a greater density of MAP2 labelled dendrites in AD cases, as compared to preclinical cases. Quantitatively, this is also suggested by the higher proportion of MAP2 labelling found in the neuropil surrounding all plaque-types in AD cases, relative to preclinical cases (Table 3.1).

Labelling for ß-amyloid identified three plaque-types in the preclinical and clinical AD cases, including dense-core (~30-60 μm in diameter), diffuse (~10-180μm in diameter) and fibrillar (~20-70 μm in diameter) types (Figure 3.2). Dense-core plaques had a central mass of ß-amyloid surrounded by more loosely aggregated fibrils, whereas fibrillar plaques appeared similar, but without the central core. Diffuse plaques had no central core or distinct edges, and were comprised of more punctate ß-amyloid labelling. The various plaque types were homogeneously distributed throughout all cortical layers, and showed no specific localisation to any particular layer of the grey matter. Similarly, all plaque-types were present in both preclinical and clinical AD cases.

Quantitation of immunohistochemistry

Non-AD cases

Non-AD cases did not demonstrate any ß-amyloid immunoreactivity, whilst labelling for MAP2 was both extensive and uniform.

Figure 3.1. Microtubule-associate protein-2 labelling in the grey matter of a brain with no neuropathological changes. Immunoreactivity is confined to long bundles of apical dendrites, which typically appear in bundles extending towards the pial surface (arrow), and in cell bodies (arrowhead).

Scale bar = 50μm.

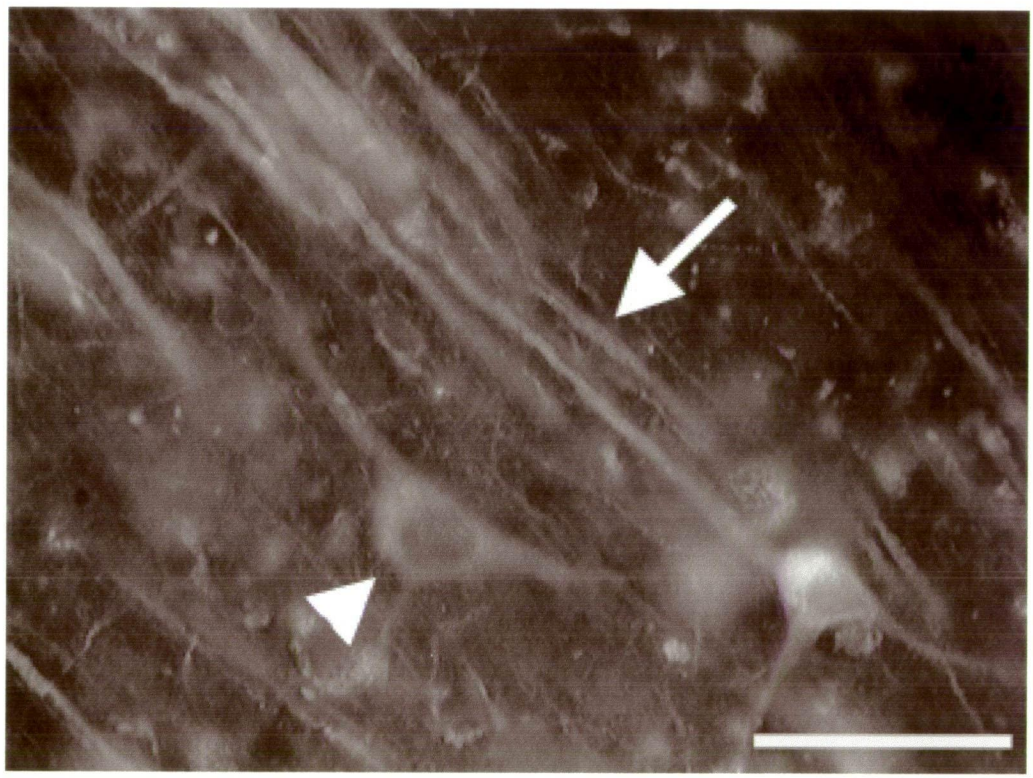


Table 3.1. Summary of quantitation of microtubule-associated protein-2 (MAP2) labelling in human material. The case types are as defined in chapter 2. The three different plaque types are as defined in the text (shown graphically in Figure 3.2), and 'Neuropil' refers to the area surrounding the plaque, whilst 'Plaque' refers to within the area occupied by the plaque. Values given are the average relative percentages, plus or minus the standard error, of the area labelled for MAP2 in each case (n=5). Statistically significant differences ($p<0.05$) between the relative amount of MAP2 labelling between the inside and the outside of the plaque are shown in all cases, except for fibrillar plaques in Preclinical Alzheimer's disease (AD) cases.

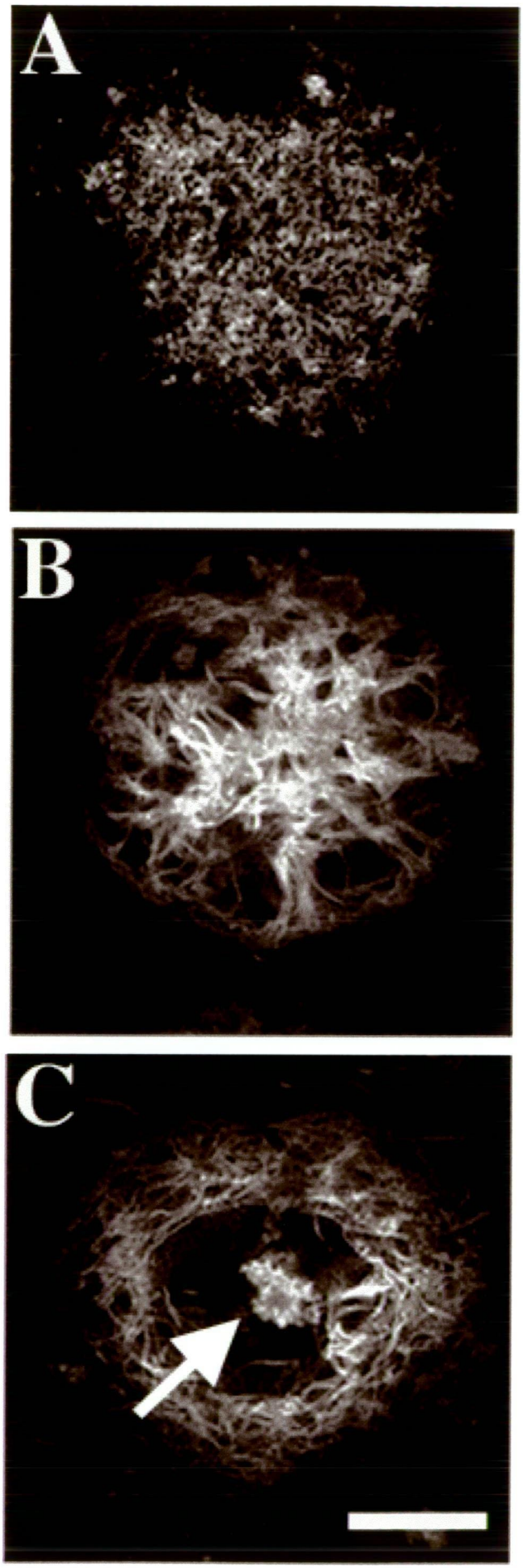
	Dense core		Diffuse		Fibrillar	
	Neuropil	Plaque	Neuropil	Plaque	Neuropil	Plaque
Case Type #						
Preclinical AD	17.2 ± 1.9*	12.4 ± 1.4*	16.8 ± 1.1*	21.2 ± 0.9*	18.8 ± 1.9	18.6 ± 1.6
AD	26.2 ± 1.9*	16 ± 1.5*	21.4 ± 2.1*	28 ± 2.1*	24.6 ± 2.5*	17.4 ± 0.9*

* Statistically significant difference ($p < 0.05$) between the 'Neuropil' and 'Plaque' value within each case type.

Quantitation of non-AD cases demonstrated that 35 ± 2.8 % of the neuropil was labelled for MAP2.

Figure 3.2. Examples of the three different categories into which plaques were grouped based on morphological findings. Human material was stained with thioflavine-S and visualised on a confocal laser scanning fluorescent microscope. (A) shows diffuse plaques, (B) fibrillar plaques and (C) dense-cored plaques (arrow shows a dense core of ß-amyloid surrounded by more loosely aggregated ß-amyloid).

Scale bar = 20μm.


A
B
C

Preclinical AD cases

Dendritic structure and organisation was not significantly affected by the presence of diffuse plaques in preclinical cases. Dendrites passed through regions occupied by diffuse plaques with little to no change in morphology (Figure 3.3). Similarly, deflection of dendrites around the perimeter of the plaque was rare, as was the termination of dendrites which entered the region occupied by the plaque. Quantitation demonstrated that there was a statistically significant ($p<0.05$) increase in the relative proportion of MAP2 labelling found within the area of the plaque, as compared to the surrounding neuropil (Table 3.1).

In contrast, there was a statistically significant ($p<0.05$) decrease in the relative proportion of MAP2 labelling found within the area occupied by dense-core plaques, as compared to the surrounding area (Table 3.1). Dendrites had a significantly altered morphology in the vicinity of dense-cored plaques, and often terminated at the periphery of the plaque or deflected around its outer margins (Figure 3.4). Dendrites occasionally passed through the outer rim of dense-core plaques (Figure 3.4).

Fibrillar plaques showed no significant change in the relative proportion of MAP2 labelling found within the plaque as compared to the surrounding neuropil (Table 3.1). Dendritic morphology was not consistently affected. Dendrites often passed through the plaque area unaffected (Figure 3.5), but were occasionally decreased in calibre as they traversed the plaque. Dendrites also terminated within plaques or were deflected around the plaque perimeter (Figure 3.5).

Figure 3.3. An example of the effect of diffuse plaques on dendritic structure. Human material was labelled with ß-amyloid (red) and microtubule-associated protein-2 (MAP2) (green), and visualised with a fluorescent microscope. MAP2-labelled dendrites are shown traversing the plaque (arrow) without morphological alteration.

Scale bar = 50 μm.

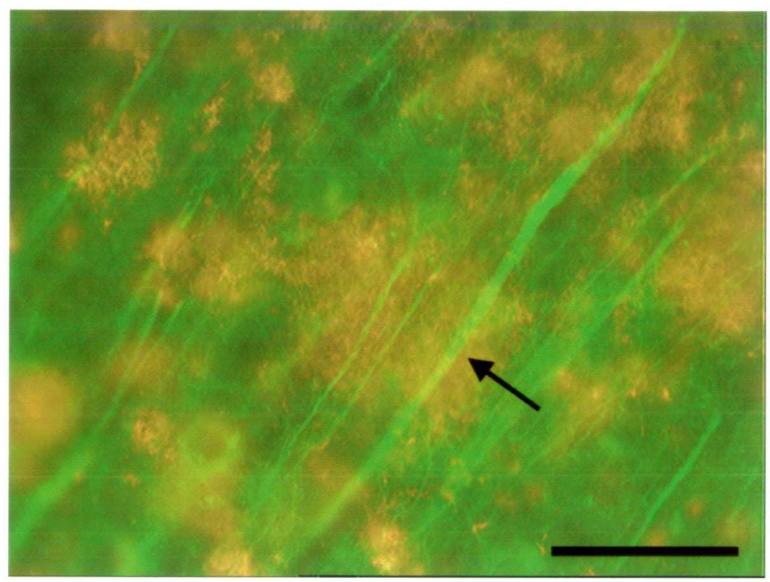


Figure 3.4. An example of the effect of dense-cored plaques on dendritic structure. Material from a preclinical Alzheimer's disease (AD) case is shown, which has been double-labelled with ß-amyloid (A) and microtubule-associated protein-2 (MAP2) (B). (A) and (B), which were visualised on a fluorescent microscope, demonstrate that there is very little MAP2 immunoreactivity within the area occupied by the plaque (which was morphologically identified as a 'dense-cored') as compared to the surrounding neuropil. Dendrites are shown both terminating within the plaque (arrowhead) and deflecting around its outer margins (arrow). (C) and (D), derived from confocal laser scanning microscopy, show material from a preclinical AD case which has been double-labelled with ß-amyloid (red) and MAP2 (green). They similarly show dendrites terminating within the dense-cored plaque (arrowhead) and deflecting around its outer margins (arrow).

Scale bar (A), (B) = 50 μm; (C) = 40μm; (D) =20μm.

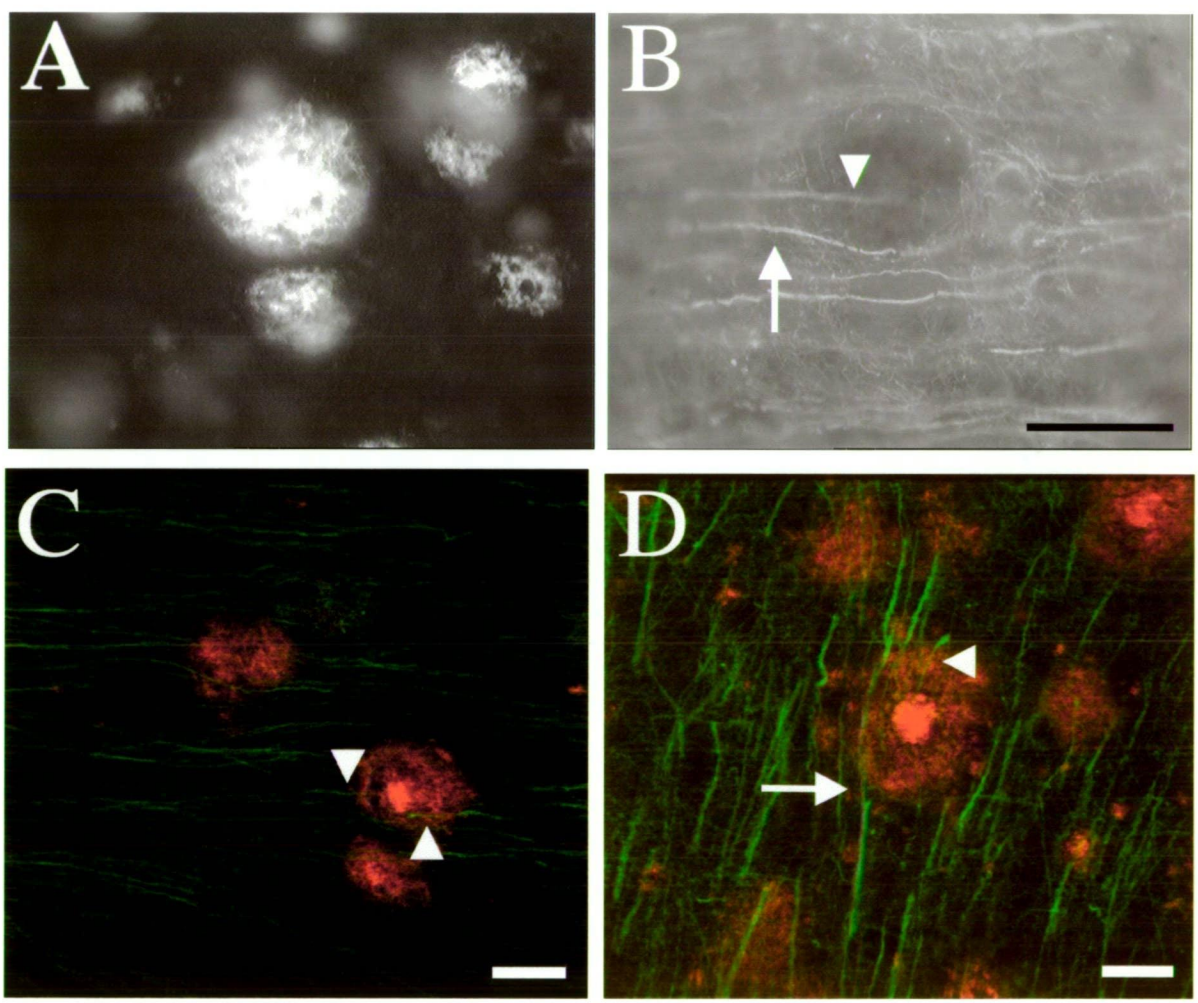
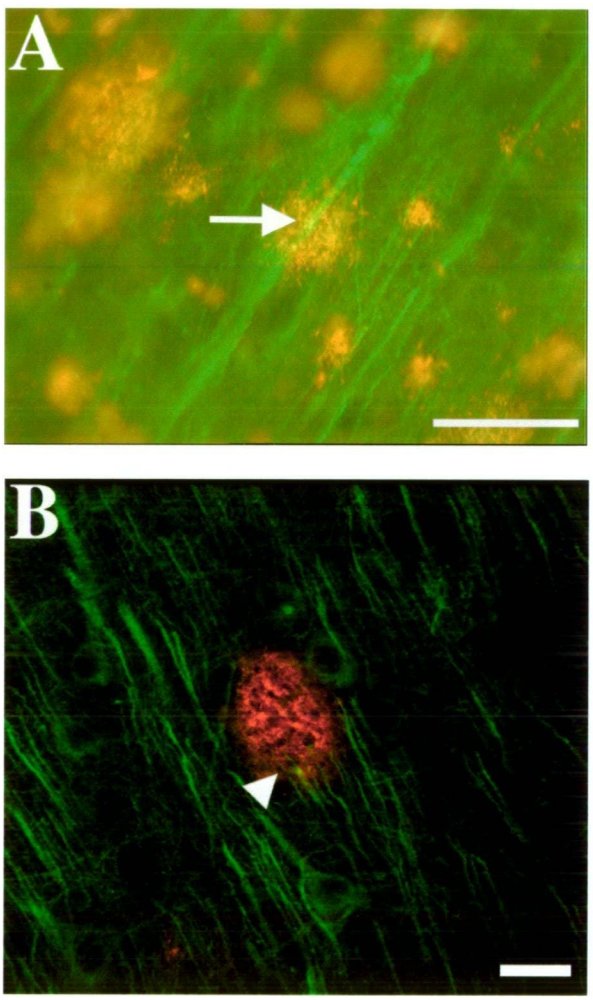

A
B
C
D

Figure 3.5. An example of the effect of fibrillar plaques on dendritic structure. Material from a preclinical Alzheimer's disease (AD) case is shown, which has been double-labelled with ß-amyloid (red) and microtubule-associated protein-2 (MAP2) (green). (A) was visualised with standard fluorescence microscopy, whilst (B) was derived by confocal laser scanning microscopy. Dendrites are shown both passing through the outer margins and the centre of the plaque (arrow) and also terminating within the plaque (arrowhead).

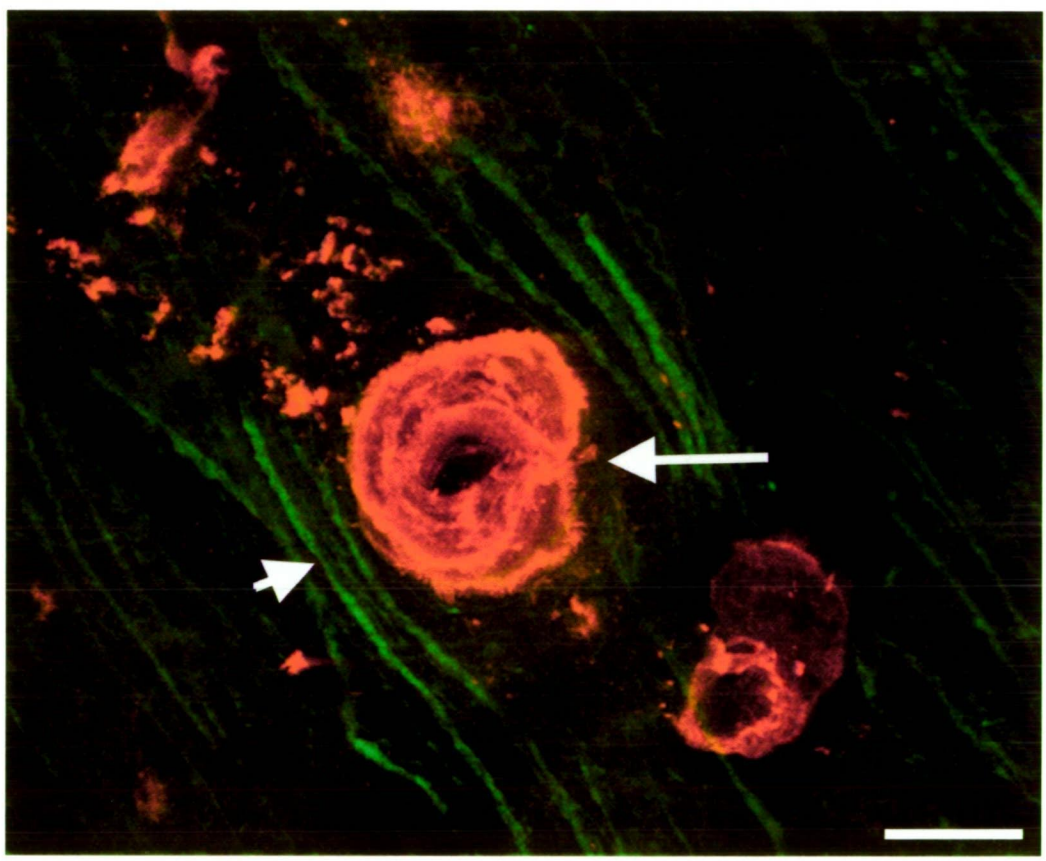
Scale bar (A) = 50μm; (B) = 40μm.


A
B

AD cases

The pattern of MAP2 and ß-amyloid immunoreactivity observed in AD material was similar to that found in preclinical AD cases. ß-amyloid labelled the same plaque-types as in preclinical AD, and MAP2 labelled dendrites were similarly affected in relation to the effect of dense core and diffuse plaques on their structure and morphology. Differences were noted, however, with respect to the fibrillar plaques found in AD material. There was a greater relative percentage of MAP2 labelled dendrites surrounding fibrillar plaques, as compared to within the plaque area (Table 3.1). However, fibrillar plaques in AD demonstrated a similar inconsistent pattern of MAP2 labelling, in association with the plaque, as observed in preclinical AD, with dendrites both unaffected and significantly altered as they traversed the plaque. Amyloid angiopathy was also observed in AD material, with MAP2 labelled dendrites deflecting around the margins of the ß-amyloid deposits associated with blood vessels (Figure 3.6).

Figure 3.6. A double-labelled image of an Alzheimer's disease brain, visualised with confocal laser scanning microscopy, showing ß-amyloid (red) and microtubule-associated protein-2 (MAP2) (green) surrounding a blood vessel (long arrow). This is an example of amyloid angiopathy, with dendrites deflected around the ß-amyloid deposit (short arrow). Scale bar = 40 μm.



3.3 Discussion

We have demonstrated that dendrites within the AD brain are subject to dramatic structural and morphological changes as a result of ß-amyloid plaque formation in both the early and late stages of AD. These findings are consistent with previous reports which show that the presence of neocortical ß-amyloid plaques in AD results in an abnormal morphological change in dendrites which encounter them (Knowles *et al.*, 1998, 1999). Knowles and colleagues (1998 and 1999) noted that neurofilament-immunolabelled dendrites lose their normal straight, aligned appearance and become more 'curvier' within regions occupied by plaques. They hypothesised that this change was a response to slowly evolving damage evoked by the presence of the plaque. Whilst our results support the notion that plaques impact upon the normal cytoskeletal network present within neurites, our investigations qualify that observation. Depending upon both the stage of the illness and the type of plaque encountered, dendrites will be differentially affected, and in fact, can remain unaffected as they pass through plaques.

Both clinical, or late-stage, and preclinical, or early-stage, AD cases were examined in this investigation, as well as non-AD controls. The same definition of plaque type was utilised across all case types. Previous investigations both in this laboratory (unpublished) and others (Wisniewski and Terry, 1973; Ulrich, 1985; Masliah *et al.*, 1993a; Schmidt *et al.*, 1995; Dickson, 1997; Armstrong, 1998) have shown that there is a degree of heterogeneity in plaque morphology. We have, however, demonstrated that plaques can be grouped into one of the three morphologically distinct types, diffuse, fibrillar or dense core. These three plaque types were identifiable in all cases examined, and these groupings were used to draw a distinction between the relative effect of plaque-type on dendrites.

Dendritic morphology and organisation was demonstrated with the microtubule-associated-protein, MAP2. This protein is a marker for microtubules, and is selectively localised within dendrites in the mature central nervous system (Hirokawa *et al.*, 1996). There are a number of existing studies that have investigated the change in MAP2 in AD, but the majority of these have examined the possible relationship between MAP2 and the formation of neurofibrillary tangles (NFT). It has been reported that NFT are labelled for antibodies against MAP2 (Kosik *et al.*, 1984; Neve *et al.*, 1986; Dammerman *et al.*, 1989; Mulvihill and Perry, 1989), and may form integral components of NFT. It has, however, been demonstrated that MAP2 is not one of the major structural components of the NFT (Rosemblatt *et al.*, 1989; Six *et al.*, 1992), although recent studies have shown that small fragments of MAP2 molecules, the microtubule-binding region, can form PHF-like structures (DeTure *et al.*, 1996; Zhang *et al.*, 1996).

Preclinical AD cases demonstrated statistically significant differences in the preservation of MAP2 labelling between the three different plaque types. That the area occupied by dense-cored plaques had statistically fewer MAP2 labelled processes in it than the surrounding neuropil is perhaps not surprising. We have previously shown that dense cored plaques are the most likely to be neuritic, and often contain dystrophic, or abnormal, axons (unpublished). These plaque types are, therefore, hypothesised to be destructive with regards to their effect on neuronal structure. The morphology of the dendrites associated with dense-cored plaques also suggests that these plaques damage the neuronal cytoarchitecture of the brain. We have demonstrated a similar phenomena to that reported by Knowles and colleagues (1998, 1999), whereby dendrites appear 'curvy' and seem to deflect around the outer margins of the plaque, suggesting that the dendrites have either

actively 'avoided' the plaque, or have been excluded from it. The background level of MAP2 labelling in AD cases was also markedly decreased relative to that found in controls. Previous studies have highlighted the paucity of microtubules in the AD brain (Terry *et al.*, 1964; Gray, 1986; Gray *et al.*, 1987; Paula-Barbosa *et al.*, 1987), which has been attributed to a displacement of microtubules, and associated proteins such as MAP2, from dendrites by the formation of PHF (Geddes *et al.*, 1994; Ashford *et al.*, 1998). The diminished MAP2 labelling may, therefore, reflect a reduction in normal dendritic structure due to an overall loss of microtubules within affected neurons. Similarly, McKee and colleagues (1989) have also demonstrated that, even in unaffected neurons in AD, there are significant dendritic changes observed with MAP2 labelling, including the complete degeneration of apical dendrites.

In contrast to the effects of dense cored plaques on dendrites, the reverse was observed in diffuse plaques. There was a statistically significant increase in the density of MAP2 labelling present within plaques relative to the surrounding neuropil. This finding, though, is not without precedent. *In vitro* studies on the effect of the ß-amyloid protein on cultured hippocampal neurons have demonstrated that Aß-1-42 can increase both the number of dendrites and their arborization (Whitson *et al.*, 1990). Similarly, further *in vitro* experiments on hippocampal cultures have shown that, when exposed to sera from AD patients, there is a significant increase in the relative amounts of MAP2 fluorescence, as compared to neurons exposed to sera from young people (Brewer and Ashford, 1992). This apparent increase in MAP2 labelling within diffuse plaques in AD cases may be due to local dendritic sprouting, which has been proposed to occur both in the neuropil (Ihara,

1988; McKee *et al.*, 1989; Geddes *et al.*, 1991; Masliah *et al.*, 1992) and within the area occupied by plaques (Probst *et al.*, 1983).

With regards to fibrillar plaques, there was no difference between the density of MAP2 labelling within the plaque, as compared to the surrounding neuropil in the preclinical AD cases. A similar result, but in clinical AD cases, has been shown by Nieto and colleagues, who demonstrated that both tubulin and microtubule-associated-protein-2 (MAP2) protein levels in the AD brain were no different to control brains. However, in this investigation, quantitation revealed that in the AD cases there was a significant difference between the MAP2 labelling within the area occupied by plaques as compared to the surrounding neuropil.

AD cases demonstrated a statistically significant decrease in MAP2 density within fibrillar plaques as compared to the surrounding neuropil. This is perhaps not surprising, as we have previously shown that fibrillar plaques are as equally neuritic as dense cored plaques in end stage AD (unpublished). This suggests, therefore, that fibrillar plaques are more disruptive to neuronal architecture in the late stage of AD, and perhaps, therefore, undergo some kind of transition from the early to the later stages of AD.

An intriguing observation has been that, both within the area occupied by a plaque and also within the surrounding neuropil, with only one exception, there is a consistently greater density of MAP2 labelling within the AD brain relative to the preclinical cases. This may be a response to the increased deposition of plaques, which may result in structural deformation to surrounding neurons, which in turn may stimulate the neuronal response to

physical injury (Vickers *et al.*, 2000) and cause attempted neurite outgrowth. Alternatively, it may be due to the formation of other abnormal AD structures, including PHF, in all aspects of the neuron, again resulting in cytoskeletal disruption, stimulation of the injury response, and attempted resprouting. There can also be significant atrophy in the AD brain, as mentioned in chapter 1, and this may contribute to a 'compressing' of the neuronal architecture and subsequent apparent increase in the density of cytoskeletal elements.

We have demonstrated, therefore, that morphologically distinct classes of plaques differentially affect dendritic structure and organisation in both the early and late stages of AD. That such dendritic disruption may form a key component underlying the pathogenesis of AD has been previously hypothesised, and suggests that therapies based on the stabilisation of the cytoskeleton against such alterations may prove efficacious in the treatment of AD.

Changes in microtubule-associated-proteins, MAP2 and tau, following both *in vitro* and *in vivo* neuronal injury

4.0 Introduction

Microtubule associated proteins (MAPs) are essential cytoskeletal components which contribute to microtubule stability, cellular architecture, form cross bridges with other filamentous proteins and which have a host of other putative functions (Hirokawa *et al.*, 1988). There are several major MAP isoforms that can be isolated from the mammalian brain (see chapter 1), including those investigated in this chapter, MAP2 and tau. The expression and localisation of the different MAPs is developmentally regulated. Within immature neurons, MAP2 is present within the axon and dendrite, but then as development continues, it is selectively lost from the axonal domain (Caceres *et al.*, 1986; Pennypacker *et al.*, 1991), and within the mature system is localised to the somatodendritic domain (Hirokawa *et al.*, 1991; Litman *et al.*, 1994). Tau is enriched in the axon (Kanai and Hirokawa, 1995; Hirokawa *et al.*, 1996), with tau mRNA localised to the cell body and axon (Litman *et al.*, 1994). In the previous chapter, it was demonstrated that dendritic architecture, as evidenced with MAP2 labelling, is subject to significant alterations, including physical deformation and even transection, in both the early and late stages of Alzheimer's disease (AD), as a result of the deposition of insoluble ß-amyloid 'plaques' in the cortex. Similarly, the role, and alterations in, tau in AD has been extensively investigated (e.g. Johnson and Jenkins, 1996). These changes may contribute to the pathogenesis of the disease, and in this respect, it has been hypothesised that they may represent therapeutic targets for the treatment of AD.

Dendritic and MAP changes can be studied experimentally, and in this chapter, the changes in MAP2 and tau have been investigated following both *in vivo* and *in vitro* cortical injury. Whilst there are existing reports detailing the change in these proteins following a variety of insults to the central nervous system (CNS), they have not been investigated in the models utilised in this chapter, which have principally been used to study the role of neurofilaments in the development of neuronal pathology (King *et al.*, 1997; Dickson *et al.*, 2000). The advantage of these models is that they have been shown to replicate the neuropathological changes associated with the early stages of AD, and they allow for the introduction of agents into the damaged cortical tissue. It is possible, then, to examine the aspects which may contribute to the development of the neuropathology which characterises these models, and AD, as well as to examine the efficacy of different agents in preventing the evolution of these neuronal changes.

4.1 Materials and methods

Animal Procedures

Methods utilised throughout this chapter are detailed in chapter 2. All procedures involving animals were approved by the Ethics Committee (Animal Experimentation) of the University of Tasmania and are consistent with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Briefly, for *in vivo* studies, animals, in groups of five, received experimentally induced cortical injuries, and were examined at one, four, seven and fourteen days post-injury (PI). Coronal sections through the injury site were cut at 50μm with a vibratome. Standard immunohistochemical techniques for double labelling were utilised for visualisation of antibody immunoreactivity (chapter 2). In all fluorescence-double-labelling investigations, a monoclonal antibody which recognises MAP2 was utilised in combination with a rabbit polyclonal antibody against tau. Antibodies were visualised with a rat-adsorbed, horse anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) and a goat anti-rabbit IgG conjugated to biotin followed by avidin Texas Red.

For *in vitro* studies, neuronal cell cultures were established as outlined in chapter 2. Neurite bundles between clusters of cells were transected at twelve hours PI, one, three and seven days PI. Standard immunohistochemical techniques for double labelling of coverslips were utilised (chapter 2). In all fluorescence-double-labelling investigations, a polyclonal antibody against tau was alternately utilised in combination with both mouse monoclonal antibodies which recognise MAP2, and the growth associated protein, GAP43. Antibodies were visualised with a rat-adsorbed, horse anti-mouse IgG conjugated to FITC and a goat anti-rabbit IgG conjugated to biotin followed by avidin Texas Red.

4.2 Results

In vivo investigation

MAP2

In the normal rodent brain, MAP2 primarily labelled long bundles of apical dendrites, which extended from the pial surface to the deeper layers of the cortical grey matter (Figure 4.1). The dendrites appeared 'smooth' and evenly labelled. MAP2 labelling was also localised to cell bodies. Following cortical injury, induced by the insertion of a 25 gauge needle, there were dramatic changes in MAP2 immunoreactivity surrounding the injury site. At one day PI, normal MAP2 labelling was completely absent from the margins of the injection tract in all animals examined (Figure 4.1). There was, however, the occasional 'wispy fibre' which appeared beaded and MAP2 positive (Figure 4.1). The loss of normal MAP2 immunoreactivity extended for a distance of approximately 200 μm on either side of the tract, past which MAP2 labelling appeared normal. The contralateral cortex, at both this and all other timepoints, did not exhibit any morphological change with MAP2 labelling, suggestive of a disruption to the normal cytoarchitecture of the brain. At four days PI, there was, similarly, a complete loss of normal MAP2 labelling at the margins of the injury site in all animals examined (Figure 4.1). At seven days PI, however, there was an inconsistent pattern of MAP2 immunoreactivity. Unlike earlier timepoints, where the margins of the tract were devoid of MAP2 labelling, in 40% (2/5) of animals examined at seven days PI, there were MAP2 labelled processes in this region (Figure 4.2). These fibers were of differing lengths, often beaded and not typically 'bundled'. None of the structures demonstrated normal dendritic morphology. In the remaining 60% of animals (3/5) the margins of the tract were devoid of MAP2 immunoreactivity (Figure 4.2). At fourteen days PI, 100% (5/5) of animals demonstrated MAP2 immunoreactivity adjacent to, and up to the

Figure 4.1. Microtubule-associated protein-2 (MAP2) labelling in the rodent brain. (A) shows the uninjured cortex, where MAP2 immunoreactivity is primarily localised to cell bodies and long apical dendrites, the latter which extend from the deeper layers of the cortex through to the pial surface. These dendrites typically appear in bundles (short arrow). (B) shows the area adjacent to the injury tract (*) at one day post-injury (PI). There is a complete loss of normal MAP2 labelling, however, there is the occasional fine, beaded fibre (long arrow). Similarly, at four days PI (C), the area surrounding the lesion site (*) is devoid of MAP2 labelling, with the exception of occasional fine fibres (long arrow).
Scale bar = 50 μm.

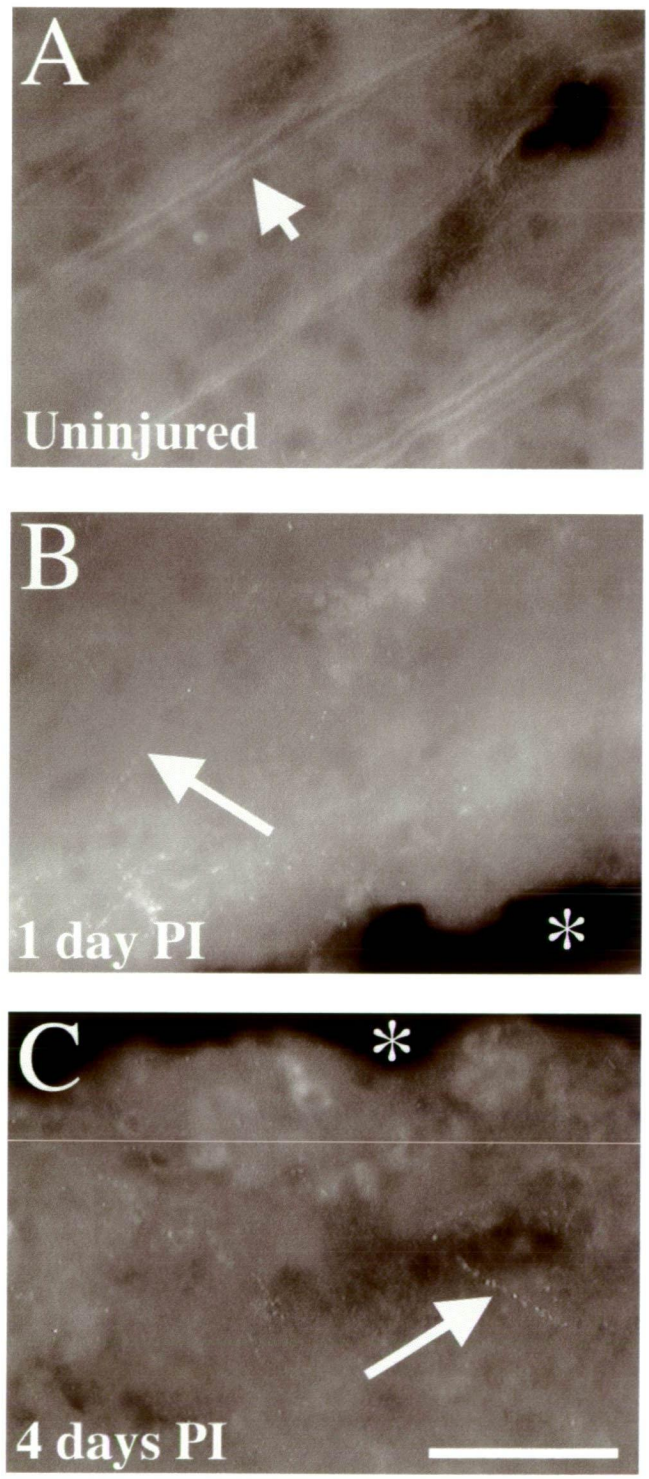
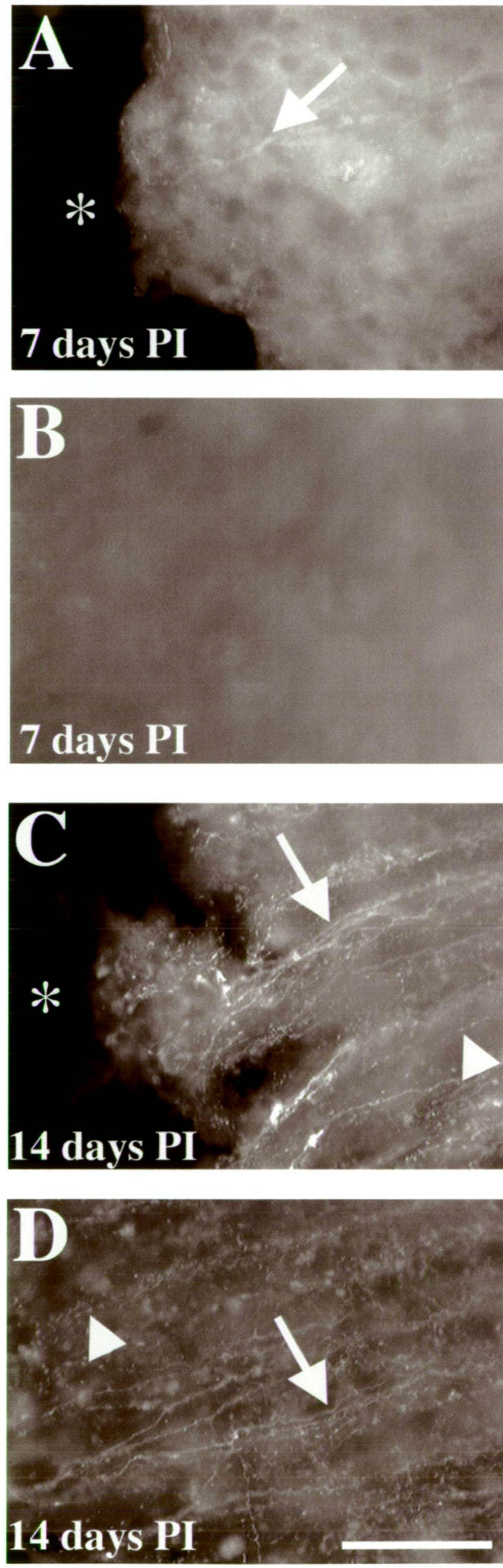

A
Uninjured
B
1 day PI
*
C
*
4 days PI

Figure 4.2. Microtubule-associated protein-2 (MAP2) labelling in the rodent brain at seven and fourteen days post-injury (PI). (A) and (C) are taken from the injury site (*), whilst (B) and (D) show areas immediately adjacent to the injury tract. At seven days PI there is the occasional MAP2 labelled process (long arrow) at the injury site (A). This was demonstrable in 40% of animals examined. The remaining 60% of animals examined showed no MAP2 labelling at, or adjacent to, the tract (B). At fourteen days PI, there are beaded processes (arrowhead) as well as processes of varied length which are labelled with MAP2 (long arrow) (C). This was observed in 100% of animals examined. (D) shows an area adjacent to the tract in which there is also extensive MAP2 labelling of dendrites (long arrow), as well as finer, beaded processes (arrowhead). This was also observed in 100% of animals examined at this timepoint.

Scale bar = 50 μm


A
*
7 days PI
B
7 days PI
C
*
14 days PI
D
14 days PI

margins of, the injury tract (Figure 4.2). Processes were again of varied length, but were often continuous with processes outside the tract area. Dendrites were not typically 'smooth', but were occasionally 'bundled', as in the uninjured cortex, and often appeared beaded along their length (Figure 4.2). There was significantly more MAP2 immunoreactivity surrounding the tract at fourteen days PI relative to earlier timepoints.

Tau

In the uninjured cortex, tau labelling primarily appeared punctate throughout the grey matter, with no labelling of discrete structures (Figure 4.3). At one, four and seven days PI, there was a decrease in the intensity of tau labelling in the area surrounding the injury site (example, Figure 4.3). However, at fourteen days PI, 100% (5/5) of animals demonstrated tau labelling similar to that found in the normal cortex (example, Figure 4.3).

In vitro investigation

MAP2

Based on light microscopic observations, *in vitro* transection involved cutting bundles of neurites extending between clusters of cells. Fluorescence microscopy revealed that the site of injury was normally devoid of MAP2 labelling, suggesting that the majority of these neurites were not dendrites. MAP2 labelling was normally confined to cell bodies and small calibre neurites extending from the periphery of individual, and clusters of, cells. There was, however, the occasional MAP2 positive fiber observed at the site of transection at all timepoints examined. Interestingly, these neurites were also invariably labelled with tau (e.g. Figure 4.4).

Figure 4.3. Tau immunoreactivity in the rodent brain. (A) shows the uninjured cortex, where there is a high density of tau immunoreactivity, which appears punctate throughout the cortex. (B) is an example of the area adjacent to the injury tract at seven days post-injury (PI), where there are few tau-positive structures (arrow) relative to the uninjured brain. At fourteen days PI (C), however, tau labelling appears similar to that found in the uninjured cortex.

Scale bar = 50μm.

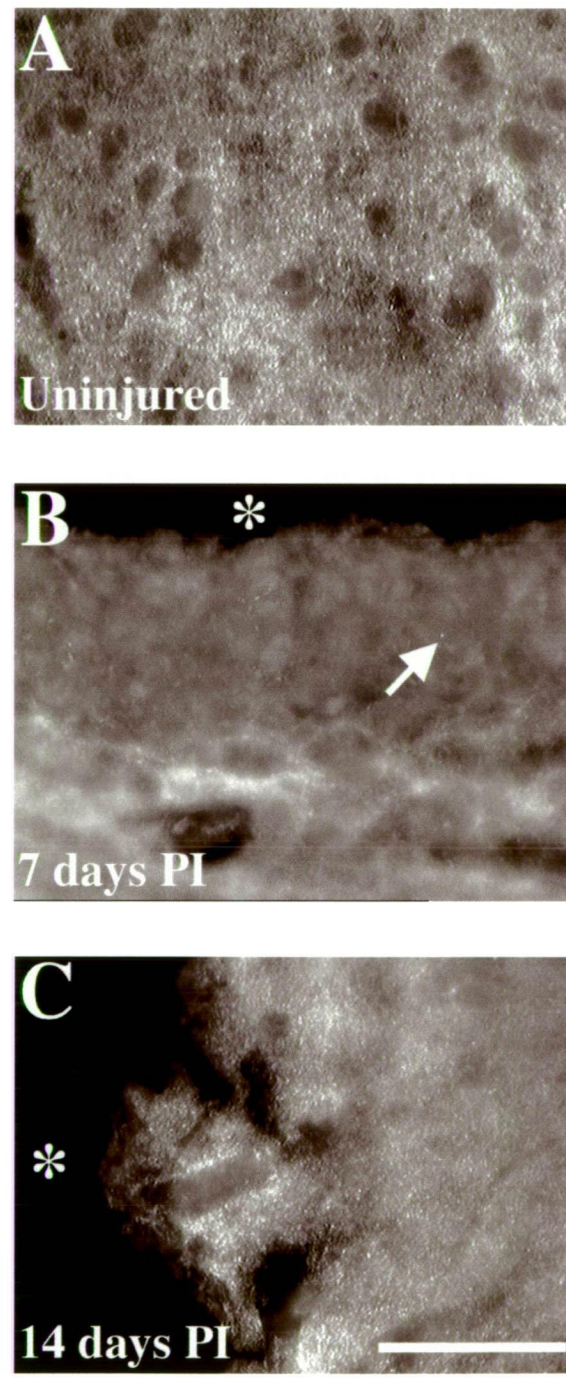
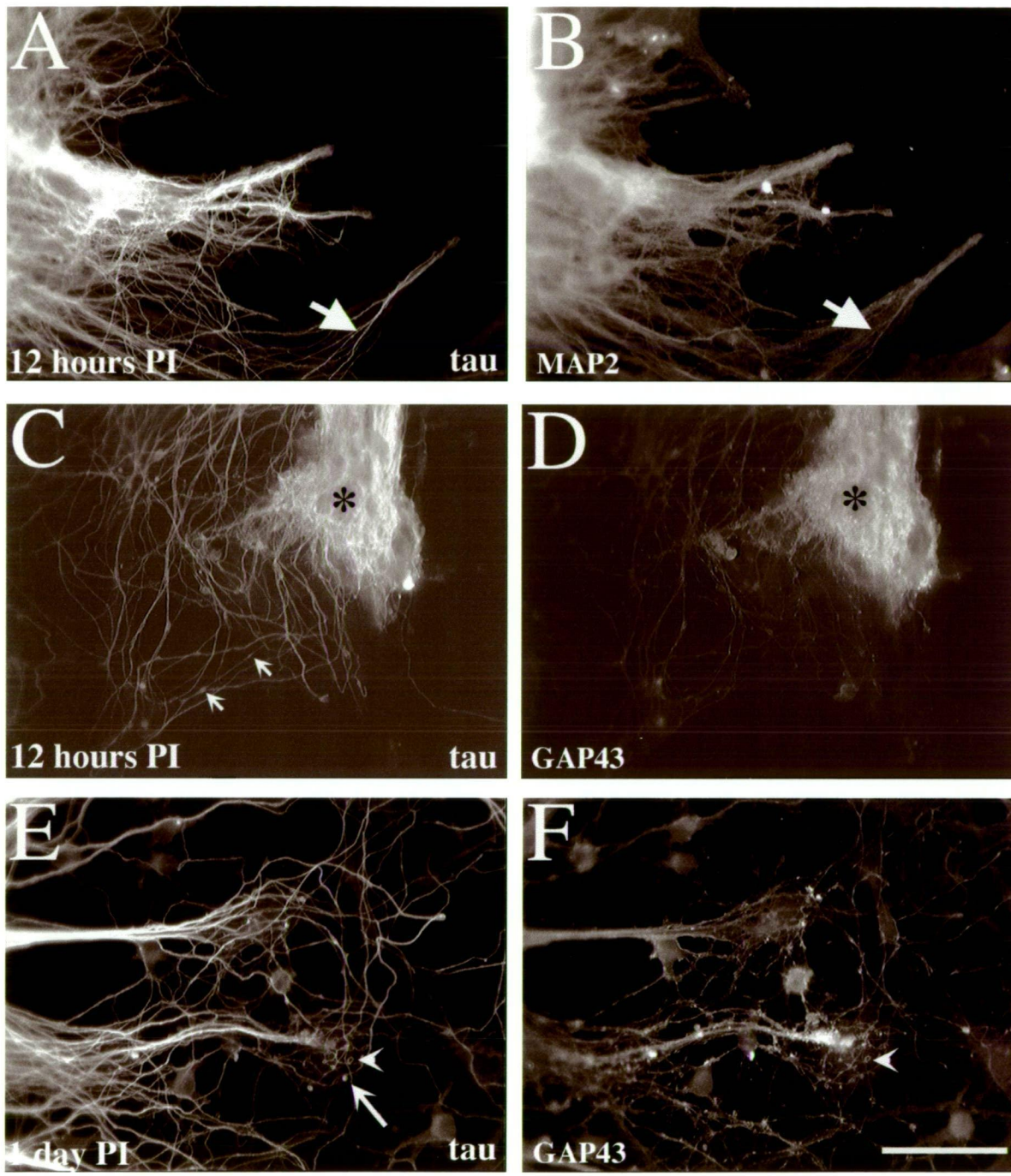

A
Uninjured
B
*
7 days PI
C
*
14 days PI

Figure 4.4. Double-labelled images of transected mature cultured cortical neurons. Processes at the site of transection are occasionally labelled for both tau (A) and microtubule-associated protein-2 (B) (short arrows). The distal portion of the transected axonal stumps (*) are consistently labelled for tau (C) and growth-associated protein-43 (GAP43) (D). Neurites also appear beaded (small concave arrows, C) at 12 hours PI. At one day PI there is the appearance of tau-positive bulb- (concave arrow, E) and ring-like (concave arrowhead, E) structures, the latter which are double-labelled with GAP-43 (concave arrowhead, F).

Scale bar = 50μm.


A
12 hours PI
tau
B
MAP2
C
12 hours PI
tau
D
GAP43
E
1 day PI
tau
F
GAP43

Tau

Tau labelling was extensive in all cases, and was primarily localised to axons, which extended from individual cell bodies and from the margins of cell clusters. At twelve hours PI there was little change in tau labelling, with axons remaining immunoreactive. There was the appearance, however, of beaded axons which were labelled for tau (Figure 4.4). At one day PI, the pattern of labelling was similar to that observed at twelve hours PI. There was, however, the appearance of both 'bulb-like' and 'ring-like' structures labelled for tau (Figure 4.4). Three day PI material again demonstrated a similar pattern of tau immunoreactivity as observed at twelve hours and one day PI. Abnormal tau positive bulb- and ring-like structures, however, were less frequent than at earlier timepoints, and tau positive fibers appeared to cross the injury tract uninterrupted (Figure 4.5). At seven days PI there was little evidence of transection between cell clusters, with tau positive fibers extending between the cell clusters (Figure 4.6). There was no evidence of abnormal tau-positive structures as observed at earlier timepoints.

GAP43

GAP43 labelling was punctate at all time points examined. At twelve hours and one day PI, GAP43 labelling was intense within the end of the transected axonal stump, and it also selectively labelled discrete sections of a number of tau positive fibers which emerged from that stump (Figure 4.4). There were also GAP43-positive growth-cone-like structures which were immunoreactive for tau. At three days PI, GAP43 labelled the majority of neurites which extended across the lesion site (Figure 4.5). A similar pattern was observed at seven days PI, however, GAP43 labelled fewer of these tau-positive neurites.

Figure 4.5. Transection of mature cultured cortical neurons. The arrow shows the transection of neurite bundles between two clusters of nerve cells (A) (image unlabelled). (B) shows the same group of cells at three days post-injury (PI), with tau-positive neurites traversing the site of axonal transection (short arrow, B). (C) shows the transection of neurite bundles (image unlabelled). This site is shown at three days PI in panel (D) and (E), with tau-positive neurites (short concave arrow, D) double-labelled with GAP43 (short concave arrow, E).

Scale bar (A), (C) = 150μm; (B) = 100μm and (D), (E) = 50μm.

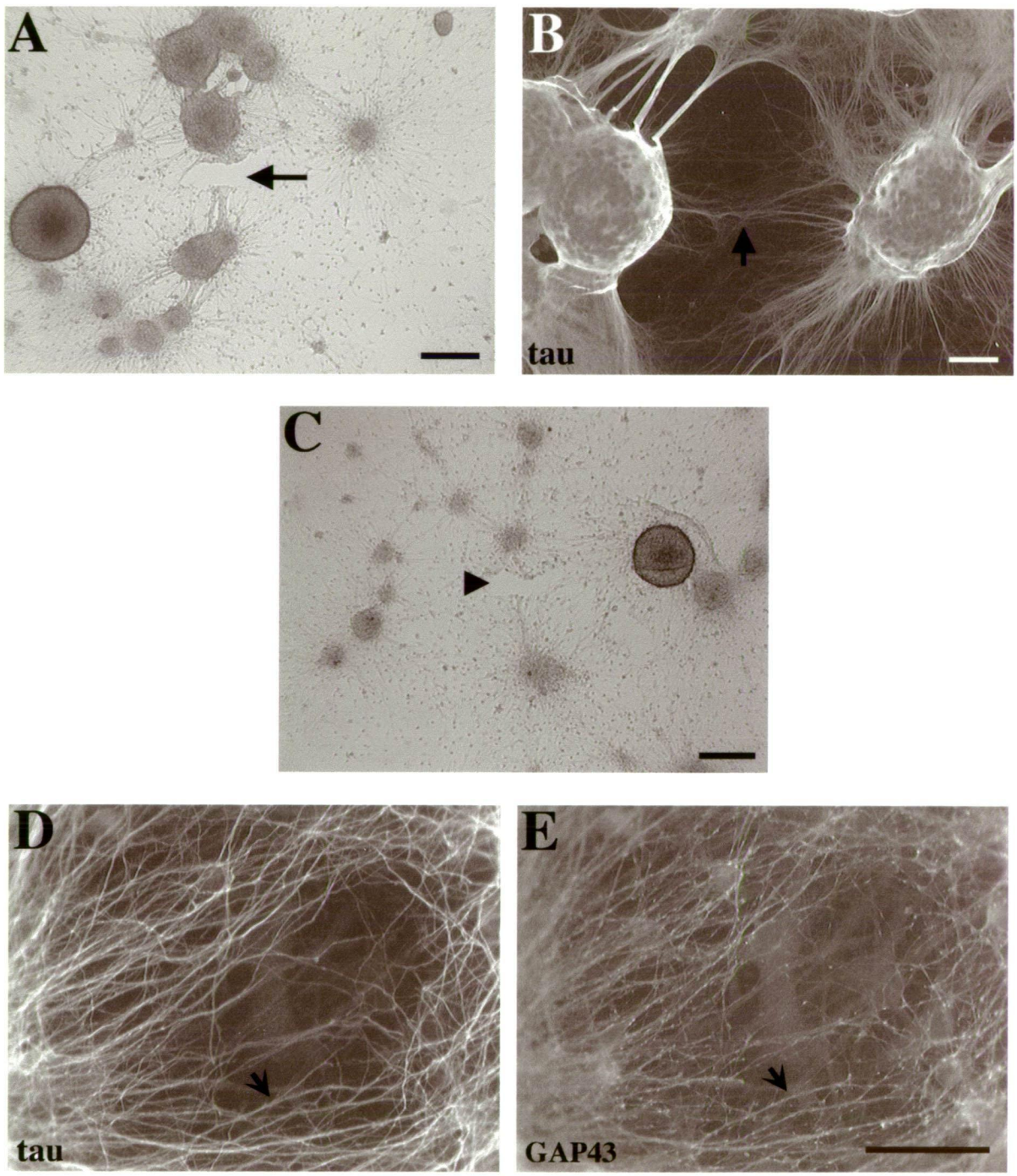
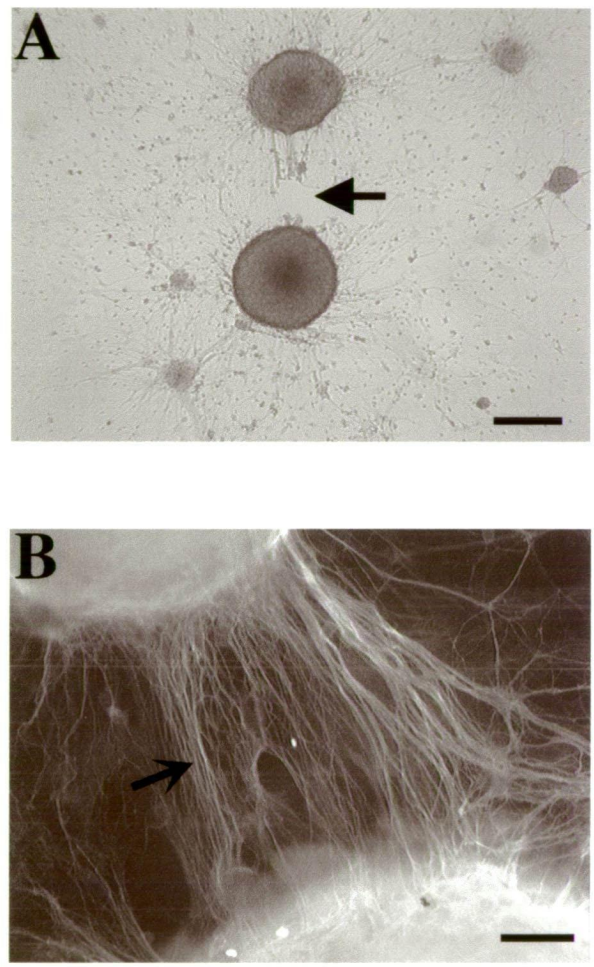

A
B
tau
C
D
tau
E
GAP43

Figure 4.6. Transection (arrow) of neurite bundles between clusters of cortical neurons at 21 days *in vitro* (A) (image is unlabelled). At seven days post-injury (PI) tau-positive neurites (concave arrow, B) are shown crossing the previously transected area.

Scale bar (A) = 150μm; (B) = 50μm.


A
B

4.3 Discussion

This investigation has demonstrated that the neuronal cytoskeleton undergoes a series of alterations following both *in vitro* and *in vivo* cortical injury, which primarily involve the loss of normal cytoskeletal protein labelling at the site of injury, and morphological alterations in the elements that remain. We have also shown that at a number of days post-injury, the neuropil begins to appear morphologically normal, which may be due to local sprouting of injured neurites.

In this investigation, *in vivo* cortical injury resulted in significant cytoskeletal abnormalities at one, four, seven and fourteen days post injury, as evidenced by reduced labelling for the different cytoskeletal proteins. Such a loss of immunoreactivity is a characteristic finding following different forms of neuronal injury. It has been demonstrated, for example, that within hours following ischaemia, there is a decrease in normal dendritic MAP2 labelling (Kwei *et al.*, 1993; Matesic and Lin, 1994; Blomgren *et al.*, 1995; Raley-Susman and Murata, 1995; Gilland *et al.*, 1998), and MAP2 protein levels (Matesic and Lin, 1994). Different forms of traumatic brain injury (TBI) have been shown to result in a decrease in MAP2 immunoreactivity within the first ten minutes post-injury (Hicks *et al.*, 1995), as well as at other times and up to seven days PI (Taft *et al.*, 1992; Hicks *et al.*, 1995; Lewen *et al.*, 1996; Posmantur *et al.*, 1996; Li *et al.*, 2000). Cortical lesions also result in the phosphorylation of tau (Janke *et al.*, 1998), which can subsequently interfere in its ability to bind to microtubules (Garver *et al.*, 1994). Following lesions of the spinal cord (Li *et al.*, 2000; Zhang *et al.*, 2000) and sciatic nerve (Chambers and Muma, 1997), there are also marked losses in proteins such as MAP2 and tau. In the case of tau, however, this may be

due to a relative shift in the expression of different tau isoforms (Chambers and Muma, 1997).

It should also be noted, however, that more mild forms of brain injury have been shown to result in accumulations of both MAP2 (Kanayama *et al.*, 1997) and tau (Smith *et al.*, 1999) in damaged axons, and particularly in the perikarya, in both the grey and white matter.

Morphologically, our *in vivo* results are in accordance with that found in other investigations. MAP2 labelled dendrites, following ischaemia, have an altered morphology (Kwei *et al.*, 1993), with dendrites appearing beaded (Matesic and Lin, 1994). TBI similarly results in injured dendrites becoming misaligned, swollen and fragmented (Posmantur *et al.*, 1996; Folkerts *et al.*, 1998) and they often lose their arborization (Posmantur *et al.*, 1996). These factors are hypothesised to contribute to the early destruction of dendrites following injury (Gallyas and Zoltay, 1992; Gallyas *et al.*, 1992). Following lesions to the spinal cord, where similar morphological alterations have been demonstrated, the resolution of this beading correlated with behavioural recovery (Zhang *et al.*, 2000). Whilst we did not perform any behavioural analysis, our results confirmed that the abnormal dendritic morphology does resolve over time, although there still remained significant differences in the cytoarchitecture of the brain at fourteen days PI, as compared to the uninjured cortex. A similar phenomenon was observed in the *in vitro* model.

In the *in vitro* model of cortical injury, which involves the transection of neurite bundles, there were not the typical MAP changes as observed *in vivo*. However, there were significant changes in tau, with fragmentation of labelling as well as the formation of abnormal bulb- and ring-like structures, the latter which were colocalised with labelling for

the growth associated protein GAP43. At a number of days PI there was the appearance of tau positive fibres crossing a previously transected area. Whether such an apparent regrowth of tissue is the result of injured neurites sprouting, or a compensatory growth of adjacent neurites has remained controversial, as it has been proposed that the CNS presents an inhibitory environment to cortical sprouting such that functional regeneration is not possible (Ramon y Cajal, 1928). In the previous chapter, however, we demonstrated that there is a greater density of MAP2 labelling present within the area occupied by diffuse plaques, as compared to the surrounding neuropil, which may be the result of local dendritic sprouting. It is possible, therefore, that in the cell culture environment, where many of the inhibitory factors normally associated with the brain milieu, are not present, that injured neurites may be better able to resprout. This is particularly true if the correct plating conditions are supplied, as Brewer (1999) has demonstrated that greater than half of adult neurons retain the ability to regenerate and proliferate in specific circumstances. It is possible, therefore, that the neurites which cross previously transected areas are the result of local sprouting. Newly formed axons express both MAP2 and tau during the initial stages of growth both *in vivo* and *in vitro* (Cambray-Deakin, 1991). That these tau-positive neurites are initially GAP43 positive, but consistently remain negative for MAP2, suggests that they may represent an altered, perhaps sprouting, response of previously injured axons.

In this chapter, therefore, it has been demonstrated that, following experimentally induced cortical injury, there are dramatic alterations to a number of different cytoskeletal elements present within neurites soon after injury. These same proteins are subject to significant changes in AD. Cytoskeletal disruption, therefore, may be a key process in the pathogenesis of both AD and the neuropathological changes following cortical injury. This suggests that

drugs which specifically target such cytoskeletal alterations may be efficacious in the treatment of the neuropathological sequelae of cortical injury. Given the similarities between the neuropathology of AD and that which occurs following cortical injury (King *et al.*, 1997), the drugs which prove effective in the *in vivo* model may also prove effective in the treatment of AD. The use of drugs that target these changes is explored in the following chapter.

The effects of taxol on the central nervous system response to physical injury

5.0 Introduction

Taxol, a diterpene alkaloid originally isolated from the Pacific Yew tree, *Taxus brevifolia*, is a cytoskeletal stabilising agent which has been used extensively in the treatment of refractory ovarian and metastatic breast cancer (Huizing *et al.*, 1995). Taxol binds to the ß subunit of tubulin, causing an increase in the polymerisation of soluble tubulin to form microtubules and also stabilising existing microtubules to prevent their breakdown (Gotaskie and Andreassi, 1994; Huizing *et al.*, 1995). In cancer therapy, taxol prevents the breakdown of the microtubules comprising the mitotic spindle present during mitosis, thereby disrupting the normal microtubule dynamics of the cell cycle, eventually causing growth arrest (Tishler *et al.*, 1992). There have also been a number of studies on both the acute and chronic effects of taxol administration on the peripheral nervous system (PNS) (Röyttä *et al.*, 1984; Röyttä and Raine, 1985; Röyttä and Raine, 1986; Vuorinen *et al.*, 1988; Vuorinen *et al.*, 1989; Vuorinen and Röyttä, 1990). There have been no such similar studies on the effect of taxol on the central nervous system (CNS), however, recent reports have suggested that it may be of use in both the treatment of Alzheimer's disease (AD) (Michaelis *et al.*, 1998) and Multiple Sclerosis (MS), with clinical trials involving taxol analogues currently being undertaken in both conditions. Degenerative diseases of the nervous system, such as MS and AD, as well as the sequelae of events following head trauma, are all characterised by some degree of neuronal damage involving the cytoskeleton (Raine and Cross, 1989; Povlishock and Christman, 1995; Trapp *et al.*, 1998; Vickers *et al.*, 2000). Changes which are common to these conditions include alterations in both neurofilaments (NF), intermediate filaments found within nerve cells, and microtubules, integral components of the filamentous network comprising the cytoskeleton (Burgoyne, 1991). Specifically there is an accumulation of both organelles and NFs at sites of axonal damage, resulting in impairments in many cellular functions, including axoplasmic transport (Yaghmai and Povlishock, 1992; Pettus and

Povlishock, 1995; Povlishock and Christman, 1995; Vickers *et al.*, 2000). This can further result in axonal transection and the formation of reactive axonal swellings which appear as bulb- and ring-like NF accumulations (Guillery, 1965; Meller *et al.*, 1993; Meller *et al.*, 1994; Vickers *et al.*, 1996; King *et al.*, 1997; Dickson *et al.*, 1999; Vickers *et al.*, 2000). Axonal damage also results in microtubule perturbations, with an apparent loss of microtubules around lesion sites in AD (Paula-Barbosa *et al.*, 1987), MS (Raine and Cross, 1989) and head injury (Blomgren *et al.*, 1997; Jafari *et al.*, 1997; Maxwell and Graham, 1997) resulting in structural alterations to, and impairments in the function of, the cytoskeleton. Similarly, in numerous models of brain injury (Hicks *et al.*, 1995; Vanciky *et al.*, 1995; Dawson and Hallenbeck, 1996; Maxwell, 1996; Pettigrew *et al.*, 1996; Posmantur *et al.*, 1996a, 1996b; Maxwell and Graham, 1997; Jafari *et al.*, 1998; Saatman *et al.*, 1998; Schmidt-Kastner *et al.*, 1998), there are also profound changes in Microtubule Associated Protein-2 (MAP2), as detailed in chapter 4.

In this respect, the use of taxol to stabilise microtubules in damaged or degenerating neurons could be efficacious in delaying or even preventing the sequence of deleterious events which follow axonal damage and microtubule disruption in these conditions (Lee *et al.*, 1994). *In vitro* studies have demonstrated that taxol treatment may protect against both calcium-mediated neuronal death in cortical neurons (Burke *et al.*, 1994; Furukawa and Mattson, 1995) and the development of cytoskeletal pathology in AD (Mattson, 1992; Michaelis *et al.*, 1998). In an *in vitro* study by Bird (1984), however, it was demonstrated that continued taxol treatment of neural tissue resulted in significant microtubular changes, including the accumulation, and abnormal morphological appearance, of microtubules.

To further examine the effect of taxol on the neuronal response to trauma we have utilised an *in vivo* animal model of physical injury which replicates the NF alterations and microtubule loss which occurs in conditions such as AD and head injury (King *et al.*, 1997). Similar NF changes have been reported in MS (Raine and Cross, 1989; Trapp *et al.*, 1998). We have

examined the effect of taxol, and docetaxel (Taxotere®), treatment on the presence of the abnormal pathological structures, which are characteristic of these conditions, and on the maintenance of the microtubule network surrounding areas of cortical damage. The results of this study will have important implications for the use of taxol-like drugs in the treatment of disorders of the CNS.

5.1 Materials and methods

Animal procedures

All procedures involving animals were approved by the Ethics Committee (Animal Experimentation) of the University of Tasmania and are consistent with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Briefly, twenty animals received experimentally induced cortical injuries as outlined in Chapter 2. In all animals, the needle was left in place for a total of 10 minutes before being slowly removed. 1 μl of either vehicle or vehicle plus taxol (1mM, Paclitaxel, ICN) was intracortically injected at a rate of 0.2 μL/minute. 10 animals received intracortical injections of the vehicle alone (0.01 M phosphate buffered saline (PBS)), and another 10 rats received intracortical injections of a solution of 1 mM taxol in PBS (0.01 M). At both one day and four days post-surgery, five control and five test animals were perfusion fixed, as detailed in chapter 2.

Coronal sections through the injury site were cut at 50 μm with a vibratome and labelled with mouse monoclonal antibodies to both phosphorylated (SMI312) and dephosphorylated (SMI32) epitopes on the medium and high molecular weight neurofilament subunits. A mouse monoclonal antibody to Microtubule Associated Protein-2 (MAP2) was also used. Antibodies to neuronal markers were visualised with a rat-adsorbed, horse anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC).

Microscopy

For quantitative purposes, the number of NF immunoreactive abnormal neurites were counted in 5 random fields of view (using a rectangular counting frame and the 50x objective of a Leitz Dialux 22 EB fluorescence microscope) taken from around the needle tract and within two fields of view extending out from the tract. Quantitation of these structures in both one-day and four-day post-injury (PI) animals was conducted in a blind fashion by an investigator who was not involved in the preparation of tissue. Quantitation of MAP2 changes in both one and four day animals was conducted by digitally capturing five fields of

view, in the same orientation (using a rectangular counting frame and the 50x objective of a Leitz Dialux 22 EB fluorescence microscope), adjacent to the needle tract. A counting frame consisting of five equally spaced horizontal lines was placed over each image and the number of MAP2 positive fibers crossing those lines quantitated. Statistical analyses of all results (unpaired t-test) were carried out using StatView 4 for Macintosh.

Other drugs

A preliminary investigation was also undertaken with a second microtubule stabilising drug, and taxol analogue, docetaxel (12mM, Taxotere®, Rhône-Poulenc Rorer). The same protocol was utilised as outlined above. The docetaxel solution was prepared according to the manufacturer's instructions, and the solvent solution provided comprised a mixture of polysorbate 80 (0.5ml) and ethanol (191.1mg in 1.5ml water). This solvent was also utilised as the control, vehicle solution. The same method of quantitation as used above was utilised to assess the density of abnormal neurofilament-positive structures surrounding the injury tract at one and four days PI.

5.2 Results

All animals injected with either vehicle alone (PBS) or taxol plus vehicle exhibited normal behaviour following surgery.

MAP2 Labelling

In non-injured cortex, MAP2 labelling was localised to cell bodies and dendrites, including long bundles of apical dendrites throughout the cortex, which extended from deeper layers to the pial surface.

At one day PI there was a distinct region around the needle tract (up to 200μm on either side) in the vehicle-treated animals in which there was statistically significantly ($p < 0.01$) fewer MAP2 labelled processes, compared ~~to~~ with uninjured cortex. In contrast to vehicle-treated animals, taxol-treated animals demonstrated statistically significantly ($p < 0.01$) more MAP2 labelled processes in the region directly surrounding the injection tract (Figure 5.1). In this respect, there was not a statistically significant difference in the number of MAP2 positive fibers surrounding the injection site in taxol-treated animals, as compared to normal uninjured cortex ($p > 0.01$) (Figure 5.1). The MAP2 labelled structures surrounding the injection site in taxol-treated animals appeared as processes of varied length, some which were continuous with processes outside the tract area. Whereas MAP2 labelled structures in the uninjured cortex appeared primarily as long smooth projections, the MAP2 labelled processes in the taxol-treated cortex often appeared beaded and swollen along their length and did not appear typically 'bundled' (Figure 5.2).

At four days PI, there was not a statistically significant difference in the degree of MAP2 labelling between vehicle-treated and taxol-treated animals ($p > 0.01$) (Figure 5.3). The area directly surrounding the injection tract remained largely devoid of MAP2 labelling in both cases, and again, the labelling was sporadic, with processes appearing of varied length and morphology. There ~~was~~ were, however, statistically significant differences between the degree of

Figure 5.1. Graph demonstrating the density of MAP2 labelled processes surrounding the injection site in both control- and 1mM taxol-treated animals, at one day post-injury, compared to normal cortex. Statistically significant differences are noted between control-treated and taxol-treated ($P=0.0064$) and control-treated and normal ($P<0.0001$).

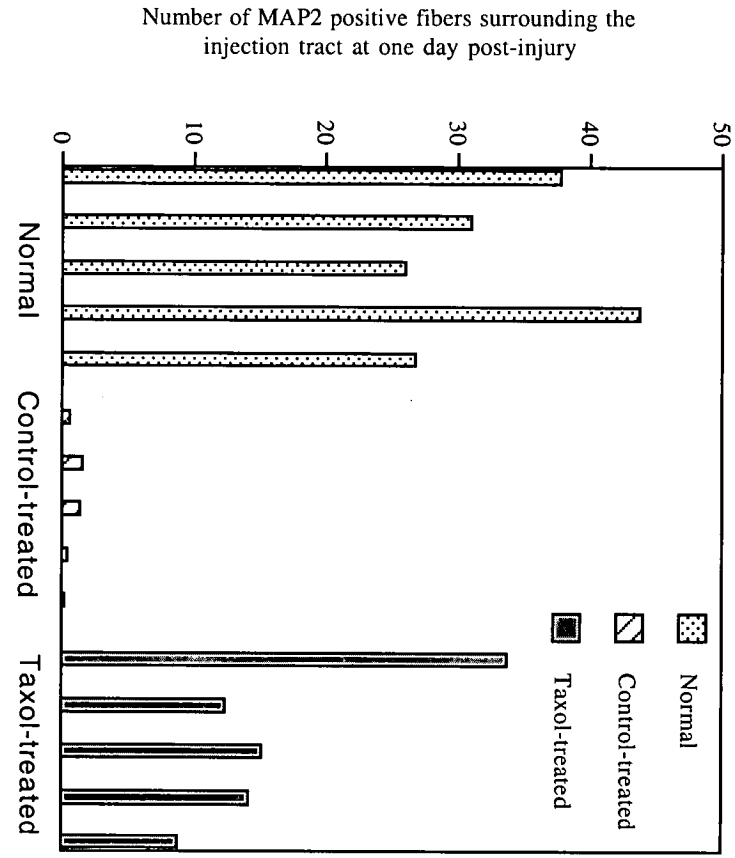

Number of MAP2 positive fibers surrounding the injection tract at one day post-injury
0
10
20
30
40
50
Normal
Control-treated
Taxol-treated
Normal
Control-treated
Taxol-treated

Figure 5.2. MAP2 labelling in the rodent brain. (A) shows labelling in the uninjured cortex, with long bundles of MAP2 positive processes (arrow). (B) is from a control-treated animal at one day post-injury (PI), and shows a complete absence of MAP2 labelling surrounding the injection tract (*). (C) and (D) both demonstrate MAP2 labelling (arrows) surrounding the injection tract (*) in taxol-treated animals at one day PI.

Scale Bar = 50 μm.

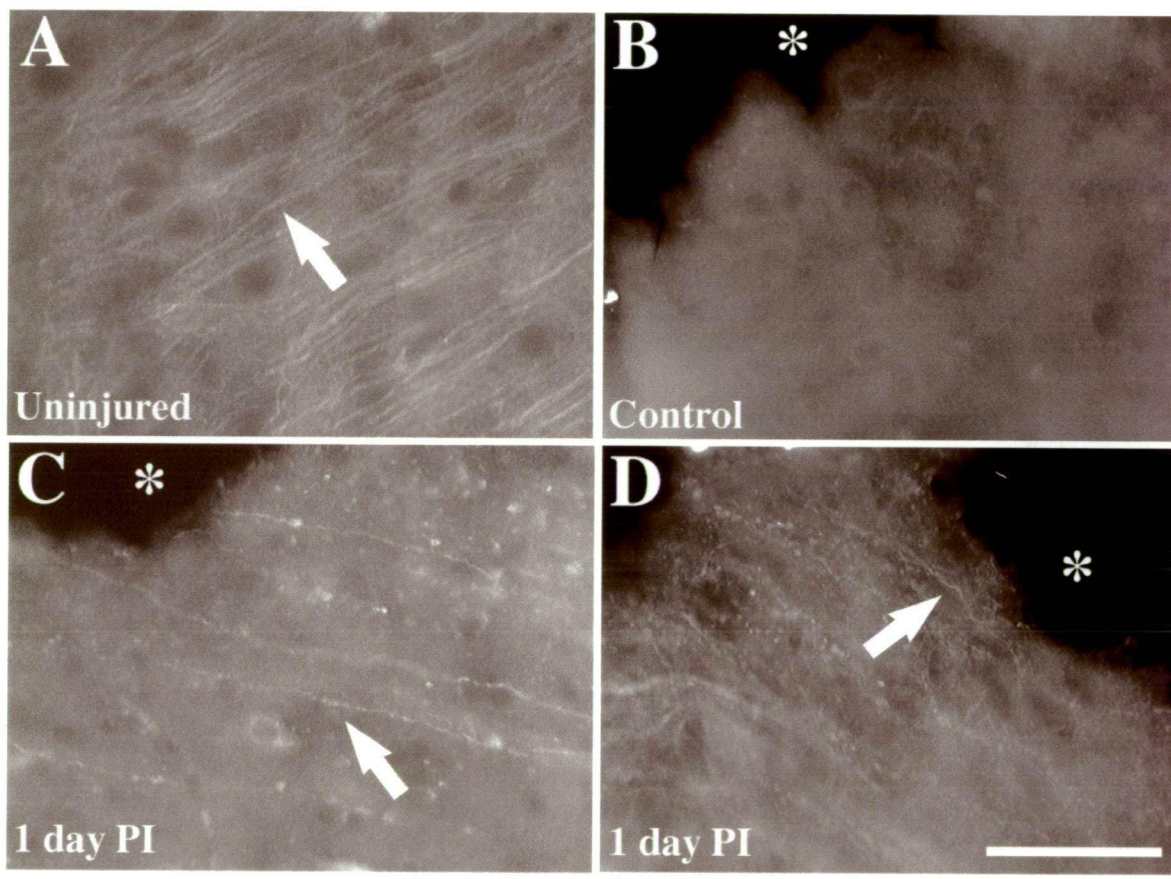
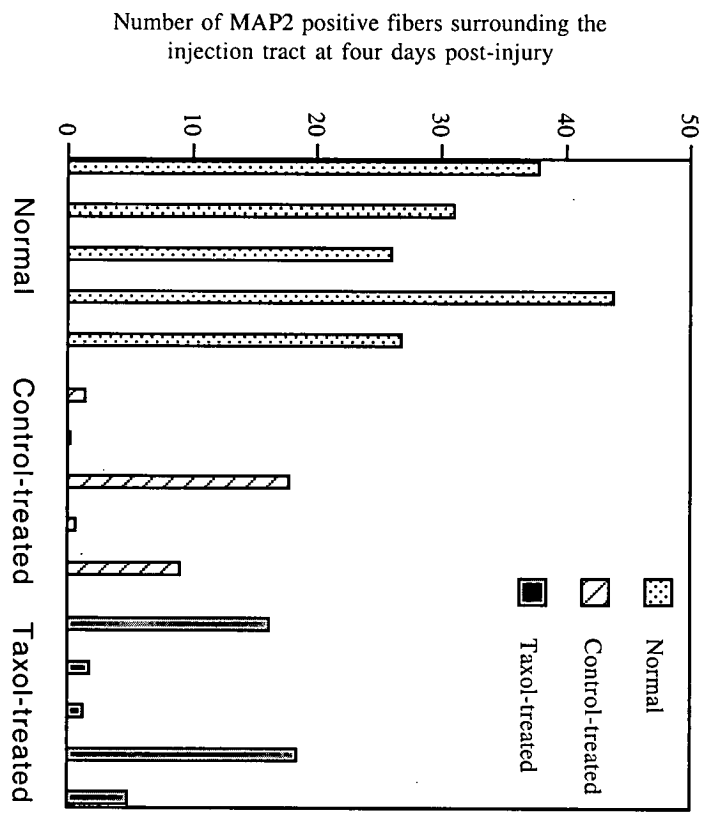

A
Uninjured
B
*
Control
C
*
1 day PI
D
*
1 day PI

Figure 5.3. Graph demonstrating the density of MAP2 labelled processes surrounding the injection site in both control- and 1mM taxol-treated animals, at four days post-injury, compared to normal cortex. Statistically significant differences are noted between both control-treated and normal (P=0.0005) and taxol-treated and normal (P=0.0012).


Number of MAP2 positive fibers surrounding the injection tract at four days post-injury
0
10
20
30
40
50
Normal
Control-treated
Taxol-treated
Normal
Control-treated
Taxol-treated

MAP2 labelling surrounding the injection site in both vehicle- and taxol-treated animals as compared to normal, uninjured cortex ($p < 0.01$) (Figure 5.3).

Neurofilament Labelling

In the contralateral cortex of both vehicle-treated and taxol-treated animals, labelling for dephosphorylated neurofilament epitopes (SMI32) was localised to cell bodies and dendrites in a subset of pyramidal-like neurons, whereas labelling for phosphorylated neurofilament epitopes (SMI312) was localised primarily to axons. Surrounding the lesion there was substantial labelling of abnormal structures (Figure 5.4). Consistent with a previous report (King *et al.*, 1997), these abnormal axons appeared primarily as ring- and bulb-like neuritic structures which were often continuous with both fine and thicker calibre fibers. These structures extended to a distance of 200 μm out from the tract edge and there was no difference in the morphology of these structures between either vehicle- or taxol-treated animals. SMI312 labelled more abnormal neuritic structures than SMI32 (Table 5.1).

At one day PI, there were fewer abnormal neurites labelled with both SMI32 (dephosphorylated) and SMI312 (phosphorylated) in the taxol-treated animals than the vehicle-treated animals. However, statistical analysis indicated that the density of SMI32 labelled bulb-like structures present in vehicle-treated animals ~~was~~ were not statistically significantly different from taxol-treated animals. There was, however, statistically significantly ($p < 0.05$) fewer SMI32 labelled ring-like structures present in the taxol-treated brain (Table 5.1). There was no statistically significant difference between the density of SMI312 labelled bulb-like or ring-like structures surrounding the injection tract in control- and taxol-treated animals (Table 5.1).

At four days PI, the density of SMI32 labelled bulb-like structures present in the taxol-treated animals was significantly increased over vehicle-treated animals ($p < 0.05$), whereas the density of SMI32 labelled ring-like structures was not significantly different. Relative to

Figure 5.4. Labelling for phosphorylated neurofilaments (NFs) in the injured rodent cortex at one day post-injury. NF-labelled structures appear primarily as 'bulb-like' (arrowhead) and 'ring-like' (arrow) accumulations surrounding the injection tract.

Scale Bar = 100μm.

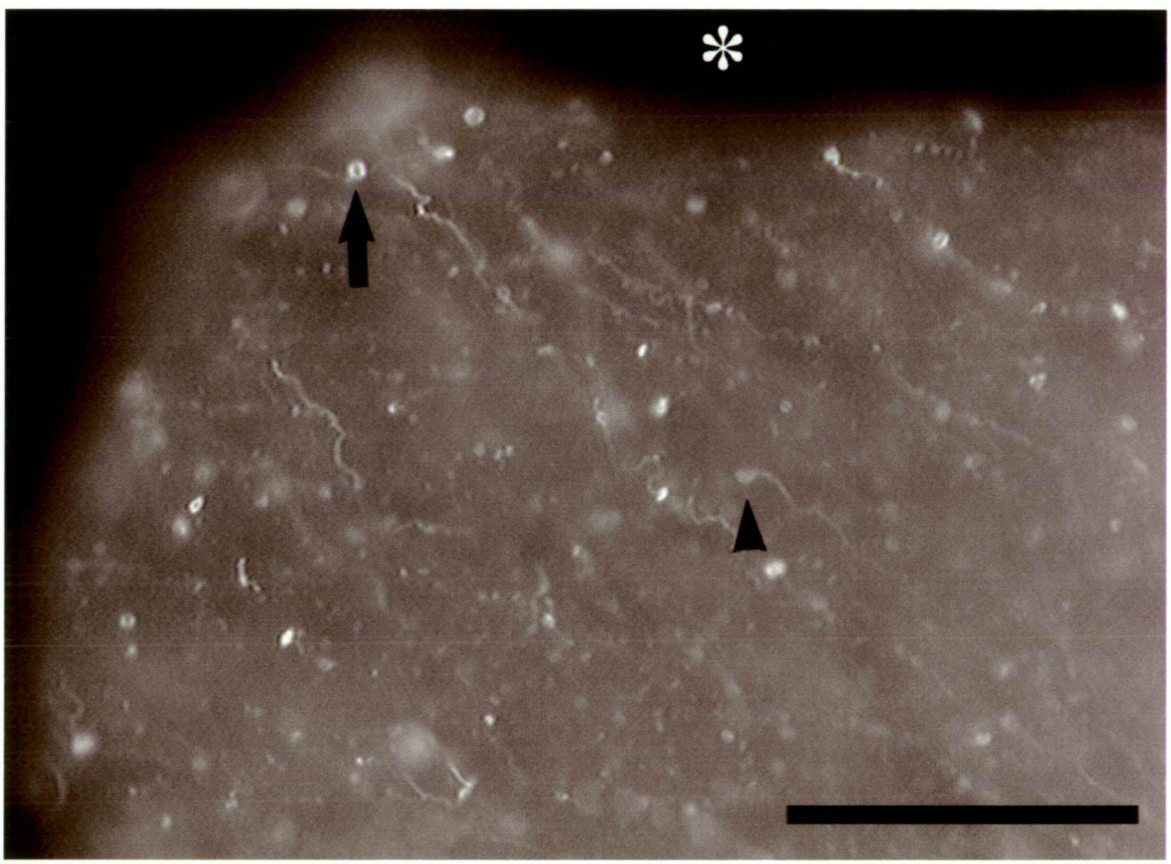


Table 5.1. Mean density (± standard deviation) of pathological structures present at both one and four days post-injury in the cortical grey matter of 1mM taxol- and vehicle-treated animals. Significant ($p<0.05$) differences were noted in the number of SMI32 labelled ring-like structures at one day (*) and the number of SMI32 and SMI312 labelled bulb-like structures present at both one ($^{\#}$) and four (†) days respectively.

Group	Antibody	Number of pathological structures (mm^2) around the injection tract			
		Ring-like		Bulb-like	
		1 day	4 days	1 day	4 day
Control	SMI32	294 ± 44*	59 ± 29	265 ± 44	157 ± 20#
Taxol	SMI32	88 ± 54*	98 ± 15	226 ± 74	363 ± 34#
Control	SMI312	1461 ± 142	559 ± 98	1049 ± 93	314 ± 59†
Taxol	SMI312	1373 ± 289	608 ± 123	794 ± 123	657 ± 108†

animals examined at one day PI, the density of SMI32 labelled structures present in the vehicle-treated group at four days PI had significantly decreased ($p < 0.05$), while the density of structures present in the taxol-treated animals demonstrated a slight increase (Table 5.1).

Similarly, the density of SMI312 labelled bulb-like structures present at four days PI in the taxol-treated animals was significantly increased ($p < 0.05$) over the vehicle-treated animals. The density of SMI312 labelled ring-like structures in the taxol-treated animals, however, was not statistically different to vehicle-treated animals. Relative to one-day animals, the density of bulb- and ring-like structures labelled with SMI312 in both vehicle- and taxol-treated animals was decreased (Table 5.1).

Docetaxel -treated animals

There was little change in the density of abnormal structures present in the docetaxel-treated animals as compared to vehicle-treated animals (Table 5.2). There ~~was~~ were, however, statistically significantly ($p < 0.05$) fewer SMI312 labelled ring-like structures present in docetaxel-treated animals at one day PI relative to controls.

Table 5.2. Mean density (± standard deviation) of pathological structures present at both one and four days post-injury in the cortical grey matter of 12mM docetaxel- and vehicle-treated animals. Significant ($p<0.05$) differences were noted in the number of SMI312 labelled ring-like structures present at one day (*) post-injury.

Group	Antibody	Number of pathological structures (mm^2) around the injection tract			
		Ring-like		Bulb-like	
		1 day	4 days	1 day	4 day
Control	SMI32	382 ± 72	88 ± 39	402 ± 109	392 ± 82
Docetaxel	SMI32	441 ± 245	186 ± 61	588 ± 98	470 ± 87
Control	SMI312	1578 ± 307	578 ± 156	1196 ± 283	764 ± 266
Docetaxel	SMI312	941 ± 231*	745 ± 194	833 ± 291	843 ± 126

5.3 Discussion

This study has demonstrated that the administration of taxol into the damaged CNS results in a significant change to the acute cellular alterations which normally follow physical injury to the cortex.

In this investigation brains were examined at both one and four days PI. At one day PI, there is a peak in neuronal pathology, whilst at four days a period of rapid reactive change has begun within the lesion site. At one day PI, quantitation of the density of pathological structures present in taxol-treated animals, relative to vehicle-treated animals, demonstrated a decrease in the number of bulb- and ring-like structures labelled with antibodies to both dephosphorylated and phosphorylated NF epitopes. Taxol treatment has, therefore, resulted in a decrease in abnormal neuritic structures surrounding the injection site, and this was statistically significant in some cases, as shown in Table 1. The MAP2 labelling surrounding the injection site in taxol-treated animals was also statistically significantly different from controls, as shown in Figure 1. The preservation of MAP2 labelling in apical dendrites throughout the injured cortex in taxol-treated animals is indicative of a stabilisation of microtubules.

At four days PI, all taxol-treated animals showed an increase in density of NF-positive structures above control values, with the density of bulb-like structures labelled for dephosphorylated and phosphorylated NF epitopes demonstrating a statistically significantly increase above controls. This apparent increase in neuropathology may be due to the toxicity of the compound. Taxol is a recognised cytotoxic agent, with demonstrated activity against a number of different cell lines, including neuronal cells (Bird, 1984; Huizing *et al.*, 1995). After four days *in vivo*, then, the compound may be exerting a toxic effect on the cell population surrounding the tract. This may result in a decrease in cell survival and subsequent increase in the density of neuritic structures. However, while taxol may give rise to 'new' pathology, it is more likely that it has affected the neurons damaged upon the initial lesion in

such a way as to prolong their presence within the brain. Microtubules are normally in a state of rapid turnover within the neuron (Hirokawa, 1991). The action of taxol, to alter the equilibrium in favour of microtubule production (Gotaskie and Andreassi, 1994; Huizing *et al.*, 1995), therefore disrupts the normal cytoskeletal dynamics within the neuron (Bird, 1984), and, experimentally, has been shown to result in a number of outcomes. In cell culture models, taxol treatment leads to the arrest of growth of existing neurites and inhibits further neurite outgrowth (LeTourneau and Ressler, 1984; George *et al.*, 1988). It has also been demonstrated, in both *in vitro* and *in vivo* studies, that taxol treatment can prolong neuronal pathology. In a dissociated cell culture model, taxol treatment has been shown to stabilise transected axons, resulting from the dissociation process, against collapse, leading to an abnormal persistence of axonal stumps once plated (LeTourneau and Ressler, 1984). These axonal stumps became swollen from microtubule accumulation and did not allow any neurite outgrowth. A similar phenomenon has been demonstrated in the PNS following sciatic nerve crush (Röyttä *et al.*, 1984; Röyttä and Raine, 1985; Röyttä and Raine, 1986; Vuorinen *et al.*, 1988; Vuorinen *et al.*, 1989; Vuorinen and Röyttä, 1990). An injection of taxol into the crush site significantly increased the number of axoplasmic microtubules, leading to prolonged neuronal pathology characterised by swollen axonal stumps or 'bulbs'. However, in contrast to the *in vitro* studies, this prolonged pathology did give rise to a secondary wave of regenerative growth from the bulbs. In the current study, the injection of taxol into the lesion site has most likely resulted in both the enhanced polymerisation and stabilisation of microtubules in the neurons surrounding the injury tract. This has then prevented the normal resolution of the neuropathology resulting from the initial cortical lesion, thereby prolonging the presence of neuritic structures within the brain to give a comparably higher density surrounding the injury site in taxol-treated animals at four days PI.

That the MAP2 labelling was comparable between vehicle- and taxol-treated animals at four days PI also suggests that the action of taxol was acute, and has indeed prevented the resolution of the neuritic structures resulting from the initial cortical lesion. Similarly, the administration of docetaxel resulted in an acute response at one day PI, with a decrease in the number of ring-like structures labelled with antibodies to phosphorylated NFs. This effect, however, was not long lasting. In contrast to taxol-treated animals, however, there was not a significant increase in the density of abnormal structures present at four days PI. This may be due to reduced toxicity of docetaxel, or alternatively, a result of decreased drug effectiveness and hence an increased resolution of neuritic structures as compared to taxol-treated animals.

We have, therefore, demonstrated that taxol has a profound effect on the CNS. Whilst it does not alter normal neuronal morphology within the brain, it can significantly decrease the immediate evolution of neuronal pathology occurring as a result of physical injury to the cortex. This has relevance to the neuronal pathology of head injury and MS, and also particularly to Alzheimer's disease, where physical injury is not only a key risk factor but perhaps, at a microscopic level, also the underlying cause of the disease (Vickers *et al.*, 2000). Any intervention in the cascade of cytoskeletal changes leading to neuronal dysfunction may offer protection against the progression of the disease.

Increased density of metallothionein I/II immunopositive cortical glial cells in the early stages of Alzheimer's disease

6.0 Introduction

Metallothioneins (MT) are cysteine rich (23-33 mol%) proteins of low molecular weight (6-7 kDa). The MT family now consists of at least four different isoforms which are found throughout a variety of tissues (Nakajima and Suzuki, 1995; Zheng *et al.*, 1995; Erickson *et al.*, 1997). Isoforms I and II are found in most tissues, isoform III in the brain and isoform IV in stratified squamous epithelia (Erickson *et al.*, 1997). MTs have been shown to be induced, or regulated, by a number of different factors including bacterial endotoxins, oxidative stress, heavy metals and various cytokines and polypeptide hormones (Anezaki *et al.*, 1995; Dalton *et al.*, 1995; Ebadi *et al.*, 1995; Zheng *et al.*, 1995). Within the body, MTs may have a role in sequestering heavy metals such as Zn(II) and Cu(I) (7-12 ions/MT), regulating the availability of metals to various enzymes and transcription factors. In this fashion, MTs may influence a host of biological processes such as DNA transcription and protein synthesis. MTs could also contribute towards the detoxification of heavy metals and act as an intracellular antioxidant.

Alzheimer's disease (AD) is a progressive neurodegenerative disease and is the major cause of dementia in people over 65 years of age. With respect to AD, much emphasis has been placed on the potential role of MT III in the progression of the disease. MT III possesses a unique growth inhibitory function which has been shown to suppress neuronal growth (Palmiter *et al.*, 1992; Masters *et al.*, 1994; Vallee, 1995). MT III, reported to be down regulated in AD (Uchida *et al.*, 1991; Tsuji *et al.*, 1992; Uchida and Ihara, 1995), may, therefore, potentiate the aberrant neuronal sprouting associated with this disease (Vickers, 1997). Of interest in this study, however, was the possible role that MT I/II may play in AD. These isoforms, which are expressed in similar concentrations and similar locations within the brain as MT III (Nakajima and

Suzuki, 1995; Aschner *et al.*, 1997), have not been extensively investigated in relation to AD. Existing reports show that MT I/II mRNA is upregulated in the AD brain (Duguid *et al.*, 1989; Nakajima and Suzuki, 1995). This increase was correlated with an increase in glial fibrillary acidic protein (GFAP) mRNA, and so it was postulated that elevated MT I/II expression may follow glial cell proliferation and, thus, may not have a direct role in AD pathology (Nakajima and Suzuki, 1995). We have re-examined the possible role of MT I/II in AD, with a specific focus on the cellular localisation of these MT isoforms relative to that of other glial markers such as GFAP and ferritin. In addition to AD cases, we have specifically investigated MT I/II immunoreactivity in the brains of individuals who may be in a 'preclinical' stage of AD, possessing cortical ß-amyloid plaques but not the widespread neuronal pathology that is associated with dementia (Ulrich, 1985; Crystal *et al.*, 1988; Benzing *et al.*, 1993; Coria *et al.*, 1993; Morris *et al.*, 1996; Vickers *et al.*, 1996; Vickers, 1997). Such preclinical cases typically demonstrate a mild cognitive impairment that predicts subsequent progression to dementia (Morris *et al.*, 1996). Investigation of these individuals may, therefore, give an insight into changes in MT I/II expression associated with the early stages of the disease process.

6.1 Materials and methods

Tissue source and processing

Forty μM sections of the superior frontal gyrus of each of 5 AD cases (case codes; mean age ± standard error: AD-1, AD-2, 91-10, 91-11, 91-16; 72 ± 3.4 years), 5 preclinical-AD cases (PM-6060, N-17, 15-17, 15-35, 15-90; 68.4 ± 3.1 years) and 5 cases without any AD pathological changes (N-14, 15-49, 15-69, 15-87, 15-98; 65.8 ± 5.5 years). Full case details are outlined in chapter 2. Prior to use, human material was pretreated with formic acid and autofluorescence quenched (chapter 2).

Standard immunohistochemical techniques for both single and double labelling were utilised for visualisation of antibody immunoreactivity (chapter 2). The immunohistochemical markers used in this study included rabbit polyclonal antibodies to GFAP, a major protein of intermediate filaments in astrocytes, S-100α+ß, a protein common to reactive protoplasmic and fibrous astrocytes (Kimura and Budka, 1986), and a marker for microglia, ferritin, an intracellular iron-storage protein in most eukaryotic cells and a marker for reactive astrocytes (Bignami *et al.*, 1972; Kaneko *et al.*, 1989) and pan ß-amyloid, a marker for ß-amyloid plaques. A Mouse monoclonal antibody to MT, specific for MT isoforms I and II (Jasani and Elmes, 1991) was also utilised. Thioflavine-S staining was used to visualise AD pathology, and this was combined with immunofluorescent techniques (Vickers *et al.*, 1992).

In all immunofluorescence experiments, antibodies were visualised with a horse anti-mouse IgG conjugated to fluorescein isothiocyanate and a goat anti-rabbit IgG conjugated to biotin followed by avidin Texas Red.

Microscopy

For quantitative purposes, the number of MT I/II immunopositive cells were counted in 10 random fields of view taken from layer II of the cortical grey matter (using a rectangular counting frame and the 50x objective of a Leitz Dialux 22 EB fluorescence

microscope) of all cases, and a similar number of fields of view from the underlying white matter. An identical approach was taken for the determination of GFAP labelled cell density in these cases. In addition, we also determined the extent of colocalisation of these markers from 5 random fields of view from layer II of each case using the 50X objective. Statistical analyses of results (analysis of variance and regression analysis) were carried out using StatView 4 for Macintosh.

6.2 Results

MT I/II and GFAP labelling in non-AD cases

MT I/II immunolabelling was present in glia-like cell bodies and their processes. In the five cases lacking any AD-like pathology in the neocortex, MT I/II labelling was sparse throughout layers II-VI of the cortical grey matter and uniformly distributed throughout the white matter. Layer I of the grey matter was devoid of MT I/II labelling.

Double immunofluorescence labelling showed that reactivity for MT I/II and GFAP was colocalised in approximately half of the cells labelled for each marker (Table 6.1). GFAP labelling, but not MT I/II immunoreactivity, was also present in long varicosed processes extending from layer 1 into deeper layers of the grey matter. Quantitation of the density of GFAP and MT I/II labelled cells in these cases is presented in Table 6.2. There was no significant difference ($p > 0.01$) between the density of MT I/II and GFAP labelled cells in the grey or white matter of non-AD cases.

MT I/II and GFAP labelling in preclinical AD cases

The preclinical AD cases showed similar patterns of immunostaining as the normal cases. MT I/II immunopositive cell bodies and processes were present in layers II-VI of the grey matter and the white matter was uniformly labelled. These cases, however, demonstrated a significant increase ($p < 0.01$) in MT I/II labelled cells in the grey matter, as compared to non-AD cases, which was not accompanied by a similar increase in GFAP immunoreactive cells (Table 6.2). Colocalisation of MT and GFAP immunoreactivity in cells did occur, but double labelling verified that a greater proportion of MT I/II positive cells (63%) lacked GFAP immunolabelling (Figure 6.1, Table 6.2). GFAP labelling in general was similar to that observed in the non-AD cases. There was also extensive labelling of capillaries with both MT I/II and GFAP antibodies, representing the feet of the astrocytes wrapping around the vessels. There

Table 6.1. Colocalisation of metallothionein-I/II (MT-I/II) and glial fibrillary acidic protein (GFAP) immunoreactivity in cells in layer II of the superior frontal gyrus of non-Alzheimer's disease (AD), preclinical-AD and AD cases (n=900 cells). Values shown are the percentage, plus or minus the standard deviation.

Case Type	MT I/II only	MT I/II and GFAP	GFAP only
Non-AD	34 ± 20	28 ± 12	37 ± 25
Preclinical AD	63 ± 7	35 ± 9	2 ± 3
AD	11 ± 13	63 ± 15	26 ± 8

Table 6.2. Mean density (± standard deviation) of metallothionein-I/II (MT-I/II) and glial fibrillary acidic protein (GFAP) immunopositive cells in the superior frontal gyrus of non-Alzheimer's disease (AD), preclinical-AD and AD cases (n=3267 cells).

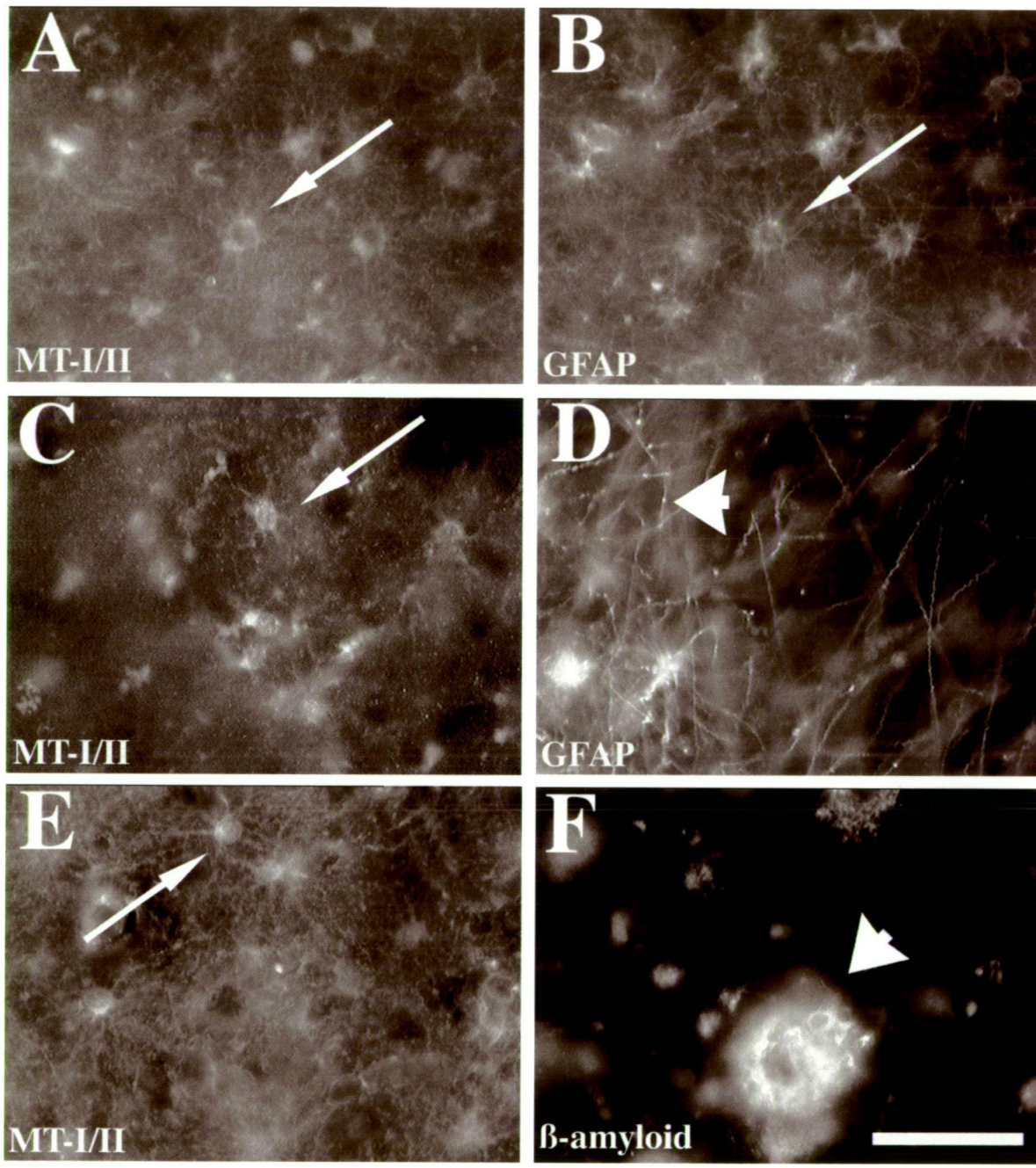
Case	Number of Cells (mm^2) in Grey matter		Number of Cells (mm^2) in White matter	
	MT I/II	**GFAP**	**MT I/II**	**GFAP**
Non-AD	69 ± 39	90 ± 60	270 ± 45	240 ± 445
Preclinical AD	209 ± 27*	109 ± 69*	284 ± 40	233 ± 62
AD	439 ± 78**	595 ± 125**	357 ± 89**	325 ± 33**

* Statistical difference ($p < 0.01$) within the case category.

** Statistical difference ($p < 0.01$) with other case categories.

Figure 6.1. Double immunolabelling of the grey matter of the superior frontal gyrus, with metallothionein I/II (MT I/II) (A, C, E), glial fibrillary acidic protein (GFAP) (B, D) and ß-amyloid (F). (A) and (B) demonstrate the colocalisation observed between MT I/II and GFAP labelling in an Alzheimer's disease (AD) case, with astrocytes (arrow) immunoreactive for both markers. (C) and (D) show a preclinical-AD case where astrocytes are labelled for MT I/II (arrow) but not GFAP. (D) also shows the fine long varicosed fibres (arrowhead) observed in preclinical-AD and non-AD cases which were labelled for GFAP but not MT I/II. (E) and (F) are taken from an AD case and show MT I/II labelled astrocytes (eg. arrow) (E) which are not associated with the plaque (arrowhead) (F).

Scale bar for (A) to (F) = 50 μm


A
MT-I/II
B
GFAP
C
MT-I/II
D
GFAP
E
MT-I/II
F
ß-amyloid

was no significant difference between the density of MT I/II and GFAP labelled cells in the white matter of these cases.

MT I/II and GFAP labelling in AD

AD cases showed extensive labelling for both MT I/II and GFAP throughout layers II-VI of the grey matter and the white matter. The majority of labelled cells were immunoreactive for both MT I/II and GFAP (Figure 6.1, Table 6.2), with a statistically significant increase ($p < 0.01$) in cell numbers above both preclinical AD and non-AD cases (Table 6.2). Interestingly, GFAP labelling in AD cases was present in cell bodies and their immediate processes, with no labelling of the long varicosed processes observed in both non-AD and preclinical AD cases. There was also a statistically significant ($p < 0.01$) difference between the density of GFAP and MT I/II labelled cells in the grey matter, but no significant difference for these markers in the white matter.

Within each case category, there was no significant correlation between the density of MT I/II labelled cells relative to the post mortem interval.

MT I/II localisation and association with AD pathology

In each case category, all MT I/II labelled cells were also immunoreactive for S-100α+ß, a marker for reactive fibrous and protoplasmic glia (Kimura and Budka, 1986). An antibody to ferritin was used as a marker for microglia (Bignami *et al.*, 1972; Kaneko *et al.*, 1989). Double labelling immunohistochemistry revealed that MT I/II immunoreactive cells were not labelled for ferritin in all cases. Ferritin immunoreactive microglia mainly occurred in clusters of >3 cells, and upon double-labelling with ß-amyloid, these clusters were shown to be localised to plaques.

In contrast, double labelling showed that MT I/II immunoreactive cells were not particularly associated with ß-amyloid plaques in either preclinical-AD or AD cases

(Figure 6.1). In addition, thioflavine-S staining with MT I/II antibody labelling demonstrated that MT I/II positive cells were not spatially localised to any other aspect of AD pathology.

6.3 Discussion

Demonstration of an increased density of MT I/II labelled cells in the AD brain in the present study is consistent with reports of elevated MT I/II gene expression in this condition (Duguid *et al.*, 1989; Nakajima and Suzuki, 1995). More surprisingly, our studies have shown significantly elevated densities of MT I/II labelled cells in the cortical grey matter of cases containing ß–amyloid plaques but not the widespread neurofibrillary pathology linked to dementia in AD. Furthermore, the increased density of glial cells showing MT I/II immunoreactivity in these cases was not associated with a concomitant increase in cell density based on GFAP labelling. In contrast to these presumable preclinical cases (Morris *et al.*, 1996), GFAP and MT immunolabelling was generally colocalised to the same cell bodies in AD material. GFAP immunoreactivity, detected primarily in fibrous astrocytes (Eng *et al.*, 1971; Bignami *et al.*, 1972; Graeber and Kreutzberg, 1986) but also in protoplasmic astrocytes under certain brain fixation protocols (Shebab *et al.*, 1990), is considered to be a marker for reactive gliosis- the proliferation of astroglial cells which represents the fundamental reaction of the central nervous system to tissue damage (Manuelidis *et al.*, 1987). GFAP immunoreactivity has been utilised as a marker for gliosis in a number of neurological disorders, including progressive dysphasic dementia (Kobayashi *et al.*, 1990); the latter stages of Creutzfeldt-Jakob disease (Manuelidis *et al.*, 1987); amyotrophic lateral sclerosis (Kushner *et al.*, 1991); scrapie (Tatzelt *et al.*, 1996) and AD (Beach *et al.*, 1989; Delacourte and Buee, 1989; Delacourte, 1990; Jorgensen *et al.*, 1990). However, other researchers have emphasised that gliosis is correlated with an increase in the size of astrocytes in the white matter, and not the density of GFAP immunoreactive cells (da-Cunha *et al.*, 1993). In addition, Pekny *et al.* (1995) have demonstrated that GFAP gene knockout mice display post-traumatic reactive gliosis, suggesting that GFAP expression is not obligatory for gliosis to occur. Therefore, whilst there is no apparent concomitant increase in glial cell density in these preclinical AD cases based upon GFAP immunolabelling, there remains the possibility that the increase in MT I/II cell density is due to a proliferation of glial cells

not labelled for GFAP. This possibility is supported by the labelling of all MT I/II cells with an antibody to S-100α+ß, a marker for reactive protoplasmic glia that are otherwise unlabelled for GFAP (Kimura and Budka, 1986). This data, therefore, does suggest a specific increase in MT I/II levels in the early stages of the disease process.

MT I/II labelled cells in both the AD and preclinical AD cases did not show a spatial relationship with ß-amyloid plaques. In contrast, microglial cells, labelled for ferritin and not immunoreactive for MT I/II, were closely associated with plaques. This indicates that increased levels of MT I/II may be more closely linked to a general aspect of AD pathology and is not a specific response localised to hallmark pathological features such as ß-amyloid plaques or neurofibrillary pathology.

MT proteins appear to play important roles in heavy metal binding and detoxification as well as in preventing free radical-mediated cellular damage, both of which have been implicated in playing a role in the pathology of AD (Bremmer, 1993; Cherian and Chan, 1993; Sato *et al.*, 1993; Vallee and Maret, 1993; Maret, 1995; Aschner *et al.*, 1997). Oxidative damage can occur either by a deficiency in normal cell antioxidant defences or an increase in the production of free radicals. Whilst alterations to antioxidant defence systems, such as glutathione, increase vulnerability to oxidative stress (Cuajungco and Lees, 1997b), there is little evidence that such alterations play any role in the pathogenesis of AD. Free radicals, however, are postulated to be increased in the AD brain, as evidenced by the high levels of antioxidant enzymes, such as superoxide dismutase, found in association with NFT and senile plaques in the AD brain (Pappolla *et al.*, 1992). Similarly, Smith and colleagues (Smith *et al.*, 1996, 1997) have demonstrated an accumulation of redox-available iron, a catalyst for oxyradical generation, in association with plaques, NFT and neuropil threads. These researchers have also demonstrated an increase in carbonyls in the neuronal cytoplasm and nuclei of neurons in AD, which is

characteristic of an increase in oxidative stress, and at least partly due to an increase in peroxynitrite- a source of hydroxyl-radical-like reactivity (Smith *et al.*, 1997). This increase in oxidative stress in the AD brain may result in the release of soluble mediators, such as glucocorticoids and cytokines (Cherian and Chan, 1993), which subsequently induce the synthesis of MT (Sato *et al.*, 1993). MT, which contains sulfhydryl groups similar to glutathione, can scavenge both free hydroxyl and superoxide radicals (Min *et al.*, 1993; Sato *et al.*, 1993; Aschner *et al.*, 1997) and also react with electrophiles to prevent cellular oxidative damage (Maret, 1995). Although the importance of MT relative to other antioxidant systems, such as superoxide dismutase and glutathione, remains uncertain (Cherian and Chan, 1993), the increased density of MT I/II cells in the early and latter stages of AD may represent a response of the surrounding tissue to increased levels of potentially damaging free radical species.

Conversely, the increased density of MT I/II labelled glial cells may be due to elevated levels of particular metal species that contribute to AD pathology. Examination of trace elements in the AD brain has demonstrated whole brain increases in a number of elements, but only a minority are significantly increased above non-AD controls (Wenstrup *et al.*, 1990; Markesberry and Ehmann, 1994). Many of the elements elevated in AD, such as mercury, cadmium, zinc and copper, can be bound by MT (Hamer, 1986; Kagi and Schaffer, 1988; Vallee, 1995), and in doing so MT can function to maintain a level of metal ion homeostasis (Cuajungco and Lees, 1997a, 1997b). This regulation can prevent potentially toxic alterations in metal levels, which can, for example, prevent the misassembly of microtubules within the cytoskeleton (Wenstrupp *et al.*, 1990) in the case of excess mercury.

One of the more widely studied metals in relation to both AD and MT has been zinc. Zinc has structural, catalytic and regulatory roles in cell biology and is crucial to >200 proteins/ enzymes (Cuajungco and Lees, 1997a, 1997b). Whilst the possibility of

increased brain concentration of zinc during pathological conditions such as AD remains controversial, there are numerous reports on the potential interactions of zinc in the pathogenesis of AD. It has been demonstrated that the amyloid precursor protein (APP), from which the ß-amyloid comprising plaques in AD is derived, contains a zinc binding site which may modulate the function of the protein (Bush *et al.*, 1993; Multhaup *et al.*, 1994). The binding of zinc at this site was shown to increase the proteins affinity for heparin (Multhaup *et al.*, 1994), which in turn could promote the binding of the protein to extracellular matrix molecules such as heparin-sulfated proteoglycans (Bush *et al.*, 1993, 1994b) and type I collagen (Aschner *et al.*, 1997), possibly affecting the processing of APP. It has also been suggested that zinc may stabilise APP and inhibit its degradation (Bush *et al.*, 1994a, 1994b; Li *et al.*, 1995; Cuajungco and Lees, 1997a).

Finally, recent *in vitro* studies have indicated that zinc may contribute to the aggregation of ß-amyloid and prevent its alpha-secretase cleavage (Bush *et al.*, 1994a, 1994b). Whilst there is some controversy regarding the concentration of zinc required to induce the aggregation of soluble ß-amyloid protein into plaques, Bush and coworkers (Bush *et al.*, 1994a, 1994b) have demonstrated that it occurs at levels of zinc below that found under physiological conditions. The appropriate maintenance of zinc homeostasis may, therefore, prevent increased levels of APP and insoluble ß-amyloid.

MTs possess a unique structure consisting of two clusters which cumulatively bind 7 atoms of zinc/ apothionein (metal-free form of metallothionein) (Hamer, 1986; Vallee, 1995), and as such, represents the major 'store' of zinc within the cell (Karin, 1985; Cherian and Chan, 1993). MT is, therefore, crucial in the maintenance of zinc homeostasis, with increases in zinc resulting in increased transcription of MT genes (Karin, 1985; Hamer, 1986) and, conversely, a decrease in intracellular zinc resulting

in increased MT degradation (Karin, 1985). MT may, therefore, serve a protective role, preventing interactions between zinc and APP/ ß-amyloid.

Another possibility, however, is that the increased levels of MT I/II may ~~infact~~ be harmful. AD plaques, comprised of ß-amyloid, may induce the formation of hydrogen peroxide resulting in an increase in oxidative stress (Maret, 1995). This oxidative stress can potentially increase both MT and glutathione disulfide (part of the glutathione antioxidant defence system), altering the glutathione redox balance (Maret, 1995). The glutathione disulfide can interact with the thiolate bonds in the MT structure causing the release of metal ions from MT for various zinc-dependent processes (Maret, 1995; Vallee, 1995). Therefore, while MT is a 'store' for zinc, possibly preventing some zinc-induced aggregation of ß-amyloid, it may also release zinc under the biological conditions present in the AD brain, perhaps potentiating ß-amyloid misprocessing and plaque formation.

We have demonstrated, therefore, that MT I/II expression appears to be upregulated in response to a specific stimulus in the early stages of AD. This raises the question of what function MT is serving in this phase of the disease, and whether elevated levels of MT may prevent or potentiate the AD disease process. The potential role of this protein is further examined in the *in vivo* model of the early neuronal pathology of AD in the following chapter.

Induction of metallothionein I/II in an *in vivo* time course of cortical injury

7.0 Introduction

Whilst the metallothionein (MT) isoform most studied within the central nervous system (CNS) has been MT III (Vallee, 1995; Aschner *et al.*, 1997; Palmiter, 1998), in the previous chapter it was demonstrated that MT I/II may have an important role in the CNS of AD sufferers, as it is specifically upregulated in the preclinical stage of the disease process (Adlard *et al.*, 1998). This phase of the disease has been experimentally replicated in an animal model of physical injury to neurons (King *et al.*, 1997), where we have demonstrated that the neuropathological sequelae of localised cortical injury mimics the early neuronal pathology associated with preclinical AD. To further our understanding of the functional role of this protein in this stage of the disease we have, therefore, examined the distribution and expression of MT I/II over a time course in this model.

7.1 Materials and methods

Animal procedures

Methods utilised throughout this chapter are detailed in chapter 2. All procedures involving animals were approved by the Ethics Committee (Animal Experimentation) of the University of Tasmania and are consistent with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Briefly, twenty nine animals received experimentally induced cortical injuries, and at one day post-injury (PI), four days PI, seven days PI and fourteen days PI, five animals at each timepoint were reanaesthetised and transcardially perfusion fixed, as outlined in Chapter 2. Coronal sections through the injury site were cut at 50 μm with a vibratome. Standard immunohistochemical techniques for double labelling were utilised for visualisation of antibody immunoreactivity (chapter 2). In all fluorescence-double-labelling investigations, a monoclonal antibody which recognises metallothionein I/II, was alternately utilised in combination with rabbit polyclonal antibodies against glial fibrillary acidic protein (GFAP) or ferritin. The antibody to MT I/II was visualised with a rat-adsorbed, horse anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) and other markers visualised with a goat anti-rabbit IgG conjugated to biotin followed by avidin Texas Red.

At one, seven and fourteen days PI, three animals were taken at each time point, reanaesthetised and transcardially perfused with saline alone. An uninjured control brain was also utilised. Brains were immediately blocked down to a 7mm square around the injection site and stored at -80°C. These animals were utilised for Western blot analyses, as detailed in chapter 2.

7.2 Results

Immunohistochemistry

MT I/II and ferritin labelling was not evident in the normal uninjured neocortex. GFAP immunoreactivity was both extensive and uniform throughout normal cortex, labelling glial cell bodies and their processes, as well as the entire pial surface.

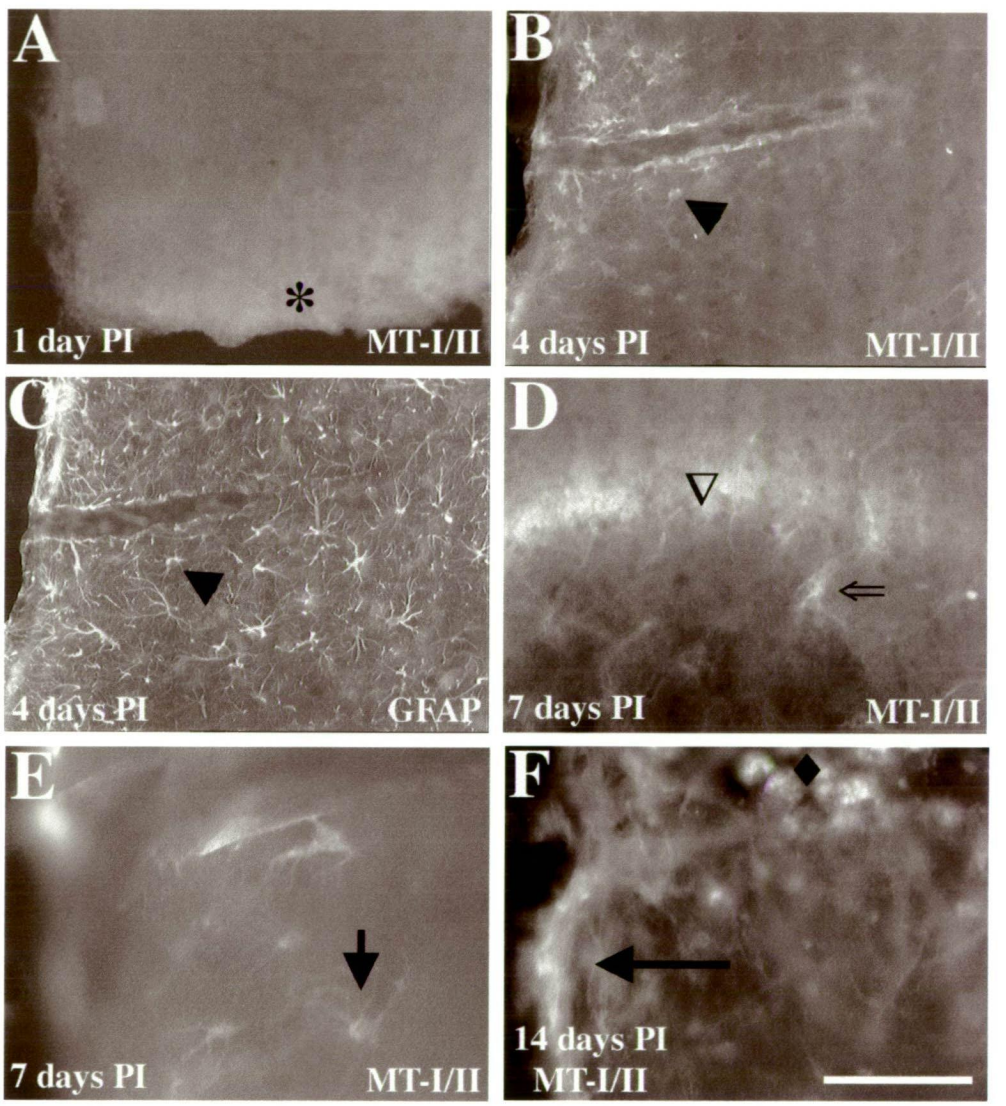
At day one PI, both ferritin (results not shown) and MT I/II (Figure 7.1A) labelling was absent. GFAP immunoreactivity remained extensive and uniform, as in the uninjured brains.

At four days PI, ferritin labelling was sparse. The MT I/II antibody, however, labelled glia-like cell bodies and processes adjacent to the injury site and surrounding blood vessels (Figure 7.1B). The pial surface, at up to 100μm on either side of the tract, was also immunoreactive for MT I/II, with labelling confined to fibrous-like processes. All MT I/II labelled structures at this, and later, time points, were immunoreactive for GFAP, but not all GFAP positive cells were labelled for MT I/II (Figure 7.1B and 7.1C). GFAP immunoreactivity was more extensive throughout the cortex than in earlier time points, and many GFAP positive structures had the morphology of reactive astrocytes.

The density of ferritin-immunoreactive cells qualitatively peaked at seven days PI and was present in cells in a bridge of tissue between either side of the injection tract, and remained distinct from the margins of the lesion, which was demarcated by an intense band of MT I/II immunoreactivity (Figure 7.1D). MT I/II labelling was primarily in fibrous-like processes, which were often thickened and aligned towards the tract. Cell bodies were also

Figure 7.1. MT I/II (A, B, D, E, F) and GFAP (C) labelling following experimental cortical injury in the rat. No labelling for MT I/II was present in the cortex at one day post-injury (PI) (A). At four days PI, MT I/II labelling was confined to glia-like cell bodies and processes (B) (arrowhead) which were often colocalised with GFAP immunoreactivity (C) (arrowhead). At seven days PI, MT I/II immunolabelling was present in a diffuse band (∇) of immunoreactivity demarcating the lesion site and morphologically appeared as reactive astrocytes (⇐) (D), whereas at a distance from the lesion, MT I/II labelled normal glia-like cell bodies and processes (e.g, short arrow, E). At fourteen days PI (F), MT I/II immunoreactivity declined and was present primarily in the external surface of the brain (long arrow). Autofluorescent material is present within the tract (♦).

Scale bar (A), (B), (C) = 100μm; (D), (E), (F) = 50μm.


A
1 day PI
MT-I/II
B
4 days PI
MT-I/II
C
4 days PI
GFAP
D
7 days PI
MT-I/II
E
7 days PI
MT-I/II
F
14 days PI
MT-I/II

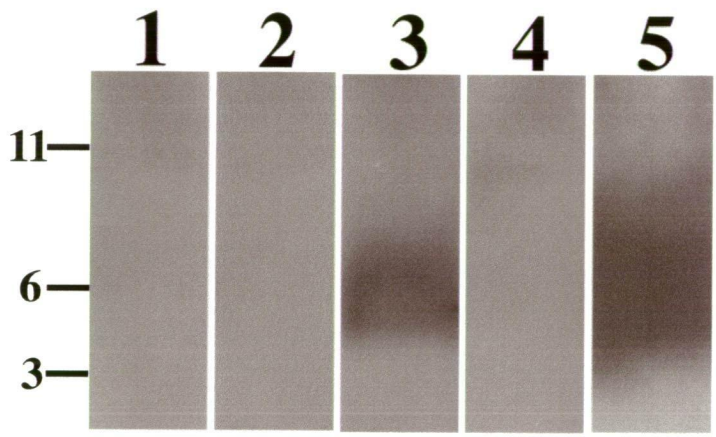
labelled for MT I/II at the lesion boundary, however, the most distinct glial (cell body and process) labelling was evident at a distance, up to 2 mm, from the injury site (Figure 7.1E). GFAP immunoreactivity was more extensive than at four days PI and primarily labelled fibrous-like processes, which were also often thickened and orientated towards the injection site.

Ferritin labelling had declined at fourteen days PI. MT I/II immunoreactivity was also reduced, being largely confined to fibrous-like processes at the pial surface, with little labelling at the edges of the tract (Figure 7.1F). There was also labelling of fibres traversing, and 'capping', the top of the tract. GFAP labelled structures were extensive, orientated towards the tract and often thickened as at seven days PI. There was also a higher density of GFAP positive structures at the tract edges.

Immunoblots

Western blot analysis (Figure 7.2) of the expression of MT I/II showed no labelling in control, one day, four days or fourteen days PI brain homogenates. Homogenates from seven day PI material, however, demonstrated labelling for MT I/II, suggesting a specific abundance of this protein at that time point. The detection of MT I/II at other time points by immunohistochemistry, and not immunoblots, reflects the relative concentration of MT I/II expressing cells in the different samples. MT I/II was concentrated enough in the cells to be seen in tissue sections, but when homogenised in a larger volume for immunoblots, the concentration dropped below detection level.

Figure 7.2. Evidence for an upregulation of MT I/II following experimental cortical injury in the rat. At each time point (1, 7 and 14 days PI), brain material from three animals was collected, homogenised and analysed with Western blotting. Normal uninjured brain as well as positive controls (Sheep MT I/II) were run concurrently in all experiments. This figure shows a representative blot, with approximate molecular weights shown on the left. Lane 1 (uninjured brain), lane 2 (1 day PI) and lane 4 (14 days PI) show no reactivity against MT I/II, whilst lane 3 (7 days PI) demonstrates strong labelling against MT I/II which also correlates with the positive MT I/II control shown in lane 5. Both bands correspond to molecular weights (M_w) of ~6-7 KDa, which is consistent with reported M_w values for MT-I/II.


1
2
3
4
5
11
6
3

7.3 Discussion

We have demonstrated that, following a focal cortical lesion, there is a specific upregulation of both MT I/II immunoreactivity and protein levels at seven days PI surrounding the injection tract. We have also shown that MT I/II immunoreactivity is detectable by four days PI, but declines at fourteen days PI.

Similar findings, but with a different temporal staging of MT I/II immunoreactivity and that of other glial markers, have also been demonstrated in two other time course studies on MT I/II expression after cortical NMDA (Acarin *et al.*, 1999b) and freeze (Penkowa *et al.*, 1999) lesions. These studies illustrate that an upregulation of MT I/II synthesis is not restricted to mechanical lesions, as described in this study, but perhaps more closely linked to generalised neuronal injury.

In this investigation, there was no expression of MT I/II in the normal and one day PI brain. MT I/II immunoreactivity was detected by four days PI, with clearly defined labelling of glia-like cell bodies and processes adjacent to the needle tract. These structures colocalised with GFAP immunoreactivity, consistent with reports on the localisation of MT I/II to astrocytes in the human brain (Nakajima and Suzuki, 1995; Adlard *et al.*, 1998). Unlike the normal human brain, where MT I/II is present in the entire pial surface (Nakajima and Suzuki, 1995), the appearance of MT I/II immunoreactivity in the pial surface in rodents was only associated with neocortical injury.

MT I/II positive structures increased noticeably at seven days PI, and, based on immunoblots, MT I/II protein content in the neocortex peaked at this time point. Based

upon their distance from the injection tract, MT I/II labelled cells had two different morphologies. At up to 2 mm distant from the injury site MT I/II labelled well defined glia-like cell bodies and processes, whereas, adjacent to the tract, labelled cells corresponded to 'reactive' astrocytes. MT I/II positive cells around the tract were distinct from central regions of the lesion containing ferritin-labelled microglia, as also demonstrated in an examination of MT I/II immunoreactivity in a variety of lesions (e.g. infarct, haemorrhage and tumour) in dogs (Shimada *et al.*, 1998). While MT I/II immunoreactivity declined by fourteen days PI, GFAP immunoreactivity was present in the normal brain and at all points PI. At fourteen days PI, reactive astrocytes showed a specific localisation to the edges of the injury tract, suggestive of the beginning of the formation of a glial scar. This is a well documented feature following CNS injury and is believed to significantly impact upon neuronal regeneration and functional recovery (Hozumi *et al.*, 1990).

We have previously demonstrated that there is increased MT I/II immunoreactivity in glial cells from the earliest stages of AD. These changes were not associated with the hallmark pathological features of the disease, but may be closely linked with a general aspect of the condition (Adlard *et al.*, 1998, chapter 6). Neuritic pathology in the animal model utilised in this investigation mimics the plaque-related neuronal changes associated with the preclinical stage of AD (King *et al.*, 1997). Thus, this investigation suggests that MT I/II changes in preclinical AD may be related to a generalised brain response to injury.

Features consistent between the sequelae of events which occur following neuronal injury and in AD, and which may be responsible for the induction of MT I/II expression, include both oxidative stress and metal accumulation. The evidence that oxidative stress may be

associated with the AD brain was detailed in the previous chapter. Similarly, following cortical injury, there is both a demonstrable increase in oxidative stress and an accumulation of harmful mediators, such as reactive oxygen species, as early as sixty minutes after traumatic brain injury (Awasthi *et al.*, 1997; Shohami *et al.*, 1999). Given that MT I/II scavenges both free hydroxyl- and superoxide radicals, the increase in MT I/II in AD and following experimental cortical injury may, therefore, be a response to increasing free radical damage (Aschner *et al.*, 1997).

It is well established that MTs also function to maintain a level of metal ion homeostasis by sequestering various metal species such as copper and zinc (Vallee, 1995). A disregulation of metal ions in both AD and following cortical injury, therefore, may result in the induction of MT synthesis. In the previous chapter, the role of various metal species in AD was outlined, and the potential importance of zinc noted. In cortical injury, it has recently been demonstrated that there is a translocation of zinc into injured postsynaptic neurons post-injury, which is an important factor controlling the fate of injured neurons (Suh *et al.*, 2000). In this regard, it was shown that the administration of a zinc chelator prior to injury resulted in significant neuroprotection. The expression of MT I/II in AD and following cortical injury may, therefore, represent an acute phase response to an increase in concentration of various metal ion species such as zinc. In this regard, the expression of MTs is also closely interlinked with the cortical pool of labile zinc (Aschner *et al.*, 1997).

We have demonstrated, therefore, that MT I/II expression is specifically and significantly upregulated at seven days PI, and, together with our earlier report on the possible role of MT I/II in preclinical AD (Chapter 6), we hypothesise that this protein may serve an

important functional role within the damaged CNS environment. These two chapters suggest that the induction of MT synthesis is likely to be a result of factors associated with neuronal injury, and may involve oxidative stress or the mismetabolism of metals, two factors which could significantly contribute to the sequelae of events which occur following both brain injury and in AD.

The effect of metallothioneins on the neuronal response to physical injury

8.0 Introduction

As outlined in previous chapters, the metallothionein (MT) family consists of a group of low molecular weight proteins which are found throughout the body, and which are reported to have a number of different functions (Karin, 1985; Cherian and Chan, 1993; Vallee and Maret, 1993; Vallee, 1995; Aschner *et al.*, 1997). With regards to the brain, it has been suggested that one of the four MT isoforms, MT-III, is a brain specific member of the group (Palmiter *et al.*, 1992; Erickson *et al.*, 1995; Aschner *et al.*, 1997). It has, however, been demonstrated that MT-III, while predominantly expressed within the brain, can also be found in other areas of the body, such as the renal system (Hoey *et al.*, 1997). MT-III was originally named growth inhibitory factor (GIF), and its isolation arose from studies of the Alzheimer's disease (AD) brain (Uchida *et al.*, 1988, 1991). Uchida and colleagues (1988) showed that extracts prepared from the AD brain could enhance the survival of cultured cortical neurons better than extracts prepared from normal human brain. This led to the proposal that there was an increase in the neurotrophic activity in the AD cortex relative to normal brain, which was postulated to be the result of a decrease in a growth inhibitory factor (Uchida and Tomonaga, 1989). This was hypothesised to account for the massive sprouting in the AD cortex, and was suggested to be responsible for the neuropathology of AD, by the repeated attempts at regrowth, subsequent exhaustion of cells and their eventual death (Uchida *et al.*, 1988, 1991; Uchida and Tomonaga, 1989). The normal brain was then examined for the presence of a protein which possessed an inhibitory action on the growth of cortical neurons. A small protein, GIF, was isolated, which was shown to be significantly decreased in the AD brain (Uchida *et al.*, 1991; Tsuji *et al.*, 1992;

Uchida, 1993; Hozumi *et al.*, 1998). This protein could also prevent the increased survival of cortical neurons cultured in the presence of AD brain extracts. This was, therefore, believed to be the candidate factor which was decreased in AD and responsible for the increased neurotrophic activity. It was later demonstrated that GIF, with the exception of a one amino acid insert in the amino terminal and a six amino acid insert in the carboxy terminal sequence of MT-I/II, demonstrated a high degree of homology with MT-I/II and was subsequently termed MT-III (Uchida *et al.*, 1991; Palmiter *et al.*, 1992; Kobayashi *et al.*, 1993).

That MT-III is significantly reduced in the AD brain, however, has remained controversial, with a number of studies suggesting that MT-III expression is either down-regulated in AD (Uchida and Tomonaga, 1989; Uchida *et al.*, 1991; Tsuji *et al.*, 1992) or not changed (Erickson *et al.*, 1994; Amoureux *et al.*, 1997). Similarly, whether the increased neurotrophic activity in the AD brain is due to an increase in a neurotrophic factor, such as nerve growth factor (Crutcher *et al.*, 1993) or fibroblast growth factor (Stopa *et al.*, 1990), or due to a decrease in an inhibitory factor, such as MT-III, also remains controversial. It is accepted, however, that MT-III does possess a unique growth inhibitory action on cortical neuron growth, which is not possessed by the other members of the MT family, and which is believed to reside in the first 32 amino acids of the ß domain of the protein (Sewell *et al.*, 1995; Uchida and Ihara, 1995).

The role of MT-III in AD, therefore, remains controversial. It has, however, been postulated that the administration of exogenous GIF may be an approach to the treatment of AD (Hozumi *et al.*, 1998). It is hypothesised that MT-III may inhibit the progression of AD

in its early stages by the inhibition of aberrant neuronal sprouting (Uchida *et al.*, 1991; Hozumi *et al.*, 1998). In this chapter, then, we have utilised the *in vivo* model of cortical injury, which has been shown to replicate the early neuronal pathology of AD (King *et al.*, 1997), to determine whether the administration of MT-III will result in a significant decrease in neuropathology which characterises this model. Given the results of the previous two chapters, as well as the commencement of clinical trials examining the effect of metal chelators in the treatment of AD patients (Colin Masters, Australia, 2000), we have also undertaken a preliminary trial with MT-II to assess its effect in this model. Thus, we have established the therapeutic potential of MTs in the treatment of AD.

8.1 Materials and methods

Animal procedures

All procedures involving animals were approved by the Ethics Committee (Animal Experimentation) of the University of Tasmania and are consistent with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Briefly, twenty animals received experimentally induced cortical injuries as outlined in chapter 2. In all animals, the needle was left in place for a total of 10 minutes before being slowly removed. During that ten minutes, 1 μl of either vehicle (0.01 M PBS, 10 animals) or vehicle plus a synthetic MT-III peptide (corresponding to the amino acid sequence, MDPETCPCPSGGSCTCADSCKCEGCKCTSCKK) (50μg/ml, AUSPEP, 10 animals) was intracortically injected at a rate of 0.2 μL/minute. At both one day and four days post-surgery, five control and five test animals were perfusion fixed, as detailed in chapter 2.

Coronal sections through the injury site were cut at 50 μm with a vibratome and labelled with mouse monoclonal antibodies to both phosphorylated (SMI312, Sternberger Monoclonals Inc., 1:2000) and dephosphorylated (SMI32, Sternberger Monoclonals Inc., 1:2000) epitopes on the medium and high molecular weight neurofilament (NF) subunits. Antibodies to NF markers were visualised with a rat-adsorbed, horse anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) (dilution 1:200, Vector Labs).

Microscopy

For quantitative purposes, the number of NF immunoreactive abnormal neurites were counted in 5 random fields of view (using a rectangular counting frame and the 50x objective of a Leitz Dialux 22 EB fluorescence microscope) taken from around the needle

tract and within two fields of view extending out from the tract. Quantitation of these structures in both one-day and four-day post-injury (PI) animals was conducted in a blind fashion by an investigator who was not involved in the preparation of tissue.

Preliminary investigations

The MT-III peptide utilised for the majority of investigations was not constituted with metals. A preliminary study (n=4), however, was carried out which involved reconstituting the synthetic peptide with zinc *in vitro*, prior to cortical injection. The peptide was preincubated in hydrochloric acid (HCl), to a final concentration of 0.025N, and 5mM DTT for thirty minutes at room temperature before the addition of zinc chloride (pH 2) at a 1:3 molar ratio. Tris HCl (20mM) was added to neutralise the peptide prior to use (method courtesy of D. Winge). The same solution, but without the MT-III peptide, was utilised as a control (n=2). Animals were examined at one (n=2) and four (n=2) days PI.

Another preliminary investigation, utilising full length, metal-constituted, MT-IIA (~200μg/ml, n=4) and MT-III (~105μg/ml, n=4) was also carried out. Animals were examined at both one (n=2 for each MT-group) and four days (n=2 for each MT-group) following cortical injury. The proteins, and appropriate controls (n=2), were a kind donation from Dr. Adrian West, Molecular Biology Unit, University of Tasmania. Only a small trial could be undertaken, as these proteins were only recently produced and characterised.

8.2 Results

All animals injected with either the vehicle alone (PBS) or with the MT-III peptide solution exhibited normal behaviour following surgery, and there were no gross differences in brain structure.

Neurofilament labelling

In the contralateral cortex of both vehicle-treated and MT-III treated animals, SMI32 labelling was localised to cell bodies and dendrites in a subset of pyramidal-like neurons. Labelling with SMI312 was restricted to axons.

Surrounding the injury tract there was substantial labelling of abnormal structures, and these appeared primarily as ring- and bulb-like neuritic structures, as shown in chapter 5. These abnormal axons extended to a distance of ~200μm on either side of the tract, and were often continuous with fibers of varying calibre. The morphology of these structures was not different between vehicle-injected and MT-III injected animals.

At one day PI, with labelling for both phosphorylated and dephosphorylated neurofilament epitopes, there was no statistically significant difference between the density of either bulb-like or ring-like structures surrounding the injection tract in control- and MT-III treated animals (Table 8.1).

Similarly, at four days PI, quantitation demonstrated that there was no statistical difference in the number of abnormal structures labelled for both phosphorylated and

Table 8.1. Mean density (± standard deviation) of pathological structures present at both one and four days post-injury in the cortical grey matter of MT III (50μg/ml)- and vehicle-treated animals. No significant ($P<0.05$) differences were noted between any group at any timepoint.

Group	Antibody	Number of pathological structures (mm^2) around the injection tract			
		Ring-like		Bulb-like	
		1 day	4 days	1 day	4 day
Control	SMI32	294 ± 44	59 ± 29	265 ± 44	157 ± 20
MT-III	SMI32	382 ± 34	64	353 ± 88	167
Control	SMI312	1461 ± 142	559 ± 98	1049 ± 93	314 ± 59
MT-III	SMI312	1441 ± 353	674 ± 103	921 ± 162	490 ± 83

dephosphorylated NF epitopes surrounding the injection tract in control- and MT-III treated animals (Table 8.1).

Preliminary investigations

Neurofilament labelling surrounding the injection tract in all drug-treated (metal reconstituted MT-III and native MT-IIA and MT-III) animals was no different to that observed in any of the control-treated animals. There was, however, a difference in the gross appearance of the injection tract in the native MT-III treated animals. All animals demonstrated a significantly enlarged lesion site, relative to all other protein- and control-treated animals, at one and four days following injury.

8.3 Discussion

In this investigation we have examined the effects of MT-III on the neuronal response to physical injury. We have demonstrated that the intracortical injection of a synthetic MT-III peptide, irrespective of the metal content, does not significantly alter the normal neuropathological changes which occur within the brain following injury.

A number of studies have examined the change in MT-III following different types of brain trauma, including cortical stab wounds (Anezaki *et al.*, 1995; Hozumi *et al.*, 1995, 1996) and ablation (Yuguchi *et al.*, 1995a), excitotoxic lesions (Anezaki *et al.*, 1995; Acarin *et al.*, 1999a) and ischaemia (Yanagitani *et al.*, 1999). The balance of these studies suggest that MT-III mRNA and proteins levels are upregulated from three to four days PI, and can remain increased for several weeks PI, although there was an initial decrease in MT-III expression at one day PI in the cortical ablation model, prior to an increase at four days PI (Yuguchi *et al.*, 1995a). Cortical injury, therefore, results in an increase in MT-III. In contrast to these studies, however, it has been demonstrated that, following facial nerve transection, MT-III mRNA is suppressed from three days PI until at least five weeks PI (Yuguchi *et al.*, 1995b). It has been shown that the facial nerve can regenerate and recover function following a lesion (Tetzlaff *et al.*, 1988), and so it was hypothesised that MT-III is actively downregulated to allow neurite outgrowth and regeneration (Yuguchi *et al.*, 1995b).

As opposed to the facial nerve, where regeneration is possible, it is widely accepted that regeneration within the cortex is very limited and unlikely to result in the reformation of functional connections. The increase in MT-III expression in these situations may,

therefore, represent a protective phenomena, whereby the injured CNS actively inhibits neuronal sprouting. The timing of this increase in MT-III expression following different types of neuronal injury also correlates with the onset of sprouting. We have previously established that, following both *in vitro* and *in vivo* cortical injury, changes to the CNS occur almost immediately, and set in place a series of stereotypical changes which ultimately results in attempts by injured neurons to resprout at between three and four days PI (unpublished). That abortive sprouting still occurs, however, suggests that the endogenous MT-III levels are either insufficient, or not elevated soon enough following cortical injury to prevent this attempted outgrowth. The earlier administration of MT-III may, therefore, be efficacious in preventing the sequelae of events which follow neuronal injury.

There have been no previously published studies on the effects of exogenous MT-III administration on the brain following cortical injury. In a review by Hozumi and colleagues (1998), however, there was reference to unpublished data which suggested that the administration of native MT-III following stab wound lesions resulted a demonstrably larger cavity as a result of the lesion, as compared to controls. We have similarly shown that the administration of native MT-III into the cortex results in a larger lesion site, as compared to vehicle-treated animals. However, administration of the synthetic MT-III peptides did not result in any alteration to the microscopic or macroscopic appearance of the injured cortex. It is possible that the lack of any apparent effect, of the synthetic MT-III utilised in our investigation, may be accounted for by the nature of the peptide.

Due to the initial lack of availability of native, full length MT-III, we had a short synthetic peptide produced for the majority of these studies. The inhibitory properties of different MT-III domains have been previously examined, including MT-III(1-26) and MT-III(5-23) (Sewell *et al.*, 1995; Uchida and Ihara, 1995). Both these short proteins, prepared from native MT-III, could inhibit the neurotrophic activity of AD extract on cultured cortical neurons. However, similar experiments utilising a synthetic form of MT-III(5-23) did not result in an inhibition of cortical outgrowth, even at concentrations ten fold greater than used for native MT-III proteins. This suggests, therefore, that synthetic MT proteins have little biological effect *in vitro*. We, however, utilised a longer peptide which corresponded to the first 32 amino acids of the ß domain of the amino terminal portion of the protein. This region was selected, as it has been demonstrated that the neurite inhibitory properties of the protein reside in this portion of the molecule, with a double mutation in prolyl residues which comprise the 'cys-pro-cys-pro' sequence at the start of the protein abolishing the bioactivity of the protein (Sewell *et al.*, 1995). The inhibitory activity of the ß-domain is comparable to that exhibited by the full length protein (Sewell *et al.*, 1995), and is believed to result from a unique conformation which may crucially involve the prolyl residues. It was hypothesised, therefore, that this longer peptide would be more likely to exhibit growth inhibitory properties *in vivo* than the shorter (5-23) peptide. Another consideration in the use of this short synthetic peptide was its metal content.

Metals are known to stabilise metalloenzymes such as metallothionein (Vallee and Auld, 1990) and to give them their unique tertiary structure which is often required for bioactivity. As mentioned above, in the case of MT-III, it is believed that its biological activity may not necessarily be dictated by its metal content, but rather, be a function of a

conformation reliant on the presence of specific prolyl residues. In support of this notion, it was originally demonstrated by Uchida and Ihara (1995) that native metal-free MT-III(1-26) had similar bioactivity to that of MT-III(1-26) which was fully constituted with metals. Therefore, we utilised the synthetic peptide without first reconstituting it with metals for the majority of investigations. It is likely, however, that this peptide would reconstitute with the pool of labile cortical zinc *in vivo*. Preliminary investigations utilising this same peptide, but after it had been reconstituted with zinc *in vitro*, were also performed. Despite the different protein preparations, and the use of the peptide at a concentration which was more than five fold higher than that required for native MT-III to demonstrate almost maximal inhibition of cortical outgrowth *in vitro* (Uchida and Ihara, 1995), there was no obvious change in the neuronal pathology observed following cortical injury. It remains possible, however, that the peptide had degraded in storage.

Due to the lack of a commercial antibody against MT-III and the ineffectiveness of polyclonal antibodies generated against MT-III in this laboratory (results not shown), we have been unable to confirm results of other investigations which have examined the expression and localisation of MT-III in AD as well as following cortical injury. The results of this study do not support a role for MT-III in the prevention of the neuropathological sequelae of neuronal injury, and hence do not suggest it useful for the treatment of AD neurodegeneration. However, further studies utilising full length native MT-III peptides are warranted before it can be determined whether MT-III will represent a future therapeutic avenue for the treatment of AD.

Final discussion

9.0 Introduction

Alzheimer's disease (AD) is a major central nervous system disorder which is the major cause of dementia in the elderly population. Despite advancements in the understanding of the underlying biological mechanisms which result in neurodegeneration, there has yet to be developed a drug which will prevent, delay or even effectively treat AD.

The literature review (chapter 1) provided a background on AD, as well as the current strategies being investigated to treat it. In the description of AD, it has been hypothesised that the deposition of insoluble ß-amyloid plaques within the cortex causes physical disruption to the surrounding neuropil, and this sets in place the programmed neuronal response to injury. This response crucially involves various cytoskeletal proteins, and ultimately leads to attempts by the injured neuron to resprout, which can eventually lead to cell death. That these changes may crucially involve the cytoskeleton was highlighted by studies of preclinical AD cases, where the earliest neuropathological changes that occur have been shown to involve cytoskeletal abnormalities (Vickers *et al.*, 1996). These changes are also recapitulated in various animal models of cortical injury (Povlishock and Christman, 1995; King *et al.*, 1997), supporting the controversial notion that ß-amyloid plaques cause structural damage to neurites. With regards to current therapeutic strategies for the treatment of AD, these are heavily weighted in favour of restoring acetylcholine levels within the AD brain. Whilst such therapies have a place in treating the symptoms of AD, and indeed have proven successful in improving the quality of life for a short period of time in a subpopulation of AD sufferers, they do nothing to prevent the progression of the

disease. In this respect, the central aim of this thesis has been concerned with establishing alternative routes of therapy for AD than those currently available.

The first three experimental chapters were based on the concept that, following ß-amyloid deposition, there are very early cytoskeletal changes in affected neurons which involve microtubules, and that targeting these proteins may be an effective method of preventing the neuropathology of AD. To investigate this, studies were initially undertaken to establish whether or not dendrites, and by inference, microtubules, are significantly affected as a result of plaque deposition in the AD cortex. Utilising an animal model of the early neuronal pathology of AD, we then determined whether or not similar changes occur following cortical injury *in vivo*, and finally, established the efficacy of different drugs in preventing these changes.

The final three chapters were aimed at establishing if a family of proteins, metallothioneins (MT), have any role in AD. We examined the cellular distribution and expression of different MT isoforms in both the early and late stages of AD and following cortical injury. We also trialed various MT peptides in the *in vivo* animal model to determine whether these agents had the potential for use in the treatment of AD,

9.1 General discussion

That physical injury to neurons, as a result of ß-amyloid deposition within the cortex, is the cause of neurodegeneration in AD, and that this crucially involves cytoskeletal components such as neurofilaments, has been extensively investigated within our laboratory. We have hypothesised, however, that targeting other cytoskeletal changes, in particular, stabilising

microtubules against collapse, may be an effective method of preventing the neuropathology which leads to dementia in AD. In this thesis, the extent of microtubular change in AD was initially determined by examining the distribution of MAP2, a marker for microtubules specifically localised to dendrites, both within and around plaques in the early and late stages of AD. In contrast to existing studies by Knowles and colleagues (1998, 1999), we have demonstrated that the morphological change which occurs in dendrites in AD is a function of the type of plaque which they encounter, rather than a generalised effect of all plaque types on dendrites. In this respect, diffuse plaques result in little deformation to normal dendritic structure in both the early and late stages of AD. In contrast, the formation of dense-cored plaques within the cortex results in major structural disruption to the normal architecture of the brain. That there is no gradient of damage away from the plaque also argues against a diffusible toxic mechanism, and supports the notion that ß-amyloid plaques lead to neuronal dysfunction via a structural mechanism. It is posited, therefore, that specific plaque types structurally damage neurites which encounter them, resulting in a destabilisation and loss of microtubules within them. Such changes can significantly impact upon the functional integrity of the affected neurite, altering processes such as cellular transport, and may contribute to the formation of abnormal neuritic structures in association with ß-amyloid plaques. Ultimately, as suggested by Knowles and colleagues (1999), such a disruption to the neural networks of the brain may lead to dementia.

Previous studies, including early electron-microscopic investigations, have reported a specific loss of microtubules in the AD brain (Gray, 1986; Gray *et al.*, 1987; Paula-Barbosa *et al.*, 1987). Similarly, other studies in this laboratory have highlighted the absence of microtubules in the neuritic structures which are characteristic of AD, and which are found

in association with ß-amyloid plaques (unpublished). The work in this thesis, which represents one of the few studies on the effect of plaques on dendritic cytoarchitecture, suggests that such findings are the result of the destruction of neurites which encounter dense-cored plaques in all stages of the disease process, and also fibrillar plaques in the late stage of the disease. This further supports studies which have demonstrated axonal dystrophy in association with plaques (Dickson *et al.*, 1999).

One approach to the treatment of AD, therefore, would be to prevent plaque formation, and such an approach is currently under investigation (chapter 1). However, based on current data, such a treatment would not be of benefit once plaques have formed, and so would do nothing for the millions of people currently suffering from, or in the early stages of, AD. The strategy examined in this thesis, then, was to determine if it is possible to prevent the neurodegenerative changes which lead to dementia, by the stabilisation of cytoskeletal elements within damaged neurites.

Utilising an animal model of the early neuronal pathology of AD, we initially established that, following cortical injury, there was a significant loss of normal MAP2 labelling surrounding the lesion site, suggestive of both dendritic, and microtubular, disruption as a result of injury to the cortex. That microtubules are affected following *in vivo* cortical lesions is further highlighted by electron-microscopic investigations in this laboratory. These studies have demonstrated that the abnormal axonal structures which occur following injury, which demonstrate morphological, neurochemical and ultrastructural similarities with early forms of DNs in AD, show a complete loss of microtubules (King *et al.*, 2000). Both the dendritic and axonal changes which occur following experimentally-induced

cortical injury, therefore, closely resemble that which occurs in the AD brain following the deposition of ß-amyloid plaques. This further supports the notion that ß-amyloid is physically destructive to local neurites. The animal model utilised not only allows the investigation of the mechanisms which lead to neurodegeneration in AD, but also provides a platform on which to test the ability of different agents to prevent these AD-like alterations. That the model also mimics the early neuronal pathology of AD is also advantageous, as this represents the stage of the disease in which there has been little cortical damage, and hence is the most favourable window for therapeutic intervention. Thus, the *in vivo* model formed the basis for the investigations on the effect of taxol on the central nervous system (CNS) response to injury in the fifth chapter.

Taxol functions to increase the polymerisation of soluble tubulin to form microtubules, and also stabilises existing microtubules against collapse. This drug, therefore, was a good candidate for study in this thesis. That it has already been approved for use in humans, for the treatment of various forms of cancer, would also be beneficial should this drug be of potential use in the treatment of AD. There have been no previous studies on the effect of taxol on the CNS, although extensive investigations have been performed on its effect in the peripheral nervous system. Several reports, however, have suggested that taxol may be of use in the treatment of AD (Mattson, 1992; Lee *et al.*, 1994; Michaelis *et al.*, 1998).

We demonstrated that taxol has a profound effect on the CNS response to physical injury. Although it did not alter the normal neuronal morphology within the brain, it had demonstrable effects on the microtubule marker, MAP2, within the lesion site, such that there was a relative preservation of MAP2 labelling within the area of damage. That taxol

may have stabilised microtubules against collapse was further highlighted on quantitation of the abnormal NF-positive axonal structures which evolve, probably as a consequence of discrete cytoskeletal alterations, following cortical injury. Taxol significantly decreased the evolution of the neuritic structures which characterise both this model, and AD. This clearly demonstrated, therefore, that it is possible to intervene in the normal stereotypical response of neurons to physical injury, and that maintenance of neuronal architecture can prevent the degeneration of injured neurites.

In the animal model, the administration of taxol was only effective in the short term. Taxol-injected animals examined at four days post-injury (PI) demonstrated MAP2 labelling around the injury site that was similar to the control-treated animals, and there were significantly more abnormal axonal structures labelled for NFs surrounding the lesion site in taxol-treated animals. It was demonstrated, however, that following *in vivo* and *in vitro* cortical injury, there is a significant reduction in the appearance of abnormal neuritic pathology and, morphologically, the damaged cortex begins to resemble normal neuropil. It is likely, therefore, that taxol prevented the resolution of the initial neuropathological changes in axons.

In AD, the initial stabilisation of the cytoskeleton within neurites may ultimately be beneficial in preventing the subsequent evolution of abnormal neuritic structures. We hypothesise, therefore, that taxol may be effective in the treatment of AD. However, the eventual use of taxol for AD would require a number of issues to be addressed, including the method of drug delivery and the potential toxicity of the drug on the normal neuropil. So, whilst taxol may, ultimately, not be the drug of choice for the treatment of AD, this

thesis has demonstrated that taxol-like drugs, which specifically target the neuronal cytoskeleton and aspects of the neuronal reaction to physical trauma, can be effective in preventing the evolution of abnormal neuritic structures which occur in the early stages of AD.

An *in vitro* model of physical injury was also developed to allow for the rapid screening of potential therapeutic agents. This model has been shown to replicate the cellular changes which occur following both *in vivo* cortical injury and the deposition of ß-amyloid plaques in the early stages of AD (Dickson *et al.*, 2000). Due to the absence of a robust axonal microtubule marker, it was not utilised for drug investigations in this thesis. However, further characterisation of this model was made with respect to the microtubule associated proteins, MAP2 and tau. In particular, the alterations in tau observed following cortical injury *in vitro* may be crucial to the loss of normal microtubular structures which was demonstrated to follow such injuries *in vivo*. Also, that tau was localised to both abnormal neuritic and growth cone-like structures, where it has been proposed to participate in the formation of the growth cone (DiTella *et al.*, 1994), suggests that it may have a role in the apparent regrowth/sprouting of transected neurites observed *in vitro*. That similar observations have also been made in AD, where there is a mobilisation of tau in dystrophic neurites, perhaps in preparation for sprouting, further supports the notion that this *in vitro* model replicates the early neuropathology of AD.

The final three experimental chapters centred on the role which metallothioneins may have in AD and brain injury. We have demonstrated that ß-amyloid plaques physically damage local neurites. This may cause injured cells to enter into a cycle of repeated futile attempts

at sprouting, where the subsequent accumulation of proteins may be harmful. We hypothesised, therefore, that following cortical injury, sprouting may result in cytoskeletal changes and eventual neuronal cell death. With regards to the metallothionein family of proteins, then, the brain specific isoform of this family, MT-III, was reported to possess growth inhibitory properties. Originally isolated from the normal human brain, it was shown to be specifically down regulated in AD and hypothesised to be the protein ultimately responsible for aberrant neuronal sprouting (Uchida and Tomonaga, 1989; Uchida *et al.*, 1988, 1991). Whilst we could not obtain antibodies to MT-III, and hence examine the localisation and distribution of MT-III in AD and following cortical injury, we were able to assess the effect of this protein on the neuronal response to physical injury.

There have been no previously published *in vivo* investigations on the effect on exogenous MT-III administration on the brain, its response to injury or its potential use in the treatment of AD. In this thesis, then, these questions were answered by examining the effect of a synthetic MT-III peptide in the *in vivo* animal model of cortical injury previously described. The results of this study suggested that MT-III has little effect on the injured cortex and they do not support a role for MT-III in the treatment of AD. A number of factors, as outlined in chapter 8, need to be considered before this protein can be ruled out as a potential therapeutic agent. Also, further studies of the expression and localisation of MT-III in both the early and late stages of AD are required to conclude the controversy surrounding the regulation of MT-III in AD.

Other members of the MT family are also expressed within the cerebral cortex, in particular, MT-I and MT-II. Both these proteins share a high degree of homology with MT-

III, and are also expressed in similar locations and concentrations within the brain as MT-III. Despite the presence of these other MT isoforms within the brain, they had not been extensively investigated in relation to AD. As part of our investigation on the role of MTs in AD, therefore, we examined the distribution and localisation of MT-I/II in both the early and late stages of AD, as well as in normal brain. Existing reports demonstrated that MT-I/II mRNA, as well as GFAP mRNA, is upregulated in the AD brain (Duguid *et al.*, 1989; Nakajima and Suzuki, 1995). The concomitant elevation in GFAP suggested that the increase in MT-I/II was unrelated to the disease process. These previous studies, however, only examined end-stage AD cases. In reexamining these proteins in this thesis, then, both end stage as well as preclinical AD cases were examined, thus giving a better perspective of MTs role relative to the staging of AD.

Our studies supported the findings of the previous reports on the cellular localisation of MT-I/II, as well as the determination that MT-I/II increases in the end-stage of AD. Importantly, however, we demonstrated that there was a significant upregulation of MT-I/II in the preclinical phase of the disease which was not associated with a concomitant proliferation of glial cells. This suggests, therefore, that MT-I/II is specifically upregulated in this early stage of the disease process. MT-I/II positive cells did not show any particular association with specific AD pathological hallmarks, and so the inducing factor is likely to be a general aspect of the disease process. I also demonstrated that there was a significant increase in MT-I/II expression in the rat brain at seven days following localised injury to the cortex. The induction of MT-I/II may, therefore, be due to a factor which is common to both AD and the brain response to injury. In this respect, the increased levels of reactive oxygen species or other free radical mediators are possible candidates, as outlined in

chapter 6 and 7. There is an extensive literature on the disregulation of metal species within the AD brain (Wenstrup *et al.*, 1990; Markesberry and Ehmann, 1994), and recent *in vivo* work suggests that a disruption in homeostatic mechanisms controlling zinc ions may be an important factor in controlling the fate of injured neurons following cortical injury (Suh *et al.*, 2000). That MT-I/II is a major intracellular store for metal ions such as copper and zinc, therefore, suggests that this protein may be responding to harmful levels of particular metals in both conditions. It is of note, then, that a clinical trial on the use of metal chelators in the treatment of AD has recently begun. Cumulatively, therefore, these data support a role for MT-I/II in the early stages of AD, perhaps as an acute phase reactant to a disregulation of metals. To further examine this possible link, the distribution of MT-I/II in relation to areas of oxidative stress and metal accumulation should also be examined. Whilst the initial interest in metallothioneins was for the therapeutic potential of MT-III, we have clearly demonstrated that other members of the MT family, MT-I/II in particular, should be considered for their potential use in the treatment of AD.

9.2 Conclusions

As the world's population ages, the prevalence of late onset neurodegenerative disorders such as AD will increase significantly. In the absence of a cure for AD, it is necessary to establish novel means of treating the cause of the disease, with symptomatic relief only applicable in the short term. With a greater understanding of the biological mechanism underlying the disease, it will become increasingly likely that more effective drugs will be developed to treat the disease process. It is imperative, therefore, that the scientific community be guided by the strength, rather than the perceived 'popularity', of new hypotheses. The results of this thesis, then, have highlighted new therapeutic avenues for

the treatment of AD, some of which have already entered into clinical trial throughout the world. The major conclusions from this thesis, then, are as follows.

*** ß-amyloid plaque deposition results in structural deformation to the AD brain**

The cytoarchitecture of the brain was shown to be differentially affected by morphologically distinct types of ß-amyloid plaques. That there were such similarities between the microtubular changes that occur following cortical injury and in AD further supports the notion that ß-amyloid plaques result in structural deformation to the neuropil.

*** Drugs that stabilise the cytoskeleton may be effective in treatment of AD**

We demonstrated that targeting the cytoskeletal abnormalities which occur following cortical injury could significantly reduce the subsequent evolution of neuropathology in an animal model which produces neuritic changes characteristic of the early stages of AD. The implications are, therefore, that drugs such as taxol, which specifically stabilise the neuronal cytoskeleton, may be effective in the treatment, and perhaps prevention, of AD.

*** The MT family of proteins may be important mediators in the neuropathology of AD**

Specific MT proteins expressed within the brain were shown to be induced, perhaps by a disregulation of metal species, in both the early stages of AD and following cortical injury. This supports a role for metal chelators, such as MT-I/II, in limiting the neuropathological sequelae of AD. The use of growth inhibitors in stalling the stereotypical response to physical trauma also needs to be further investigated.

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Appendix

11.0 General solutions

0.01M PBS, pH 7.4 (4°C):

100 ml	10X saline stock (90 g NaCl (ASTRAL) per litre MilliQ® water)
40 ml	Di-sodium hydrogen orthophosphate (Na_2HPO_4, BDH) (28.4 g per litre MilliQ® water)
10 ml	Sodium di-hydrogen orthophosphate ($NaH_2PO_4 2H_2O$, UNILAB) (31.2 g per litre MilliQ® water)
850 ml	MilliQ®

Diluent (4°C)

300 µl	Triton X-100 (Sigma)
100 ml	PBS (0.01M)

Tissue storage solution (4°C)

100 ml	0.01 M PBS
0.01 g	Sodium azide (BDH)

11.1 Solutions for human investigations

Tissue storage solution (4°C) **- as in section 11.0**

Tissue storage solution (-20°C)

300 ml	glycerol (BDH)
300 ml	ethylene glycol (BDH)
300 ml	MilliQ®
80 ml	Di-sodium hydrogen orthophosphate (BDH) (28.4 g per litre MilliQ® water)
20 ml	Sodium di-hydrogen orthophosphate (UNILAB) (31.2 g per litre MilliQ® water)

11.2 Solutions for animal investigations

4% Paraformaldehyde fixative

40 g	Paraformaldehyde (BDH)
100 ml	Sodium di-hydrogen orthophosphate (UNILAB) (31.2 g per litre MilliQ® water)
400 ml	Di-sodium hydrogen orthophosphate (BDH) (28.4 g per litre MilliQ® water)
500 ml	MilliQ®

Solution heated, but not boiled, until dissolved.

Tissue storage solution (4°C) **- as in section 11.0**

11.3 Solutions for cell culture

'Initial' plating media (4°C)

500 ml	Neurobasal® media (Life Technologies)
10 ml	B-27 supplement® (2%) (Life Technologies)
0.0365 g	L-Glutamine (0.5 mM) (Life Technologies)
0.0018 g	Glutamate (25 μM) (Life Technologies)
50 ml	Foetal calf serum (CSL)
0.5 ml	Gentamicin (David Bull Laboratories)

All additives are filter sterilised into the Neurobasal® media in a laminar flow hood.

'Subsequent' plating media (4°C)

500 ml	Neurobasal® media (Life Technologies)
10 ml	B-27 supplement® (2%) (Life Technologies)
0.0365 g	L-Glutamine (0.5 mM) (Life Technologies)
1.5 ml	Gentamicin (David Bull Laboratories)

All additives are filter sterilised into the Neurobasal® media in a laminar flow hood.

4 % Paraformaldehyde fixative

40 g	Paraformaldehyde (BDH)
40 g	Sucrose (UNILAB)

100 ml	Sodium di-hydrogen orthophosphate (UNILAB) (31.2 g per litre MilliQ® water)
400 ml	Di-sodium hydrogen orthophosphate (BDH) (28.4 g per litre MilliQ® water)
500 ml	MilliQ®

Solution heated, but not boiled, until dissolved.

Borate buffer (4°C, pH 7.4)

~115 ml	disodium tetraborate ($Na_2B_4O_7.10H_2O$, BDH) (38.16 g per litre MilliQ® water)
4 L	Boric acid (H_3BO_3, M & B Lab Chemicals) (6.18 g per litre MilliQ® water)

Add disodium tetraborate solution to boric acid solution until pH reaches 7.4.

10mM HEPES buffered saline (HBS) (4°C)

2.38 g	HEPES (N-2-Hydroxyethyl piperazine-N-2-ethane sulphonic acid) (BDH).
1 L	PBS (0.01M)

The solution is autoclaved at 121°C for 15 minutes.

11.4 Solutions for Western blotting

Blocking Solution 2.5 and 5%

2.5 or 5 g	Skim milk powder (Diploma)
100 ml	PBS-T

PBS-T

1 L	0.001M PBS
1 ml	Tween 20 (Bio-Rad Laboratories)