# Microtubule-associated protein tau in oligodendrocytes following acute brain injury

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A thesis submitted for the Degree of Doctor of Philosophy to the Faculty of Medicine, University of Glasgow.

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## List of Abbreviations

| | |
|---|---|
| AD | -Alzheimer's disease |
| APES | - Amino propyltriethoxy-silane |
| AMPA | - 2-amino-3-hydroxy-5-methylisoxazole-4-propionic acid |
| APP | - amyloid precursor protein |
| BFGF | - basic fibroblast growth factor |
| BSA | - bovine serum albumin |
| CNS | - central nervous system |
| CSF | - cerebral spinal fluid |
| CNP | - 2',3'-cyclic nucleotide 3'phosphodiesterase |
| DEPC | - di-ethyl pyrocarbonate |
| DTT | - dithiothreitol |
| GFAP | - glial fibrillary acidic protein |
| GTE | - glucose Tris EDTA |
| H & E | - haematoxylin and eosin |
| HBSS | - Hank's balanced salt solution |
| HRP | - horse-radish peroxidase |
| IF | - intermediate filament |
| MABP | - mean arterial blood pressure |
| MAP | - microtubule-associated protein |
| MCA | - middle cerebral artery |
| MCAO | - middle cerebral artery occlusion |
| MSA | - multiple system atrophy |

| | |
|---|---|
| MBP | - mylein basic protein |
| MK 801 | - dizocilpine |
| NFT | - neurofibrillary tangle |
| NMDA | - N-methyl-D-aspartate |
| PHF | - paired helical filament |
| PD | - Parkinson's disease |
| PBN | - α-phenyl-tert-butyl-nitrone |
| PBS | - phosphate buffered saline |
| PDGF | - platelet derived growth factor |
| PSP | - progressive supranuclear palsy |
| PLP | - proteolipid proteins |
| SDS-PAGE | - sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SDW | - sterile distilled water |
| TBI | - traumatic brain injury |
| TAE | - Tris acetate EDTA |
| TBS-T | - Tris buffered saline |

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I have thoroughly enjoyed the time I have spent working at the Wellcome Surgical Institute and would like to take this opportunity to thank everyone in the department for their support and friendship throughout the duration of my PhD.

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## Preface and Declaration

This thesis presents results from investigations conducted within 4 broadly defined areas :

1) To investigate the effect of acute brain injury on the distribution of the microtubule-associated proteins tau, MAP2 and MAP5 in oligodendrocytes.

2) To asses the mechanisms underlying the accumulation of tau immunoreactivity in oligodendrocytes following acute brain injury.

3) To determine if increased tau immunoreactivity within these cells reflected increased synthesis or protein modification.

4) To compare the distribution of tau, MAP2 and MAP5 within oligodendrocytes with that in neurons following acute brain injury.

Results from these studies are presented and discussed separately. The thesis comprises my own original work and has not been presented previously as a thesis in any form. Dr. K. Yatsushiro performed the permanent middle cerebral artery occlusions. In vitro studies were carried out in collaboration with Dr. S.C. Barnett who kindly supplied the oligodendrocyte cultures. In situ hybridisation studies were carried out in collaboration with Dr. P. Dickinson. Both the electron microscopy and confocal microscopy were performed by Prof. I.R. Griffiths.

## Abstract

With the growing evidence supporting glia-neuronal signalling it is becoming increasingly apparent that it is crucial to gain an understanding of the mechanisms underlying the degeneration of both neurons and glia to fully understand the pathogenesis of acute and chronic degenerative diseases of the brain. Breakdown of the cytoskeleton is thought to represent a common pathway mediating irreversible neuronal damage in a variety of both acute and chronic neurodegenerative conditions. One of the most well documented alterations to the cytoskeleton, occurring in various neurodegenerative disorders including Alzheimer's disease, Parkinson's disease and progressive supranuclear palsy, is the formation of neurofibrillary tangles. The formation of neurofibrillary tangles is thought to involve alterations in the microtubule-associated protein tau, the protein being hyperphosphorylated compared to its normal form. Tau is traditionally believed to be a neuron-specific protein where it is predominantly located within the axonal compartment. Supporting this specific localisation of tau, immunoreactivity was found only within axons in histologically normal rat or human brain tissue throughout the investigations carried out in this thesis. However, the novel finding of this thesis was the presence of tau immunoreactivity within oligodendrocytes in both rat and human brain tissue following acute brain injury. The studies described in this thesis were aimed at characterising the increased tau immunoreactivity present in oligodendrocytes in animal models of acute brain injury, in human post-mortem brain tissue from patients who died following a stroke or head injury and in an oligodendrocyte cell line.

In the initial study of the thesis tau-positive oligodendrocytes were present 4 h following glutamate-induced toxicity in the rat. In order to confirm that the induction of tau immunoreactivity in these cells following injury was not a phenomenum characteristic to the rat, human post-mortem brain tissue from acute brain injured patients was examined for the presence of tau-positive oligodendrocytes. Tau-positive oligodendrocytes were detected within areas of tissue, as defined by decreased haematoxylin and eosin staining, affected by brain injury, but not in tissue obtained from control patients who had no previous history of neurological or psychiatric disorders. Tau-positive oligodendrocytes were detected as early as 2 h following head injury, suggesting that this is a rapid response of these cells to acute brain injury. In support of this, the induction of tau immunoreactivity in oligodendrocytes was detected as early as 40 min following the induction of permanent focal cerebral ischaemia in the rat.

Glutamate toxicity has been shown to be involved in neuronal degeneration following ischaemic brain injury, and tau-positive oligodendrocytes were detected 4 h following intracortical perfusion of 1M monosodium glutamate, both within the resulting cortical lesion and in the white matter immediately underlying this area. This result implicated glutamate in the mechanisms underlying the accumulation of tau in oligodendrocytes following injury. However, the ability of NaCl to induce a similar response in oligodendrocytes suggested that mechanisms other than glutamate toxicity may be involved. Using pure oligodendrocyte cultures exposed to monosodium glutamate or NaCl it was shown that alteration of tau in these cells was a direct effect, not mediated through neuronal degeneration. Taken together the results of these studies indicated that some aspect of the brain injury *in vivo* other than glutamate receptor activation may be involved in the alteration of tau in oligodendrocytes. In order to investigate further the mechanisms which may be involved in this oligodendrocyte response, the effects of 3 pharmacological agents on the density of tau-positive oligodendrocytes following focal cerebral ischaemia in the rat were determined. Pre-treatment with the spin trap agent a-phenyl-tert-butyl-nitrone reduced the number of tau-positive oligodendrocytes by 55% in the subcortical white matter of the ischaemic hemisphere compared to untreated animals at 40 min after middle cerebral artery occlusion. In contrast, pre-treatment with glutamate receptor antagonists dizocilpine or 2,3-dihydroxy-6-nitro-7-sulpfamoyl-benzo(F) quinoxaline, failed to reduce the number of tau-positive oligodendrocytes following 40 min of ischaemia. Thus free radial mediated mechanisms were involved in the induction of tau immunoreactivity in oligodendrocytes following ischaemic brain injury.

In both the animal models of brain injury and the human post-mortem brain tissue obtained from patients who died following a stroke or head injury, tau-positive oligodendrocytes were detected with a range of antibodies to different epitopes of the protein. This suggested that there may be increased levels of the full-length protein. In order to investigate whether this increased immunoreactivity was due to increased protein synthesis, *in situ* hybridisation studies were performed in rat brain tissue obtained from the focal cerebral ischaemia model. However, these *in situ* hybridisation studies failed to detect increased tau mRNA within the ipsilateral white matter up to 4 h after the induction of focal cerebral ischaemia. This suggests that increased tau immunoreactivity in oligodendrocytes does not reflect increased *de novo* synthesis of the protein.

In contrast to the accumulation of full-length tau in oligodendrocytes, neuronal tau was dephosphorylated and/or degraded following acute brain injury in the rat. Dephosphorylation of tau in neurons was detected as early as 20 min following the induction of focal cerebral ischaemia in the rat. In addition, degradation of tau in neurons

but not in oligodendrocytes suggests that tau present in neuronal perikarya may be more susceptible to ischaemic brain damage than that in oligodendrocytes. However these results demonstrate that tau present in both neuronal perikarya and oligodendrocytes undergoes rapid alteration following ischaemic challenge. Glutamate excitotoxicity induced changes in tau similar to those occurring following focal cerebral ischaemia in the rat suggesting that while the accumulation of tau in oligodendrocytes is initiated through free radical mediated mechanisms, dephosphorylation and/or degradation of tau in neurons appears to be mediated through glutamate mediated mechanisms.

The results presented in this thesis provide compelling evidence that tau is present in oligodendrocytes and therefore can no longer be considered as a neuron-specific protein. Moreover these results show that tau undergoes rapid alteration within oligodendrocytes in response to acute brain injury which is initiated through free radical mediated mechanisms. Alterations in the distribution of microtubule-associated proteins in neurons following injury are thought to represent altered cytoskeletal integrity which may result in irreversible neuronal damage. It is possible to speculate therefore that alterations to tau in oligodendrocytes, following acute brain injury may also represent early stages of cytoskeletal breakdown within these cells. Oligodendrocytes have been until recently considered to be relatively unresponsive to ischaemic brain injury, and neuroprotective agents have been evaluated solely on their ability to reduce neuronal damage. One of the most successful strategies involves the blockade of glutamate receptor activation which was without effect in the reduction of the induction of tau immunoreactivity in oligodendrocytes following focal cerebral ischaemia. The results of this thesis may therefore have important consequences for the development of new therapeutic strategies, since protection of neuronal elements without the protection of glial cells would lead to delayed neuronal death rather than complete protection of the brain following acute brain injury.

# CHAPTER 1 INTRODUCTION

## 1.1 General introduction

Until recently neurons were believed to be the most important cells in the functioning of the central nervous system (CNS) with neuroglia providing a purely supportive role. With this view much attention has been directed at elucidating the mechanisms underlying neuronal degeneration, in both acute and chronic degenerative diseases of the brain. Assuming that similar mechanisms underlie neuronal degeneration in both acute and chronic disorders, animal models of acute brain injury have been used to gain insight into such mechanisms in order to uncover suitable targets for therapeutic intervention. As a result, neuroprotective agents have been evaluated on their ability to reduce the extent of neuronal death following acute brain injury.

Glutamate is a major neurotransmitter in the CNS and is essential for neuronal signalling under normal conditions (Palmer and Gerhon, 1990; Salt and Herrling, 1991). In acute brain injury, including stroke and head injury, excessive glutamate receptor activation, subsequent calcium influx and free radical formation plays a pivotal role in neuronal degeneration (see section 1.2.3). It is now widely accepted that both astrocytes and oligodendrocytes possess glutamate receptors (for review see Gallo and Russell, 1995), suggesting that receptor activation in these cells may also occur during the presence of elevated extracellular glutamate concentrations. In neurons glutamate toxicity whether via calcium release and/or free radical toxicity, has been shown to be mediated largely through the activation of N-methyl-D-aspartate (NMDA) receptors and therefore many of the most effective neuroprotective agents have been directed towards NMDA receptor blockade. Astrocytes and oligodendrocytes however appear to lack NMDA receptor subtypes and glutamate toxicity has been shown to be mediated through non-NMDA receptors in these cells (Gallo and Russell, 1995; Patneau, et. al. 1994; Puchalski, et. al. 1994; Yoshioka, et. al. 1994). In light of this it is possible to envisage that although neurons are protected successfully by NMDA antagonists, glial cells remain at risk in conditions where there are high concentrations of extracellular glutamate.

The role of astrocytes in the uptake of glutamate and other neurotoxic substances and the proliferation of these glial cells following acute brain injury has been well documented. In contrast however, oligodendrocytes have been considered to be relatively unresponsive to acute brain injury. With the exception of the undisputed role of these cells in the pathology of multiple sclerosis, the elucidation of possible roles for oligodendrocytes in the pathogenesis of both acute and chronic neurodegenerative conditions has therefore on the whole been neglected. However, the discovery that these glial cells possess glutamate

receptors suggest that oligodendrocytes may have a function in neuron-glial signalling and therefore play an important role in disease pathology (see section 1.5.5). A limited number of investigators have provided evidence that in addition to astrocytes and neurons, oligodendrocytes may have the ability to respond rapidly to ischaemic, hypoxic and traumatic brain injury (see section 1.5.7).

Cytoskeletal breakdown is thought to represent a final common pathway of neuronal degeneration in a variety of both acute and chronic degenerative conditions. Accumulation of tau within neuronal perikarya and altered distribution of the microtubule associated proteins tau, MAP2 and MAP5, may illustrate alterations in cytoskeletal integrity within these cells. Abnormal cytoskeletal profiles have recently been detected in both oligodendrocytes and astrocytes in various chronic degenerative diseases such as multiple system atrophy (MSA) and progressive supranuclear palsy (PSP) (see section 1.4.4). The distribution and density of these oligodendrocytes in MSA closely parallels the severity of the disease, in addition these cells can occur in areas where neuronal degeneration is absent. This suggests that the formation of these cytoskeletal inclusions in oligodendrocytes may represent an early and important event in the pathogenesis of this chronic degenerative disease.

Together these findings suggest that oligodendrocytes, once thought to be relatively inactive in disease, may play an important role in the progression of both acute and chronic disease of the CNS. In order to fully understand the pathogenesis of acute and chronic brain injury and to highlight areas for new therapeutic intervention, it is crucial to determine the response of these cells to brain injury, the possible mechanisms underlying such responses and the consequence of these responses to the structural integrity of the neuron. Any similarities between mechanisms underlying neuronal and oligodendroglial degeneration may lead to a target for drug intervention protecting both neuronal and glial elements of the CNS essential for effective and prolonged neuroprotection. The main focus of this thesis therefore was to examine the effect of acute brain injury on one particular cytoskeletal protein; tau in oligodendrocytes and the mechanisms through which such effects are mediated.

## 1.2 Acute brain injury

### 1.2.1 Stroke

Stroke results from a reduction of blood flow to a given area of the brain, normally caused by the blockage of a major artery, however, spontaneous intracerebral haemorrhage or decreased cardiac output are also minor causes of stroke. Cerebrovascular disease, accounts for approximately 10% of all deaths in the United Kingdom surpassed only by cancer and

heart disease. The incidence of stroke increases with age, however strokes can occur in all age groups (Bamford, et. al. 1990). For those that survive a stroke lasting for 24 hr, 50% remain permanently disabled with only 10% returning to normal activity.

Structural damage resulting from stroke or hypoxia can be limited to neurons (selective neuronal necrosis) or may also affect glia, nerve fibres and blood vessels (cerebral infarction) (Nedergaard, 1988). It is now widely accepted that neurons followed by astrocytes and oligodendrocytes are most vulnerable to hypoxia and stroke. The degree of infarction is dependant on the reduction of cerebral blood flow and the duration of hypoperfusion. A threshold for infarction of 35 % of normal cerebral blood flow or 17ml/100mg tissue has been found in sustained ischaemia (Jones, et. al. 1981). Normally infarction occurs within the territory of a given arterial supply, the extent of which is dependant on the efficiency of collateral blood supply to the affected area. Through the use of post-mortem brain tissue, the evolution of ischaemic brain damage has been well documented in man (Adams and Graham, 1988). At post-mortem, recent infarcts result in soft very swollen brain tissue and can be anaemic or haemorrhagic depending on whether or not some blood flow has been restored or if blood vessel necrosis has occurred. Histological assessment by haematoxylin and eosin (H&E) staining shows dead neurons present in the grey matter, recognised by intensely eosinophilic cytoplasm and a poorly stained nucleus with haematoxylin 4-6 h after cessation of blood flow. Areas of ischaemic brain damage are extremely difficult to define at survival times of less than 4 h following the onset of stroke. Within 12-25 h the infarct has a sharply delineated boundary due to decreased H&E staining of neuropil caused by a combination of the swelling of astrocyte processes and axons. The next stage of development occurs within 24 h and involves proliferation of microglia and astrocytes this proliferation extends to lipid phagocytes 1-2 weeks later which phagocytose the affected tissue. The grey matter becomes shrunken and granular and if the patient survives for several months following stroke the dead tissue is removed by phagocytosis and the infarct presents as a shrunken cystic lesion.

### 1.2.2 Head injury

Trauma is the largest cause of death of under 45 yr olds in the United Kingdom with head injury being largely responsible. In non-missile head injury a sudden acceleration or deceleration of the head is usually involved with or without skull fracture. The brain moves within the skull resulting in a number of shearing forces and impact of the brain against the bony protruberances of the skull. The majority of head injured patients make an uneventful recovery, however, others sustain significant irreversible brain damage to the extent that may leave them dependant on family members or institutions for the rest of their lives. In the UK alone 1 in 300 families has a member permanently disabled by head injury.

Brain damage resulting from head injury is classified by describing the damage as focal or diffuse or as primary or secondary. Primary damage involves fracture of the skull, cerebral contusions and diffuse axonal injury while secondary damage includes that due to intracranial haematoma, raised intracranial pressure, brain swelling, hypoxic brain damage or infection. Diffuse axonal injury is now widely accepted to represent the most important factor in the outcome of patients following non-missile head injury (Graham, et. al. 1995) often responsible for concussion up to and including post-traumatic coma (Blumbergs, et. al. 1989; Gennarelli, 1993). In this type of injury there is widespread disruption of axons due to the shearing stresses of acceleration/deceleration injury resulting in discrete lesions in the dorsolateral quadrant of the rostral brain stem and in the corpus callosum as well as diffuse damage to axons throughout the brain. Axonal damage can be detected histologically by H&E staining or silver staining and immunologically with anti-amyloid precursor protein antibodies (Gentleman, et. al. 1993; 1995; McKenzie, et. al. 1995) or anti-neurofilament antibodies (Christman, et. al. 1996; Grady, et. al. 1993). In patients surviving only a short time following head injury axonal swellings and axonal bulbs can be detected, however as survival time increases (several weeks) the most conspicuous feature is the presence of multiple clusters of microglia with a few axonal bulbs still detectable 2 months following injury.

Approximately 90% of fatal head injured patients show ischaemic cell change of varying degrees at post-mortem (Graham, et. al. 1978) suggesting that ischaemic brain damage plays pivotal role in patient outcome after head injury. 40% of these patients spoke following head injury implying that cerebral ischaemia may represent a secondary and therefore treatable event (Bullock and Teasdale, 1990). Ischaemic brain injury may therefore be crucial in the degree of brain damage sustained following both moderate and severe head injury. Cerebral ischaemia may be caused by intracranial haematoma or systemic insults such as hypoxia and hypotension which may result in raised intracranial pressure and therefore reduced cerebral perfusion. Further supporting a role for ischaemic brain damage in outcome after head injury, a body of evidence now exists showing that events at the time of the primary injury may leave the brain susceptible to ischaemic injury. This may occur through altered metabolism and function or by alteration of normal vascular reactivity, such that mild ischaemic insults which would normally be tolerated, may cause major ischaemic brain damage (Jenkins, et. al. 1989; Wei, et. al. 1980).

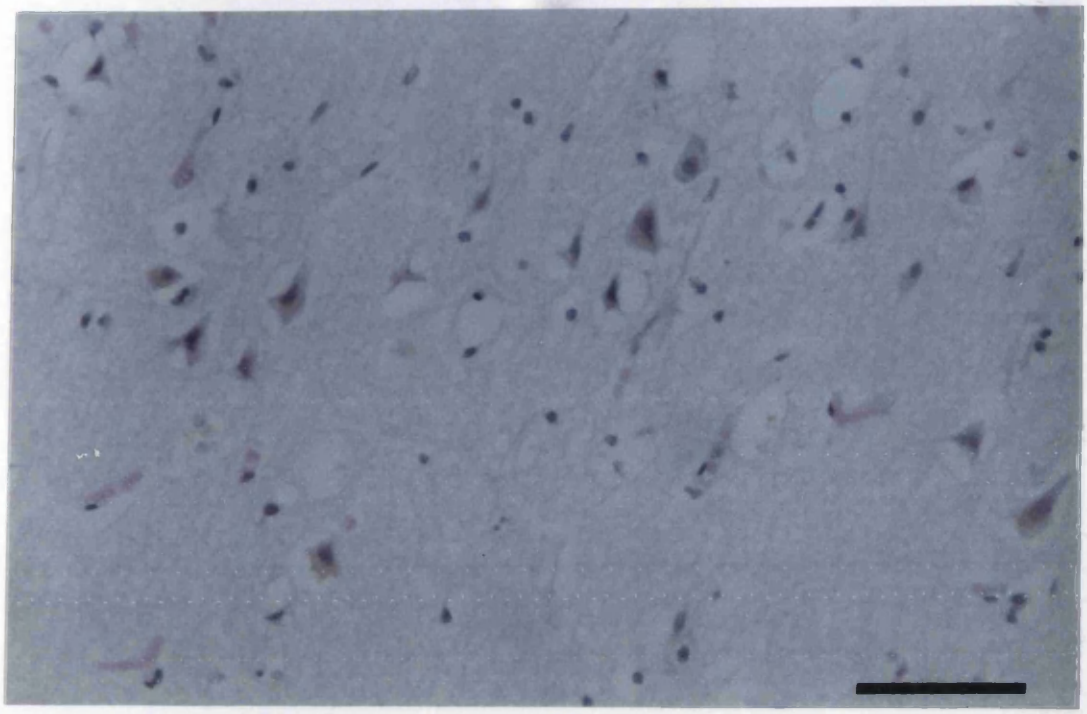


**Figure 1**. Photomicrograph of neurons displaying ischaemic cell change. Post-mortem tissue obtained from the cingulate cortex of a patient surviving 5 d following a stroke stained with H&E. Note the triangulation of the nuclei present in neurons and the eosinophilic cytoplasm of these cells. Neuropil staining is reduced, probably through swelling of axons, dendrites and astrocytic processes. This reduction in H&E staining of the neuropil clearly delineates the boundary between areas of tissue affected by ischaemic brain damage and histologically normal tissue. Scale bar = 100μm.

### 1.2.3 Mechanisms of acute brain injury

***Glutamate toxicity***

There is a large body of evidence supporting a role for glutamate excitotoxicity in the pathogenesis of cerebral ischaemia. In experimental models of cerebral ischaemia there is a marked, immediate rise in extracellular glutamate concentration, irrespective of the cause of the ischaemic episode (Beneviste, et. al. 1984; Bullock, et. al. 1990; Butcher, et. al. 1990; Faden, et. al. 1989). The largest elevation of 20 fold in the cerebral cortex and 80 fold in the caudate nucleus occurred following focal cerebral ischaemia in the rat (Butcher, et. al. 1990; Graham, et. al. 1990). This elevation peaks within 40 min after middle cerebral artery occlusion (MCAO) in the rat and probably results due to an increase release of glutamate from neurons and reduced uptake into both neurons and astrocytes (Drejer, et. al. 1985). This release appears to be dependent on the severity of blood flow reduction, elevation being triggered by blood flows below 20ml/100g/min (Shimada, et. al. 1989).

Levels of glutamate released within 10-30 min of focal cerebral ischaemia have been shown to be comparable with a glutamate concentration (60-500μM) which is toxic to neurons *in vitro* (Choi, 1987).

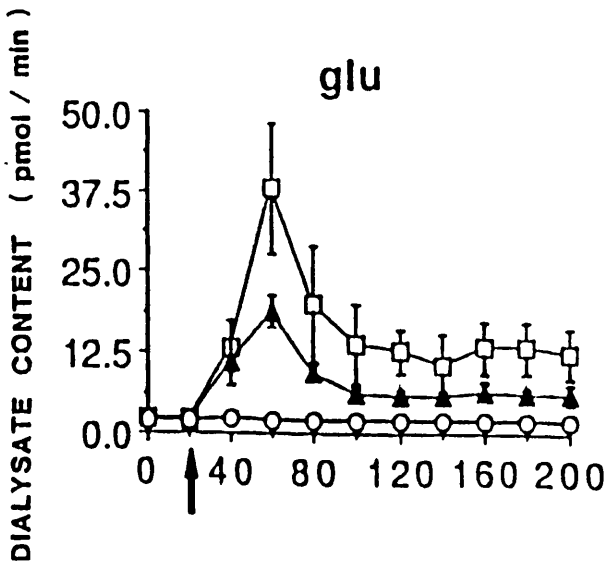



**Figure 2.** Extracellular glutamate levels are elevated following permanent MCAO in the rat. Microdialysis probes were placed in the cerebral cortex and dialysates collected every 20 min. Extracellular glutamate concentrations were increased approximately 20-fold 40 min after MCAO. Arrow respresents the onset of focal cerebral ischaemia by permanent occlusion of the middle cerebral artery (MCA). o represent sham operated controls; represent proximal occlusion of the MCA; ▲ represent distal occlusion of the MCA. Reproduced from Butcher, et. al. (1990).

Since the initial discovery of the toxic potential of glutamate by Lucas and Newhouse in (1957), when they described the degeneration of retinal neurons in neonatal mice injected systemically with glutamate, it is now widely accepted that high extracellular concentrations of glutamate are neurotoxic (Rothman and Olney, 1986; Choi, 1991). Glutamate acts on 4 major types of receptor, the NMDA receptor, the 2-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor, the kainate receptor and metabotropic receptors (Collingridge and Lester, 1989; Monaghan, et. al. 1989; Watkins, et. al. 1990). The NMDA, kainate and AMPA receptors are linked to receptor activated ion channels while metabotropic subtypes are thought to be linked to phosphoinositide metabolism as a second messenger system (Schoepp, et. al. 1990). Under normal conditions, AMPA and kainate receptors which are permeable to $Na^+$ and $K^+$ (there are also some reports of $Ca^{++}$

permeability, see below) are responsible for fast excitatory synaptic transmission in the CNS since NMDA receptors are blocked through a voltage dependant blockade by $Mg^{++}$ (MacDermott and Dale, 1987; Mayer and Westbrook, 1987).

Glutamate toxicity has been shown to involve both a sodium chloride and a calcium dependant component (Dessi, et. al. 1994). Cellular swelling following glutamate toxicity is thought to result from the influx of $Na^{+}$ through the activated NMDA receptor channel, which subsequently leads to an influx of $Cl^{-}$ and water through this channel due to the osmotic properties of these ions. Excessive activation of NMDA receptors leads to toxic levels of intracellular calcium (Choi, 1987; 1991; MacDermott, et. al. 1986; Michaels and Rothman, 1990). Depolarisation frees the $Mg^{++}$ channel block allowing the passage of $Ca^{++}$ through the receptor mediated channel, voltage-gated calcium channels and results in the mobilisation of intracellular $Ca^{++}$ stores. Although, originally believed to be impermeable to $Ca^{++}$, a large body of evidence has accumulated showing that certain configurations of non-NMDA receptor subunits form ionophores permeable to $Ca^{++}$ (Hollman, et. al. 1991; Murphy and Miller, 1989; Murphy, et. al. 1992; Ogura, et. al. 1990; Ozawa, et. al. 1991). Activation of non-NMDA receptors therefore may also contribute to calcium influx in pathological circumstances. Although the exact mechanisms involved in glutamate excitotoxicity are not fully understood, most evidence to date suggests that the influx of $Ca^{++}$ is probably largely responsible (Siesjö, et. al. 1991; Miller, et. al. 1992).

Calcium plays an important role in many normal cell functions including the control of membrane permeability, modulation of neurite extension and synapse formation, triggering neurotransmitter release and the regulation of gene expression such as the induction of immediate early genes. In addition $Ca^{++}$ modulates the activities of various enzymes including calcium-dependant phosphatases, kinases and proteases and therefore plays a role in the maintenance of neuronal structure, since cytoskeletal proteins are substrates for these enzymes (for review see Siesjö, 1988). Intracellular and extracellular $Ca^{++}$ concentrations of neurons are around $10^{-7}$ and $10^{-3}$M respectively (Choi, 1988) the intracellular concentration being strictly controlled by an ATP-dependent $Ca^{++}/Na^{+}$ exchange (Mayer and Miller, 1990) for normal cellular function. Under pathological conditions such as ischaemia or head injury however, where excessive glutamate receptor stimulation is thought to occur, the resultant influx of $Ca^{++}$ results in the loss of calcium homeostasis. Calcium "overload" in the cell results in abnormal activation of proteases, such as calpain, resulting in the breakdown of cytoskeletal proteins, activated nucleases result in DNA fragmentation, while activated lipases result in membrane damage and the production of free radicals. Together these events ultimately result in cell death (Siesjö, 1988; Siesjö and Bengtsson, 1989).

In addition to the increase in glutamate release, the most compelling evidence for a role of glutamate receptor activation in the pathogenesis of cerebral ischaemia is the large number of studies carried out, in various species, which show significant reduction in infarct size following treatment with glutamate receptor antagonists (for review see McCulloch, et. al. 1991). It is now widely accepted that glutamate antagonists reduce the volume of infarcted tissue following cerebral ischaemia independent of the species or model of ischaemia involved. The non-competative NMDA antagonist dizocilpine (MK801) and the 2,3-dihydroxy-6-nitro-7-sufomyl-benzo(F)quinoxaline (NBQX) have shown the largest and most reproducible reduction in infarct volume (Gill, et. al. 1992; Park, et. al. 1988). In the rat, pretreatment with MK801 reduced the volume of ischaemic brain damage by more than 50%, a similar reduction was also seen when administered up to 2 h following the onset of ischaemia (Gill, et. al. 1992; Park, et. al. 1988). Pre-treatment with MK801 (0.5mg/kg) 30 min prior to MCAO in the rat significantly reduced the volume of infarcted tissue (Park, et. al. 1988). Similarly pre- or post- treatment of rats with NBQX reduced the volume of infarcted tissue following MCAO in the rat (Gill, et. al. 1992) however this reduction was not a great as that of MK801. NBQX is selective antagonist at the AMPA and kainate prefering receptors with little or no affinity for the NMDA receptor complex (Sheardown, et. al. 1990).

***Free radical mediated toxicity***

Free radicals are atoms that have an orbital with an unpaired electron and are formed under normal conditions during reduction and oxidation reactions. Some free radicals are highly reactive and are able to extract an electron from neighbouring molecules in order to fill the vacancy in their orbital. Reactions such as these can damage a variety of critical biological molecules including DNA, proteins and lipids (Halliwell & Gutteridge, 1985). Superoxide radicals ($O_2^{\cdot}$), hydrogen peroxide ($H_2O_2$) and the hydroxyl radical ($OH^{\cdot}$) are produced through the reduction of molecular oxygen to water by oxidative phosphorylation. Free oxygen can accept ions from transition metals such as iron that can exist in many different valence states and therefore donate, or accept, electrons in redox reactions resulting in radical formation. Neither the superoxide radical or hydrogen peroxide is very reactive, and it is the hydroxyl radical, formed from hydrogen peroxide in a reaction catalysed by a transition metal such as iron, which is thought to be the mediator of radical toxicity. The hydroxyl radical has the ability to combine with most biological molecules, and therefore results in widespread oxidative damage (for review see Olanow, 1992).

Under normal conditions, a series of naturally occurring antioxidant defense mechanisms, prevent or limit free radical production and tissue damage (Rose and Bode, 1993). In pathological conditions such as ischaemia and trauma, an imbalance of the equilibrium of

production and protection, favouring free radical production is defined as a state of free radical stress. Cellular injury appears to increase the availability of metal ions, therefore once cellular injury has begun, damaging radical reactions tend to be amplified, especially during reperfusion when oxygen is exposed to an excess of metal ions (Choi, 1988; Hall and Braughler, 1989; Siesjö, et. al., 1989). For several biochemical, physiological and anatomical reasons the nervous system may be particularly susceptible to free radical injury compared to other organs of the body. These include a high rate of metabolic activity, high concentrations of readily oxidisable substrate, in particular membrane lipid polyunsaturated fatty acids, and low levels of intrinsic free radical scavengers such as catalase and glutathione peroxidase. Membrane disruption and destabilisation of calcium homeostasis have been proposed as major mechanisms by which free radicals induce neuronal damage (Boobis, et. al. 1989; Braughler, et. al. 1985; Orrenius et. al. 1989; Siesjo, et. al. 1981). However, the mechanisms of free radical release, the mode of action of radicals, including the molecular targets and the sequence of events that lead to cell degeneration remain largely undefined.

Although the concept of free radical mediated brain damage is widely accepted, it has proved difficult to obtain definitive evidence of free radical-mediated damage following ischaemia. Watson et. al. (1984) provided some evidence of diene conjugation in unsaturated fatty acids, which is generally accepted as the first structural manifistation of free radical damage, however, other studies failed to show significant levels of lipid peroxidation. Negative results obtained could be explained by the fact that lipid peroxidation is neither a prominent nor early sign of free radical damage (Halliwell, 1987). Superoxide ions and $OH^{\cdot}$ have been shown to be generated in trauma and ischaemia (Hall, et. al. 1993; Kontos, 1989; Sen and Phillis, 1993; Zini, et. al. 1992). An important role for free radicals in ischaemic brain damage was suggested by Floyd, et. al. (1990) and by Oliver, et. al. (1990) who described the presence of oxidated proteins in gerbils subjected to transient ischaemia, however no oxidated proteins were detected in the dog (Folbergrová, et. al. 1993). Pretreatment with free radical scavangers such as dimethylthiourea and allopurinol and the enzymes superoxide dismutase or catalase have been shown to moderately reduce the extent of ischaemic brain damage (Liu, et. al. 1989; Martz, et. al. 1989). However the most compelling evidence implicating a role for free radical release in the genesis of ischaemic brain injury comes from studies showing significant reductions in brain damage following both transient (Folbergrová, et. al. 1995; Phalmark and Siesjö, 1996; Phillis and Clough-Helfman, 1990) and permanent focal (Cao and Phillis, 1994) cerebral ischaemia by the spin trap agent α-phenyl-tert-butyl-nitrone (PBN).

PBN is one of the most widely used compounds for the detection of reactive free radicals *in vitro*. Spin trap agents are nitrone or nitroso compounds with which all forms of free radicals can react producing long lived nitroxide compounds. PBN is rapidly absorbed when injected intraperitoneally in rats, with the plasma level peaking within 15 min. Brain levels of PBN peak around 30 min after administration and decrease steadily over the next 8 h (Chen, et. al. 1990). The brain concentration of PBN is much higher than that in the blood due to the lipophilicity of the compound (Cheng, et. al. 1993). PBN has been shown to facilitate post-ischaemic reperfusion (Schultz, et. al. 1995) and improve energy status following transient ischaemia in the rat (Folbergrová, et. al. 1995) both of which could be involved in the mechanisms of tissue salvage following ischaemic injury.

***Interaction of glutamate toxicity and free radical damage***

Both glutamate excitotoxicity and free radical release have been implicated in the pathogenesis of cerebral ischaemia as described above and have until recently been independently investigated. However, observations suggest that the mechanisms of free radical damage and glutamate excitotoxicity may be at least partially interdependent. Glutamate toxicity has been shown to be markedly reduced *in vitro* (Bondy and Lee, 1993; Dykens et. al. 1987; Murphy, et. al. 1990; Miyamoto, et. al. 1989; Nakao, et. al. 1996) by various antioxidant agents and *in vivo* using PBN (Nakao, et. al. 1996; Schulz, et. al. 1995) suggesting that oxygen-free radicals contribute to glutamate toxicity. Both the glutamate antagonist MK801 and the antioxidant dimethylthiourea have been shown to reduce ischaemic brain oedema in rats indicating that both excitatory and free radical mediated mechanisms are involved in this process. Combination therapy with these two agents failed to show any significant additive effect in oedema reduction (Oh and Betz, 1991) further suggesting that glutamate toxicity and oxidative stress may be acting through similar mechanistic pathways.

Increased extracellular glutamate has been shown to inhibit the uptake of cystine from the surrounding medium into neurons through the action of the cystine/glutamate transporter (Bannai, 1986). Cystine is metabolized to cysteine within the cell which is a precursor for glutathione synthesis, reduced cystine uptake therefore results in glutathione depletion within these cells. Glutathione is an intrinsic free radical scavanger (for review see Bast, 1991) therefore its depletion renders the cell vulnerable to oxidative stress (Murphy, et. al. 1990; Miyamoto, et. al. 1989). Similar mechanism have been postulated to mediate glutamate excitotoxicity in oligodendrocyte cell cultures (Oka, et. al. 1993; Yonezawa, et. al. 1996) (see below). Free radicals have been shown to inhibit the uptake of glutamate into astrocytes (Volterra, et. al. 1994a; 1994b). Astrocytes are responsible for the uptake of glutamate and other substances that are toxic to the neuron (Hertz, et. al. 1992; Schousboe,

et. al. 1988), therefore inhibition of glutamate uptake will have profound effects on the extracellular glutamate levels in pathological conditions. In addition, free radical generating systems such as xanthine/xanthine oxidase, have been shown to cause increased release of glutamate from rat hippocampal slices *in vitro* (Pellegrini-Giampietero, et. al. 1990). Together these results give evidence for the existence of a "vicious circle" between glutamate toxicity and oxidative stress in the pathological conditions such as cerebral ischaemia.

### 1.2.4 Animal models of acute brain injury

As described above stroke and head injury are multi-factorial insults, it is impossible therefore to encompass all the events in a single animal model. However many models have been devised in an attempt to explore individual events. These models include fluid percussion, cortical impact, optic nerve stretch, subdural haematoma, glutamate toxicity and global and focal ischaemia. For the purpose of this thesis however I will focus on a model of permanent focal cerebral ischaemia and a model of glutamate toxicity, as these models were used in the studies reported here.

***Permanent focal cerebral ischaemia***

Models of focal cerebral ischaemia in the rat have gained increasing recognition due to their relation to events occurring in human stroke. In humans the most common cause of stroke is temporary or permanent occlusion of a major artery, usually the middle cerebral artery (MCA) (Brierly and Graham, 1984). Permanent and transient occlusion of the MCA in the rat can be carried out by a variety of methods (for review see Ginsberg and Busto, 1989). The subtemporal approach of Tamura, et. al. (1981) and electrocoaggulation of the MCA described briefly in section 2.6.2, has become the gold standard method of permanent proximal occlusion of the MCA. In the rat this results in reduced blood flow within the territory supplied by the MCA including most of the caudate nucleus, the lateral segment of the globus pallidus, much of the cerebral cortex, the internal capsule and adjacent subcortical white matter. Permanent occlusion results in cerebral infarction within this area. At the periphery of this region there is an area where blood flow is reduced, but is not below critical levels termed the "penumbra" (Nedergaard, 1988; Tamura, et. al. 1981; Siesjö, 1992). The boundary between viable and non-viable tissue in this model is well defined making volumetric analysis possible. This model is therefore favoured for the volumetric quantification of neuroprotection given by drugs and has been used in such investigations in the rat and other animal species (for review see McCulloch, et. al.1991).

Histological assessment of brain tissue can not accurately be performed less than four hours following MCA occlusion (MCAO), however immunological staining using an antibody directed towards MAP2 can be used to delineate ischaemic damage at shorter survival

periods (Dawson and Hallenbeck, 1996). Following 4 hr MCAO conventional H&E staining of tissue reveals swollen or lysed cells in the core of the MCA territory, they appear light due to their inability to stain with haematoxylin. In surrounding areas where blood flow is reduced shrunken and pyknotic neurons are apparent. Twenty-four hr following MCAO all of this territory may become infarcted (Graham, et. al. 1993). Most of the swollen cells disappear 24-48 hr after MCAO, small eosinophilic cells are found in the penumbra, and the lesion area is clearly delineated from normal tissue.

***Glutamate toxicity in vivo***

Glutamate toxicity is involved in neuronal death following cerebral ischaemia (see section 1.2.3), and many *in vitro* models of excitotoxicity have been employed to decipher the pathological progression of neuronal degeneration. However *in vitro* experiments lack the cellular complexity of the intact adult CNS, therefore various animal models have been devised to examine the effects of glutamate toxicity. These models allow the examination of the progression of neuronal death and the effect of glutamate antagonists on neuronal survival without the complications of reduced energy metabolism and blood flow present in ischaemia (for review see Meldrum, 1990). In such studies, selective excitatory amino acid receptor agonists are used to produce excitotoxic lesions, which avoids significant uptake by intrinsic mechanisms and interference with CNS metabolism. However in this thesis the use of monosodium glutamate was chosen for the induction of a cytotoxic lesion in the rat cortex to examine the effects of glutamate toxicity on cytoskeletal proteins. This model of glutamate toxicity has been described previously (Fujisawa, et. al. 1993a; 1993b; 1996; Landolt, et. al. 1993) and involves reterograde dialysis of glutamate into the cortex resulting in histological alterations consistent with excitotoxic neuronal damage (see section 3.1). The perfusion of 1M monosodium glutamate results in a sizeable lesion which extends in anterior and posterior directions and is characterised by an area of pallor as shown by haematoxylin and eosin staining. The histopathological features of the lesion include loss of H&E stained neurons, triangulation of the nucleus, and shrinkage of neurons and neuropil. A sharp boundary between the area of the lesion and histologically normal tissue can be readily detected in H&E stained sections (Fig. 15). In contrast, intracortical perfusion of the control solution, 1M sodium chloride, results in a smaller area of pallor on H&E stained sections compared to that resulting from glutamate perfusion. The boundary between the area of pallor and histologically normal tissue in the NaCl perfused brains was less well delined than in glutamate infused animals (Fig. 15). Within the core of a sodium chloride induced lesion, the number of H&E stained neurons are reduced compared to histologically normal tissue, while towards the edge of the lesion nuclei are triangulated.

## 1.3 Structure and function of the neuronal cytoskeleton

### 1.3.1 Components of the neuronal cytoskeleton

As mentioned above, cytoskeletal proteins are excellent substrates for calcium activated proteases therefore leaving the cytoskeletal vulnerable to ischaemic injury. Cytoskeletal breakdown is though to represent a final common pathway of irreversible neuronal degeneration in a variety of both chronic and acute degenerative diseases of the brain. Before I go on to discuss the effect of acute brain injury on these structural proteins I will briefly discuss the structure and function of the neuronal cytoskeleton. The cytoskeleton is a highly dynamic structure, involved in many essential cellular processes including cell movement, process elongation and intracellular transport. The cytoskeleton is unique to eukaryotic cells and consists of three major classes of cytoskeletal fibres: microfilaments; neurofilaments and microtubules (for review see Ludin and Matus, 1993).

*Microfilaments*

Microfilaments consist of actin fibres which are polymers of actin protein with a diameter of 6nm. Actin therefore plays a pivotal role in the shape and growth of axons and dendrites. At the leading edge of the elongating process is the growth cone which, through controlled polymerisation and rearrangement of the actin fibres is responsible for process extension. Microfilaments are not readily detectable in mature differentiated neurons, however actin is present concentrated beneath the axolemma.

*Intermediate filaments*

Intermediate filaments (7-11nm in diameter) are polymers of several intermediate filament (IF) proteins, which vary according to cell type, but are restricted to six classes of IF protein. Neurons of the central nervous system contain class IV proteins - the neurofilament proteins, NF-H (heavy chain; 115kDa), NF-M (medium chain; 110kDa) and NF-L (light chain, 60kDa). Axonal neurofilaments are connected together by numerous side arms in a rope like manner, in contrast to microtubules and microfilaments which are globular. Neurofilaments are present in axons often in association with microtubules, together they provide strength and rigidity to the axon.

*Microtubules*

Microtubules, so called as they are made of tubulin, are long cylindrical structures with a diameter of approximately 22nm. They are composed of tubulin heterodimers which consist of an α- and ß-monomer, alined longitudinally to form protofilaments which in turn join laterally to form a tube. In the neuron, microtubules are highly organised and have a defined polarity with respect to the microtubule organising centre, thus defining the polarity

of the cell. This results in a fast growing end called the plus end which is guanosine triphosphate-dependent and a slow growing end called the minus end (Wiesenberg, et. al. 1976). They are very stable, but generally dynamic structures, which go through periods of growth at the plus end that are interrupted by episodes of sudden collapse (Mitchison and Kirschner, 1984). In addition to tubulin, microtubules contain minor protein components called the microtubule associated proteins (MAP). These are a heterogeneous group of proteins originally described to co-assemble with microtubules, but have now been shown to be a major determinant of microtubule function. Microtubules are essential for the formation and structural maintenance of neurites. The highly complex morphology of neurons correlates with the abundance of microtubules in this cell type (Bray, et. al. 1978; Matus, et. al. 1986). Axons and dendrites lack the capability for protein synthesis, therefore in addition to pre-synthesised proteins, organelles and ribosomes carrying mRNA essential for protein synthesis have to be transported to the axons and dendrites to allow local protein synthesis. These functions are all mediated through the microtubule framework of the cell in conjunction with motor proteins (Bloom, 1992).

### 1.3.2 The microtubule associated proteins

The MAPs appear to be the major determinants of microtubule function and all share the ability to bind to polymerised tubulin *in vitro*. In the neuron they can be separated into 2 groups. Firstly, the motor proteins such as kinesins and dyneins which are responsible for intracellular transport of organelles and proteins from the cell body to the axons and dendrites. This is an ATP dependant function essential for neuronal survival since axons and dendrites cannot synthesize proteins (Bloom, 1992). Secondly, the structural MAPs: MAP1A/B; MAP2a/b/c; MAP4 and tau, all of which share the ability to promote tubulin polymerisation and stabilise microtubules *in vitro* (Tucker, 1990; Weingarten, et. al. 1975) suggesting that they may regulate the state of assembly of tubulin in neuronal processes and influence the stability of microtubules. MAP expression is developmentally regulated and persists in areas of the brain in which neurons continue to divide, suggesting that they may play a role in neuronal plasticity (Burgoyne, 1986; Matus, 1988; Viereck, et. al. 1989). The structural MAPs of interest in this thesis are described in more detail below.

*MAP2*

MAP2 consists of several isoforms, produced through alternative splicing of a single gene transcript, referred to as: MAP2a (280kDa); MAP2b (270kDa) and MAP2c(70kDa). MAP2c mRNA encodes a polypeptide lacking amino acids 152-1514 of the MAP2 sequence (Doll, et. al. 1990). MAP2 has 2 domains: a 200kDa N-terminal projecting arm that forms regularly spaced 100nm lateral projections from the microtubule surface and a C-terminal microtubule binding domain. It has been proposed that the projection domains of these molecules are involved in the arrangement of microtubules by acting as mechanical struts maintaining regular spacing between microtubules (Weishaar and Matus, 1993). Alternatively, it is possible that the projection domain may play a role in the interaction of tubulin with MAP2 during microtubule assembly (Fellous, et. al. 1994). The tubulin binding domain of both the high and low molecular weight proteins contains a series of 3 or 4 imperfect 18 amino acid repeats each separated by 13-14 amino acids (Kindler and Garner, 1994), which act in conjunction with adjacent sequences to affect microtubule binding affinity (Lewis, et. al. 1988). MAP2 is a phosphoprotein and the degree of phosphorylation affects the affinity of the protein for microtubules. Extensive phosphorylation reduces its ability to stimulate microtubule assembly and to bind to preformed microtubule (Burns, et. al. 1984; Murthy and Flavin 1983) and this may be a means whereby MAP2 can modulate neuronal plasticity. MAP2a is present at very low levels in embryonic and newborn brain, but becomes the major MAP in adult brain. In contrast, MAP2c is the major MAP in embryonic brain, persisting in adult brain where differentiation continues (Binder, et. al. 1984; Burgoyne and Cumming, 1984; Przyborski and Cambray-Deakin, 1995). In normal brain MAP2a and b are predominantly localised in the cytoplasm of dendrites and cell bodies where they are found associated with microtubules (Bernhardt and Matus, 1984; Caceres, et. al. 1988). In contrast, MAP2c is also found in axons of the developing brain (Riederer and Matus, 1985). This differential pattern of distribution can be explained by the compartmentalisation of MAP2 mRNA through selective transport which allows regulation of MAP2 synthesis (Tucker, et. al. 1989). MAP2a and b mRNA is found localised in dendrites (Garner, et. al. 1988; Landry, et. al. 1994) while MAP2c mRNA is present in cell bodies (Papadrikopoalou, et. al. 1986). The use of antisense oligonucleotides has provided evidence for the involvement of MAP2 in neurite extension and the maintenance of existing neurites (Caceres, et. al. 1992; Sharma, et. al. 1994).

*MAP5 (MAP1B)*

The earliest structural MAP to appear in the developing brain is MAP5 which is already present in axons at the time of their emergence from the cell body (Tucker and Matus, 1988; Tucker, et. al. 1988). In contrast to MAP2 and tau, MAP5 lacks the 3-4 repeat region at the C-terminus, however microtubule binding is mediated through 2 repeat regions. The first consists of a motif composed of 2 basic amino acids followed by 2 acidic amino acids (Noble, et. al. 1989) near the N-terminus. The second is a set of 12 imperfect repeats of 15 amino acids each near the C-terminus. MAP5 mRNA is detected in perikarya of developing neurons suggesting that synthesis occurs in the perikarya and the protein is subsequently therefore transported into the processes (Tucker, et. al. 1989). The expression of MAP5 is most prominent in extending axons (Calvert and Anderton, 1985; Black, et. al. 1994) however, protein levels decrease following termination of neurite outgrowth (Tucker and Matus, 1988), with the exception of areas of the brain where neuronal growth is continuous (Tucker and Matus, 1988; Viereck, et. al. 1989). Phosphorylation of MAP5 occurs during brain development and appears to correlate with axon growth (Calvert, et. al. 1987; Fischer and Romano-Clarke, 1990) suggesting that the function of MAP5 in axonal growth may be regulated by phosphorylation (Tucker, 1990; Gordon-Weeks, 1991). In PC12 cells stimulated with nerve growth factor, neurite extension was inhibited by the presence of MAP5 anti-sense oligonucleotides providing direct evidence for a role of MAP5 in process extension (Brugg, et. al. 1993).

*Tau*

The microtubule associated protein tau has gained much interest since the discovery, using immunohistochemical studies, that tau is the major component of neurofibrillary tangles (NFTs) found in Alzheimer's disease (AD) (Delacourte and Defossez, 1986; Grundke-Iqbal, et. al. 1986; Kosik, et. al. 1986) (see section 1.4.3). Tau was the first MAP to be isolated and shown to stimulate microtubule assembly *in vitro* (Weingarten, et. al. 1975) and molecular cloning of tau has now confirmed that there are at least six isoforms arising from alternative splicing of a single transcript encoded by a single gene located on the long arm of chromosome 17 (Druben, et. al. 1984; Himmler, et. al. 1989; Neve, et. al. 1986). Multiple isoforms of tau exist, ranging in size from 352-441 amino acids in the human brain (Goedert, et. al. 1988; 1989; Himmler, 1989; Himmler, et. al. 1989; Kanai, et. al. 1989; Kosik, et. al. 1989a; Lee, et. al. 1988), due to the differential expression of 0, 1 or 2, 29 amino acid inserts in the N-terminal region and the expression of 3 or 4 imperfect tandem repeats, 31 or 32 amino acids in length in the C-terminal region of the protein (Lee, et. al. 1988; Goedert, et. al. 1989a; Himmler, 1989) shown in Fig. 3. Tau expression is developmentally regulated, the shortest form containing three repeats and no N-terminal insert, being present in very low levels in adult brain although is abundant in fetal brain

(Goedert, et. al., 1989a,b). The longest tau transcript containing 4 repeats and a 58 amino acid insert is abundant in adult but not fetal brain. The switch from the short to longer forms of protein has been shown to occur in neurons *in vitro* when process extension is complete and synapses are forming, suggesting that this pattern of expression is probably involved in the maturation of neuronal processes (Smith, et. al. 1995; Pizzi, et. al. 1995a).

Recently an additional form of tau has been identified named "big tau", because of its higher molecular weight (110-120kDa). This isoform was originally found in the PNS (Geogrieff, et. al. 1991; Oblinger, et. al. 1991) and peripheral neuron-like cell lines (Drubin, et. al. 1988) and has recently been reported to be present in a wide range of peripheral tissues (Gu, et. al. 1996). Cloning of big tau cDNA showed that, compared to tau found in brain tissue, this tau contains additional inserts of 237 and 66 amino acids present in the middle of the protein (Couchie, et. al. 1992) or of 254 amino acids present in the N-terminal portion (Goedert, et. al. 1992), respectively in the mouse and rat. In addition big tau is transcribed from an 8kb mRNA, as opposed to the 6kb mRNA that encodes normal tau, generated through alternative splicing from the same gene, located on chromosome 17, that encodes normal tau. Another larger protein, 83kDa, reacting with tau antibodies has been detected within astrocytes (Couchie, et. al. 1988). Together this data suggests that there may be multiple isoforms of big tau present in neuronal and non-neuronal cells.

Tau is believed to be an elongated molecule with very little α-helix or ß-sheet structure (Cleveland, et. al. 1977b; Lee, et. al. 1988) and a highly assymetrical shape (Hirokawa, et. al. 1988). The most striking feature of the tau sequence is the presence of three or four imperfect tandem repeats of 31 or 32 residues located at the C-terminal which is thought to represent the microtubule binding domain (Brandt and Lee, 1993; Butner and Kischner, 1991; Lee, et. al. 1989). Ionic interactions between the basic residues of the tau protein within this repeat region and the acidic residues at the C- terminal of tubulin is partially responsible for the binding of tau to microtubules (Butner and Kischner, 1991). Tau isoforms containing four repeat regions have been shown to be more effective at binding microtubules than those with three repeats probably due to increased ionic interactions with tubulin (Goedert and Jakes, 1990). Recently however, studies carried out *in vitro* have implicated other regions of the tau protein in microtubule binding. Using fragments of tau, the regions flanking this repeat sequence have been shown to increase the affinity of tau for microtubules (Butner and Kischner, 1991; Gustke, et. al. 1994; Trinczek, et. al. 1995). In addition, Goode and Feinstein (1994) have demonstrated that intermediate regions between the repeat sequences have a higher affinity for microtubules than any of the repeat regions themselves. Together this data suggest that a complex interaction between tau and microtubules exists which cannot be explained purely through ionic interactions.

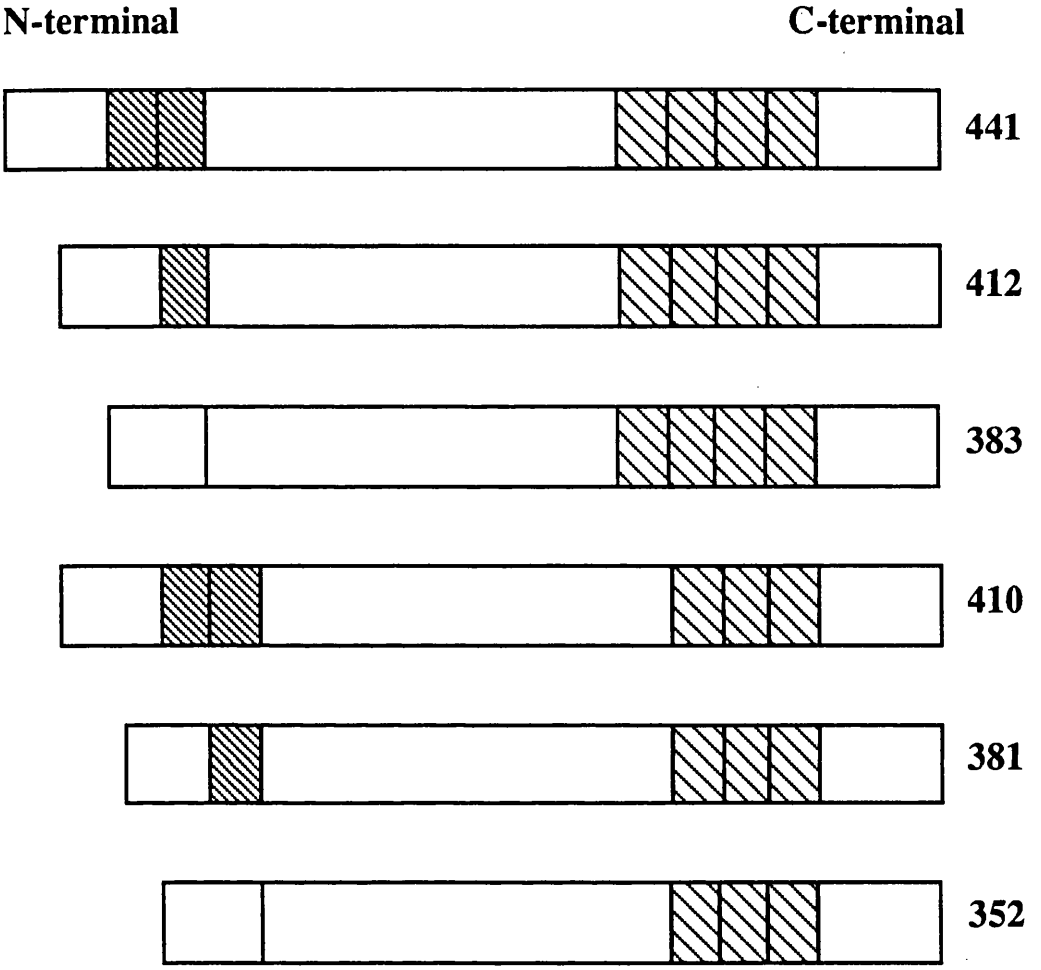



**Figure 3.** Schematic representation of the six tau isoforms found in human brain. Isoforms are formed through alternative splicing of a single tau transcript and exist due to the presence or absence of 1 or 2 inserts at the N-terminal (closely hatched shading) and the presence of 3 or 4 repeats in the C-terminal region (hatched shading). The number of amino acids in each isoform is indicated at the right of each diagram and are termed 4R, 58, 4R, 29, 4R, 0, 3R, 58, 3R, 29, 3R, 0, from top to bottom according to the nomenclature of Goedert and Jakes, (1990).

Tau contains many potential phosphorylation sites and exists as a phosphoprotein *in vivo* which contributes to the complex pattern of bands after SDS-PAGE. Phosphorylation of tau is the only post-translational modification of the protein known to date and is thought to be of functional importance, as it leads to a reduced affinity of tau for microtubules (Brandt and Lee, 1994; Lindwall and Cole, 1984; Trinczek, et. al. 1995). Fetal forms of tau which are more phosphorylated than adult forms have a decreased affinity for microtubules. Hyperphosphorylated tau present in NFTs (Gustke, et. al. 1992) lacks the ability to bind to and stabilise microtubules, however this ability can be restored following dephosphorylation of the protein (Iqbal, et. al. 1994). It has also been postulated that phosphorylation may be involved in the compartmentalisation of tau within cells (Kosik, 1990). Tau present in perikarya is phosphorylated which prevents microtubule binding and therefore allows tau to move rapidly by diffusion to the axon. However, once reaching the initial segment of the axon tau is dephosphorylated and becomes associated with microtubules, therefore entering the slow transport system that exists within axons, subsequntly leading to its predominant location within the axon.

### 1.3.3 Normal distribution of tau in the CNS

Within the normal CNS the predominant localisation of tau within the axonal compartment of neurons is widely accepted (Binder, et. al. 1985; Brion, et. al. 1988; Kowall and Kosik, 1987; Migheli, et al. 1988; Papasozomenos and Binder, 1987; Trojanowski, et. al. 1989). However the presence of tau in other cell types remains controversial. Tau has been detected in neuronal perikarya, oligodendrocytes and astrocytes following dephosphorylation of tissue sections or using phosphorylation independent antibodies (Migheli, et al. 1988; Papasozomenos and Binder, 1987). In support of its presence in oligodendrocytes, tau was detected in primary ovine oligodendrocyte cultures using western blot analysis, immunohistochemistry and *in situ* hybridisation (Lopresti, et. al. 1995). In contrast, Vouyiouklis and Brophy (1995) have failed to show the presence of tau in rat primary oligodendrocytes cultures using an antibody directed towards neuronal tau. Discrepancies between these two studies may reflect differences in the species from which the oligodendrocyte culture were derived. In addition, the absence of tau in oligodendrocytes *in situ* may suggest that cultured oligodendrocytes have different properties to those *in situ.* Electron microscopy studies of the rat brain showed that tau immunostaining is found associated with microtubules in axons and in the cytoplasm of cell bodies (Migheli, et. al. 1988). In agreement with the immunohistochemical studies, *in situ* hybridisation showed that tau mRNAs occurred in neurons and that different neuronal subtypes express different isoforms (Goedert, et. al. 1989a; Kosik, et. al. 1989a). However, tau mRNA was not detected in glial cells supporting the predominant localisation of the protein in neurons. Within the neuron, tau mRNA is localised within the cell body

and proximal part of the axon, this distribution being thought to be due to the interaction of mRNA with microtubules (Litman, et. al. 1993; 1994). Recently however, tau mRNA has been detected in ovine oligodendrocyte cultures *in vitro* (Lopresti, et. al. 1995) supporting the idea that tau protein may be present in non-neuronal cells within the CNS.

### 1.3.4 Cellular functions of tau

Information about the cellular functions of tau originated from studies employing purified microtubule preparations. These studies showed that tau promotes microtubule assembly (Cleveland, et. al. 1977a; Wiengarten, et. al. 1975) and is able to decrease the rate of transition between microtubule elongation and shortening (Dreschel, et. al. 1992; Pryer, et. al. 1992). Further evidence supporting a role for tau in microtubule binding has evolved from the use of cell lines lacking endogenous tau microinjected or transfected with human tau cDNA. In HeLa, 3T3 and rat fibroblast cells, tau expressed from transfected cDNA associated with microtubules, altered the post-translational modification of tubulin, induced microtubule bundling and protected microtubules against drug-induced depolymerisation (Drubin, et. al. 1984; Drubin and Kirschner, 1986; Lee and Rook, 1992; Lewis, et. al. 1989; Takemura, et. al. 1992). In CHO cells however, although tau expressed from transfected tau cDNA protected against drug-induced depolymerisation, tau had no effect on the monomer-polymer equilibrium (Barlow, et. al. 1994). Transfection of tau cDNA into fibroblasts was shown to change the organisation of microtubules and induced microtubule bundle formation similar to that of microtubule domains in nerve cells (Lewis, et. al. 1989; Kanai, et. al. 1992), although, no morphological changes were reported in these cells. In contrast to transfected fibroblasts, sf9 cells transfected with tau expressed in a baculovirus, produced neurite-like processes in which microtubule bundles were formed and orientated as in axons with their plus end distal to the cell body (Baas, et. al. 1991; Knops, et. al. 1991). Inhibition of tau expression with antisense oligonucleotides inhibited the development of axon-like structures in cultured neurons (Cáceres and Kosik, 1990) and in differentiated neuronal cultures resulted in the loss of axon-like structures (Cáceres, et. al. 1991). Together these reults suggest that tau is actively involved in the elongation and maintenance of neuronal processes.

As well as its presence in neuronal perikarya and axons, tau has also been shown to be localised within the nucleus of human neuroblasts (Loomis, et. al. 1990; Wang, et. al. 1993). Nuclear tau has not yet been identified in mature neuronal cells suggesting that this may play a role in neuronal differentiation. The nucleus lacks microtubules therefore these results together with the non-microtubule associated localisation of tau to ribosomes (Papasozomenos and Binder, 1987) suggests that tau may have additional roles rather than purely binding to and stabilising microtubules. It is possible to speculate that tau may target

ribosomes to microtubules for transport in the somatodendritic compartments of neurons to facilitate local protein synthesis within axons and dendrites.

As discussed in the previous section (1.3.3), tau has recently been identified in oligodendrocyte cultures. Localisation of tau at the tips of oligodendrocyte processes suggests that, as in neurons, tau may be involved in process elongation in these cells. Oligodendrocytes have abundant microtubules in their cytoplasm which have been postulated to be key elements in the transportation of myelin basic protein (MBP) mRNA and other myelin components along cellular processes to the site of myelination (Barbarese, 1991; Brophy, et. al. 1993; Colman, et. al. 1982; Trapp, et. al. 1987). Co-localization of tau with MBP within oligodendrocytes (Lopresti, et. al. 1995) suggests that tau may interact directly with MBP possibly cross-linking MBP, its RNA and the microtubule.

## 1.4 Cytoskeletal breakdown in brain degeneration

### 1.4.1 Acute brain injury

In cerebral ischaemia and traumatic brain injury (TBI), increased extracellular glutamate concentrations and the subsequent elevation of intracellular $Ca^{++}$ are thought to trigger a cascade of neurochemical events resulting in irreversible neuronal damage. Breakdown of the cytoskeleton due to the activation of calcium activated proteases, such as calpain, is thought to be one of these events. Many elements of the cytoskeleton are substrates for calpain including NF (Banik, et. al. 1987; Schlaeffer and Zimmerman, 1985), MAP2 (Johnson, et. al. 1991) and tau (Kampfl, et. al. 1996). The majority of studies investigating the effects of ischaemia and TBI have concentrated mainly on the distribution of NF, tubulin and MAP2 following injury.

Selective degradation of the neurofilament proteins NF68, NF150 and NF200 has been shown to occur following both permanent and reversible cerebral ischaemia in the rat (Nakamura, et. al. 1992; Ogata, et. al. 1989). Decreased NF 150 and NF200 immunoreactivity could be detected as early as 10 min following the induction of cerebral ischaemia, at which time degradation of other cytoskeletal components such as actin, tubulin and NF68 was not apparent suggesting that these neurofilament proteins are extremely sensitive to ischaemic injury (Ogata, et. al. 1989). The loss of NF68 immunoreactivity within the CA1 region of the hippocampus has been suggested to lead to the selective vulnerability of this area to transient global ischaemia in the rat (Nakamura, et. al. 1992). Similarly decreased NF68 and NF200 immunoreactivity was decreased in the rat 3 h following cortical impact (Postmantur, et. al. 1994). In contrast to these studies however, NF68, NF150, and NF200 immunoreactivity were increased 1-72 h following fluid

percussion induced TBI in the rat (Yaghmait and Povlishock, 1992). It is possible to speculate that the discrepancies between these studies may reflect the type and severity of brain injury used. Decreased NF immunoreactivity was detected following severe cortical impact (Postmantur, et. al. 1994) however fluid percussion resulted in a mild TBI and an increase in NF immunoreactivity (Yaghmait and Povlishock, 1992). Interestingly increased NF68 immunoreactivity has been used to show early reactive changes in axons in human diffuse axonal injury. Axonal swellings can be detected using NF68 early following head injury before significant morphological changes can be detected using routine histological staining methods (Christman, et. al. 1994; Grady, et. al. 1993).

In both transient and permanent models of cerebral ischaemia in the gerbil, MAP2 and tubulin immunoreactivity have been reported to be particularly sensitive to ischaemic damage (Hatakeyama, et. al. 1988; Kitagawa, et. al. 1989; Yanagihara, et. al. 1990). In the rat however, decreased MAP2 immunoreactivity was not detected following transient cerebral ischaemia (Geddes, et. al. 1995; Tomoika, et. al. 1992). Loss of MAP2 immunoreactivity within dendrites and neuronal perikarya was however reported following permanent focal cerebral ischaemia (Dawson and Hallenbeck, 1996). Routine histological stains fail to allow accurate estimation of lesion size less than 4 h following MCAO in the rat, however decreased MAP2 immunoreactivity was clearly delineated 1h following MCAO allowing quantitative analysis of lesion size (Dawson and Hallenbeck, 1996) showing that loss of MAP2 immunostaining can be used as a sensitive marker of early ischaemic brain damage induced by MCAO in the rat. Similarly, MAP2 immunoreactivity has also been reported to be decreased in both neuronal perikarya and dendrites following TBI (Postmantur, et. al. 1996a; 1996b; Taft, et. al. 1992) within 3 h of injury. MAP2 is associated with dendrites and may therefore reflect the sensitivity of this neuronal compartment to brain damage. Antigenic changes in tubulin and MAP2 are consistent with changes in dendrite structure observed at the electron microscope level in the gerbil following transient cerebral ischaemia (Tomioka, et. al. 1992; Yamamoto, et. al. 1986).

In contrast, although tau has gained great interest through its presence in NFTs, few studies have investigated changes in tau following cerebral ischaemia. Tau has been reported to accumulate within neuronal perikarya following transient cerebral ischaemia in the rat (Geddes, et. al. 1994) with a loss of immunoreactivity in the axon, suggesting redistribution of the protein from the axon to the cell body. Two and 6 h following permanent focal cerebral ischaemia in the rat, increased tau immunoreactivity was detected using a phosphorylation dependent antibody but not with TP70 a polyclonal antibody raised against the C-terminal of tau, immunoreactivity of which is independent of phosphorylation state. This study indicated that dephosphorylation of existing tau protein in neuronal perikarya,

rather than accumulation (Dewar and Dawson, 1995). Discrepancies between these two studies may reflect the different models of cerebral ischaemia employed. In contrast to that present in NFTs (see section 1.4.1), tau present in perikarya following both types of cerebral ischaemic injury is dephosphorylated at the Tau 1 epitope (Dewar and Dawson, 1995; Geddes, et. al. 1994). This suggests that different mechanisms may underly changes in tau occurring in acute and chronic degenerative diseases, however the possibility that tau becomes phosphorylated over time cannot be ruled out.

Glutamate excitotoxicity is involved in the pathogenesis of cerebral ischaemia and has also been implicated in Alzheimer's disease, however its involvement in the alterations of microtubule associated proteins which occur in these conditions at present remains unclear. Increased MAP2 immunoreactivity was within perikarya following exposure of neuronal cultures to high concentrations of glutamate (Bigot and Hunt, 1990, Bigot, et. al. 1991), suggesting that mechanisms other than glutamate excitotoxicity may be involved in the decreased MAP2 immunoreactivity seen following cerebral ischaemia or TBI. In support of this, pre-treatment of animals with MK801 failed to prevent decreased MAP2 immunoreactivity following TBI in the rat (Lewén, et. al. 1996). The effects of glutamate toxicity on the neuronal distribution of tau remain controversial with some groups reporting the accumulated protein to have antigenic and biochemical properties similar to tau present in neurofibrillary tangles (Mattson, 1990; Sautiere, et. al. 1992; Sindou, et. al. 1994), while others report the tau protein to be dephosphorylated at the Tau 1 epitope (Bigot and Hunt, 1990; Davis, et. al. 1995; Pizzi, et. al. 1993; 1994; 1995a), which is phosphorylated in Alzheimer's disease. Dephosphorylation of the protein following glutamate toxicity is in accordance with the dephosphorylation of tau seen following cerebral ischaemia, suggesting that glutamate may be involved in the mechanism underlying neuronal alterations to tau seen following cerebral ischaemia but not NFT formation. Discrepancies regarding the nature of accumulated tau within neurons following glutamate exposure *in vitro*, compared to those observed *in vivo* may be partially explained by the maturity of the neuronal cultures. Cells are cultured from immature tissue and immature tau differs from the adult forms of the protein being similar to those present in neurofibrillary tangles (Bramblett, et. al. 1993; Watanabe, et. al. 1993; Yoshida and Ihara, 1993). Variation in the stage of culture maturity may therefore influence the data obtained from such experiments. This highlights the care that should be exercised when extrapolating data obtained *in vitro* to events occurring in the intact adult CNS where the cellular milieu is more complex.

Together these studies show that the neuronal cytoskeleton undergoes marked alteration rapidly following acute brain injury. It is possible to speculate that altered distribution of cytoskeletal proteins following ischaemic or traumatic brain injury may reflect loss of

cytoskeletal integrity which may subsequently result in irreversible neuronal damage. The rapid alteration of cytoskeletal proteins following acute brain injury has important implications for the development of effective neuroprotective strategies, since irreversible brain damage may be triggered extremely early following injury.

### 1.4.2 Chronic neurodegenerative diseases

Alzheimer's disease, originally described by Alois Alzheimer in 1907, is the most common cause of dementia in the elderly. It is characterised by the progressive loss of memory and other cognitive functions, however diagnosis can only be confirmed by the presence of extracellular and intracellular fibrous deposits in the post-mortem brain. These deposits are amyloid plaques and NFTs respectively. Abnormal processing of the amyloid precursor protein (APP) is thought to lead to amyloid deposition (Price and Sisodia, 1994; Yankner, 1996 for reviews) and much research has concentrated on the role of this protein in the pathogenesis of AD since the discovery of familial forms of AD showing mutations in the APP gene. However recent studies have shown that the distribution and abundance of NFTs, rather than amyloid deposits, closely correlates with the severity of dementia in life (Arriagada, et. al. 1992). The density of NFTs is greatest in the nerve cells of the cerebral cortex, hippocampal formation and some subcortical nuclei, which are the areas most commonly affected in the disease. Neuronal loss results in decreased synapse numbers and therefore disruption of neurotransmission occurs. The nucleus of Meynert is severely affected by NFT formation and its disruption leads to loss of cholinergic terminals within the neocortex, which is thought to be involved in the cognitive impairment displayed by these patients. In addition to their presence in AD, NFTs have also been reported to be present in degenerating neurons of patients suffering from other chronic degenerative conditions such as progressive supranuclear palsy (PSP) (Vermersch, et. al. 1994) and Parkinson's disease (Vermersch, et. al. 1993) . In PSP neurofibrillary lesions are present in the absence of amyloid deposits suggesting that NFT formation may represent a common pathway in neurodegeneration present in a variety of chronic disorders of the brain.

### 1.4.3 Neurofibrillary tangles

Neurofibrillary lesions are found in cell bodies and apical dendrites as NFTs and in distal dendrites as neuropil threads. All the lesions consist of abnormal paired helical filaments (PHF) as their major fibrous component and straight filaments as a minor component. In electron micrographs PHF consist of two strands twisted in a helix with a periodicity of approximately 80nm and a width that varies from 8-20nm (Crowther and Wischnik 1985). The straight filaments are found as a minor component in PHF preparations, they are 15nm wide and appear to lack the variation in width of the PHF. In contrast to those present in AD, NFTs in PSP are composed mainly of straight filaments with a diameter of 13-22nm

(Montpetit, et. al. 1985). Despite the morphological differences, the biochemical properties of these two structures are very similar (Vermersch, et. al. 1994).

Immunohistochemical studies have suggested the presence of MAP2 (Six, et. al. 1992), MAP5 (Geddes, et. al. 1991; Ulloa, et. al. 1994a), ubiquitin (Morishima-Kawashima, et. al. 1993) and APP (Perry, et. al. 1993) in PHF. However the major constituent shown by both immunohistochemical and biochemical procedures, is tau protein (Brion, et. al. 1985; Grundke-Iqbal, et. al. 1986; Goedert, et. al. 1988; Wischik, et. al. 1988). Labelling of PHF with antibodies directed towards both the N- and C- terminal of tau protein suggests the presence of full length tau (Brion, et. al. 1991a; Goedert, et. al. 1992). More recently biochemical analysis showed that PHF tau runs as 3 major bands of 60, 64 and 68 kDa and a minor band of 72 kDa following sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Alkaline phosphatase treatment at high temperature resulted in the presence of 6 bands following SDS-PAGE showing that PHF contains all 6 tau isoforms in an abnormaly phosphorylated state (Brion, et. al. 1991b; Greenberg, et. al. 1990). PHF tau is hyperphosphorylated, i.e. it is phosphorylated at more sites than normal tau and for each site a higher percentage than normal of tau molecules are phosphorylated. Sites identified through the use of phosphorylation dependant antibodies include threonine residues 185,205 and 231 and serine residues 202, 396 and 404 (Biernat, et. al. 1992; Goedert, et. al. 1993; 1994; Otvos, et. al. 1995). Some of these sites are phosphorylated in fetal tau, therefore hyperphosphorylation of tau appears to occur through an exaggeration of normal phosphorylation mechanisms. Much research has therefore been concentrated on determining possible candidates for this abnormal phosphorylation. MAP kinase, GSK3 and cdc-2 kinase and calcium calmodulin dependant kinases have all been implicated in this process (Steiner, et. al. 1990; Goedert, et. al. 1995). The hyperphosphorylation of tau and subsequent lack of affinity for microtubules is thought to contribute to the formation of PHF. However, whether tau accumulation within neuronal perikarya occurs before or after phosphorylation is as yet undetermined. Loss of Tau 1 immunostaining (Tau 1 recognises tau dephosphorylated at ser 202) has been reported to occur prior to NFT formation (Bancher, et. al. 1989) suggesting that phosphorylation occurs before PHF formation. However, tau has also been shown to form PHF without prior phosphorylation (Crowther, et. al. 1994) and fragments of tau containing the repeat region which does not encompass the phosphorylation sites have also been shown to form filaments similar to PHF (Crowther et. al. 1992; Wilie, et. al 1992). In addition, phosphorylated tau transfected into COS-1 cells failed to form PHFs (Baum, et. al. 1995). The PHF core consists of the repeat region of tau leaving the N- and C-terminals in a disorganised pronase sensitive state which becomes pronase resistant following hyperphosphorylation (Crowther, et. al. 1992; Vincent, et. al. 1994). Recently Mena, et. al. (1996) have suggested that PHF formation

occurs in two stages. Firstly, the formation of tau complexes truncated at the C-terminal of the protein. Secondly, these complexes then bind together to form PHFs. It is probable therefore that phosphorylation is not the only factor involved in PHF formation.

The formation of PHF in neurons is thought to be involved in the degeneration of neurons in AD. Accumulation of NFT accompanies a decrease in the number of microtubules, decreased affinity for microtubule binding and altered microtubule morphology resulting in loss of microtubule function (Lu and Wood, 1993). As well as decreased affinity of PHF tau for microtubules, the presence of PHF tau appears to inhibit the binding of normal tau to microtubules possibly through binding to normal tau itself leaving it unavailable for microtubule binding (Alonso, et. al. 1994). Within neurons presenting NFTs there is often an accumulation of degraded mitochondria, lysosomes and multivesicular bodies. These organelles are transported along the axon by interaction with microtubules in an ATP dependant manner. This loss of vital functions is thought to lead to rapid degeneration of the cell.

In addition to genetic factors such as APP mutations leading to abnormal amyloid deposition (Price and Sisodia, 1994; Yankner, 1996 for reviews) factors including head injury (Roberts et. al. 1991), free radical damage (Evans, 1993; Friedlich and Butcher, 1994) and glutamate excitotoxicity (Choi, et. al. 1988; Greenamayre and Young, 1989) have been implicated to play a role in the pathogenesis of AD. Clinical investigations have shown that treatment of patients with antioxidant nutrients such as vitamin E and C, selenium and zinc have indicated a reduction in the progression of the disease (McLachlan, et. al. 1991). Furthermore age related increases in oxidatively-inactivated enzymes in the brain and impaired memory in aged gerbils has been reduced by the spin trap agent PBN (Carney, et. al. 1991). It has been postulated that abnormal energy production in the disease may result in increased susceptibility of cells to excitotoxic injury (Greenamyre, 1991). Thus excitotoxicty would occur at physiological levels of glutamate.

At present however, it is unknown if free radical damage and/or glutamate toxicity are involved in the mechanisms underlying NFT formation. As discussed in section 1.4.1, glutamate toxicity has been shown to increase tau in neuronal perikarya (Bigot and Hunt, 1990; Davis, et. al. 1995; Mattson, 1990; Pizzi, et. al. 1993; 1994; 1995; Sautiere, et. al. 1992; Sindou, et. al. 1994), however the antigenic properties of the accumulated protein remains controversial. Some groups report the presence of tau similar to that of PHF tau (Mattson, 1990; Sautiere, et. al. 1992; Sindou, et. al. 1994) while others report accumulated tau to be dephosphorylated at the Tau 1 epitope (Bigot and Hunt, 1990; Davis, et. al. 1995).

### 1.4.4 Glial fibrillary tangles

Argyrophilic oligodendroglial inclusions were first described by Papp, et. al. (1989) and since this time they have been used routinely in histological assessment of multiple system atrophy (MSA). These inclusions are most commonly detected within the cytoplasm, but have also been detected in the nucleus of the cell (Papp and Lantos, 1992). MSA is a chronic neurodegenerative condition comprising a combination of striatonigral degeneration (Adams, et. al. 1964), olivopontocerebellar atrophy (Duvoisin, 1984) and autonomic failure (Shy and Drager, 1960). Although glial inclusions have been used as a diagnostic marker for this disease for many years, it is only recently been recognised that the formation of these cytoplasmic inclusions may play a pivotal role in the disease pathogenesis. The density and distribution of oligodendroglial inclusions rather than neuronal degeneration correlates with the areas of affected brain and the severity of the disease (Papp and Lantos, 1994). Inclusion bearing cells are found mainly within regions in which myelinated fibres were affected by MSA lesions (Abe, et. al. 1992; Papp and Lantos, 1994) and also in areas of grey matter where little or no neuronal loss was detected, suggesting that neuronal degeneration is not required to induce inclusion formation within oligodendrocytes. The presence of these inclusion bearing cells in areas of tissue which appear normal, suggests that the formation of oligodendrocyte inclusions may be an early event in the degeneration characteristic of MSA (Abe, et. al. 1992; Papp and Lantos, 1994). Although the presence of oligodendroglial inclusions has been used in the diagnosis of MSA similar inclusions have also been described in post-mortem brain tissue of patients dying as the result of AD, PD and PSP (Iwatsubo, et. al. 1994; Nishimura, et. al. 1995; Yamada and McGeer, 1990). There are relatively few oligodendroglial inclusions compared to NFTs in AD and are therefore unlikely to be involved in the pathogenesis of this disease (Nishimura, et. al. 1995). In PSP, glial inclusions are found mainly within astrocytes, although oligodendroglial inclusions are detected in some cases (Iwatsubo, et. al. 1994; Nishimura, et. al. 1995; Yamada and McGeer, 1990). Cytoplasmic inclusions found within astrocytes in PSP are distinct from those occuring in MSA in that they can not be stained using ubiquitin antibodies (Yamada, et. al. 1992).

Neither the ultrastructure nor the biochemical composition of oligodendroglial inclusions is well defined. Some studies however have reported that these inclusions contain fibrils between 20 and 30nm in diameter, however whether these fibrils are microtubules or another form of cytoskeletal component remains controversial (Abe, et. al. 1992; Kato, et. al. 1991). In support of the presence of microtubules, oligodendroglial inclusions have been reported to stain positive for α and ß-tubulin subunits (Abe, et. al. 1992; Aria, et. al. 1992; Kato, et. al. 1991). Independent of cell type or the underlying disease, immunohistochemical studies have shown that all forms of glial inclusion can be stained

with antibodies directed against tau (Murayama, et. al. 1992; Iwatsubo, et. al. 1994; Nishimura, et. al. 1992; Yamada and McGeer, 1990; Yamada, et. al. 1992) and antibodies directed towards PHF tau (Iwatsubo, et. al. 1994). Together this data suggests that tau may undergo similar modification in both neuronal and glial cells in chronic neurodegenerative conditions. Immunohistochemical studies have also shown the presence of MAP5 with oligodendrocyte inclusions present in MSA.

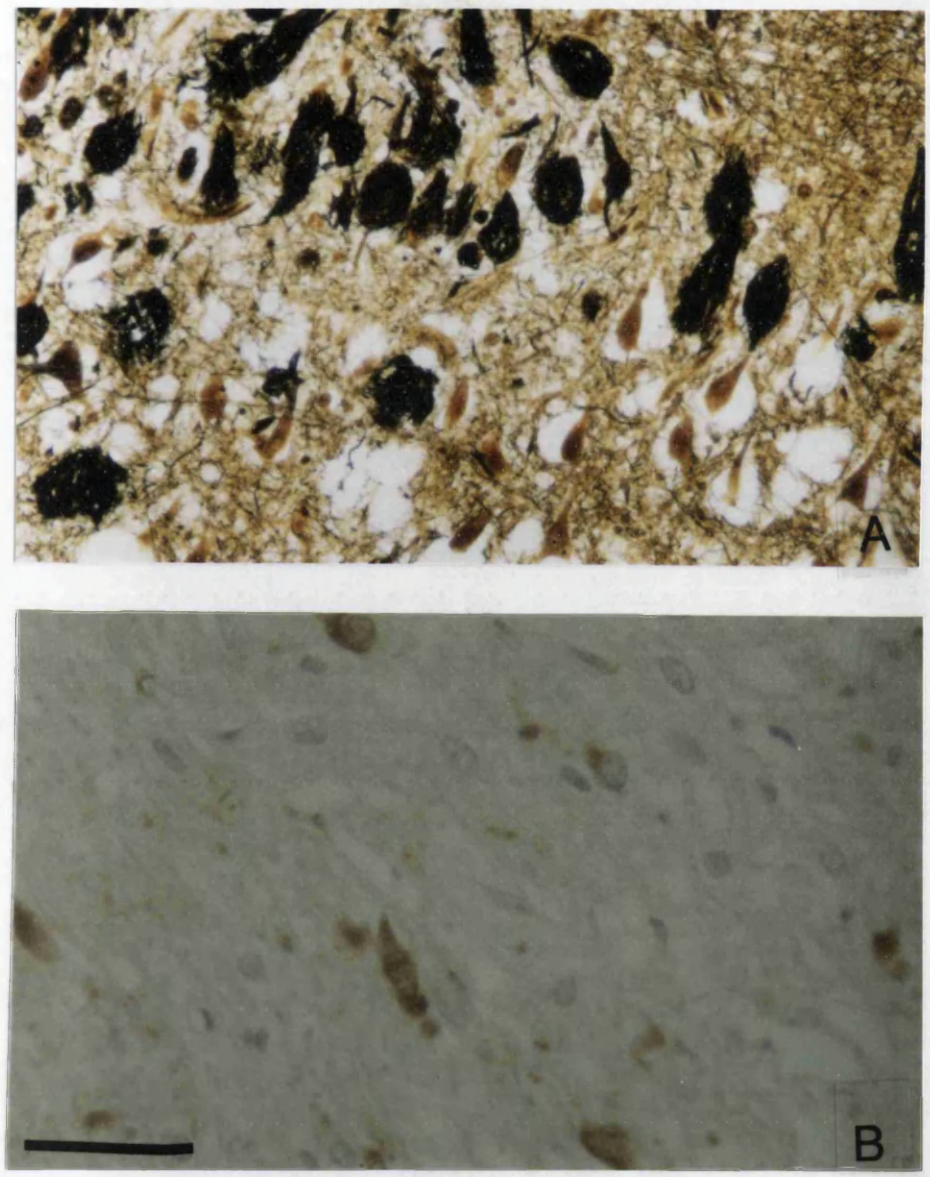



**Figure 4**. Photomicrograph showing NFT and GFT morphology. A) shows NFTs present in the hippocampus of a 85 year old female diagnosed with Alzheimer's disease at post-mortem stained with Bielschowsky modified silver stain. B) shows the morphological appearance of oligodendrocyte cytoplasmic inclusions, stained with an anti-ubiquitin antibody, from the pons of a 52 year old female who died with MSA. Scale bar = 50μm.

## 1.5 The neuroglial cells

### 1.5.1 Introduction

Recognition and characterisation of glial cells occurred long after the description of neurons. Neuroglia were first described by Virchow in 1846 as "nerve glue" which following the advent of electron microscopy on tissue from the central nervous system fixed by perfusion was found to consist of perikarya and processes. Knowledge of the morphologies of these cells emerged with the development of metallic impregnation techniques. The "astrocyte" was then identified and separated from other neuroglia and thus became the second element of the central nervous system next to the neuron (Cajal, 1913, 1916). In 1897 and 1899 Robertson was the first to identify the "mesoglia" cell, so named due to its different morphology compared to the commonly accepted neuroglial cell. This cell type was later characterised and named the "oligodendroglia" (del Rio-Hortega, 1919; 1921) comprising the third element of the central nervous system. Since these original characterisations the glial cells have been separated into the macroglia, consisting of the astrocytes and oligodendroglia and the microglia. Unlike neurons, macroglia have only one type of process and have the ability to divide throughout life.

### 1.5.2 Astrocytes

Of the 3 types of glial cells the astrocyte has been the most extensively studied. At the light microscope level astrocytes are recognised as star shaped cells whose processes extend into the surrounding neuropil. In white matter these cells have many fibrils and are referred to as fibrous astrocytes, however those in the grey matter have fewer fibrils and are thus called protoplasmic astrocytes. The astrocyte contains all normal organelles found in eukaryotic cells, however these are more sparse than in other glia. The most prominent cytoplasmic components of the astrocyte are filaments 8 nm in diameter which occur in the perikarya and extend into the processes. These filaments closely resemble intermediate filaments found in neurons, however their dimensions are smaller and they regularly appear as closely packed bundles. Glial fibrillary acidic protein (GFAP, MW 47kDa) one of the intermediate filament proteins is known to be the major component of these filaments (Eng and Bigbee, 1978). In the CNS, GFAP is unique to astrocytes and therefore antibodies raised against this protein are routinely used as specific cellular markers to distinguish these cells from other glia and neurons (Martin, et. al., 1991). Astrocytes can therefore be identified in the CNS at the light microscope level by both immunological and morphological assessment (see Fig. 5).

### 1.5.3 Microglia

Although microglia were detected in the early 1900's it was 1932 before del Rio Hortega classified the microglia. These cells are small compared to astrocytes, have an elongated or triangular nucleus and display many fine spine-like branches. Microglia are found in both white and grey matter, but are more abundant in grey matter, where they are often situated near neurons or blood vessels. In the normal adult brain microglial cells appear to be inactive, however become activated by any inflammatory or degenerative process. Once activated they move to the site of injury, where they turn into macrophages and remove the debris. At this time, the cells may assume a rounded or rodlike shape as they fill with lipids and cell constituents. They then transport the debris to the vicinity of blood vessels for clearance from the brain tissue.

### 1.5.4 Oligodendrocytes

As mentioned above the oligodendrocyte was first described by Robertson (1899) however it was decades later before this cell received its name and definitive description by del Rio Hortega (1921; 1928). The cell was described as having a small soma with sparse cytoplasm and in contrast to the astrocyte which shows an elaborate array of processes these cells have few (between 2-10) processes thus leading to the name oligodendrocyte meaning "cell with few branches". Since its original identification the oligodendrocyte has become well characterised both at the light and electron microscope level. Del Rio Hortega separated oligodendrocytes into two major classes according to the anatomical position: 1) perineuronal - where the cell body is adjacent to a neuron and located in the grey matter; 2) intrafascicular - oligodendrocytes positioned between nerve fibres in white matter. Electron microscopy has confirmed del Rio Hortega's descriptions (Stensaas and Stensaas, 1968), the mature oligodendrocyte is a small cell (10-20μm) in diameter with a dense nucleus and cytoplasm (Peters, et. al. 1976) due to the presence of heterochromatin and an unknown dense material occupying the cytosol. Oligodendrocytes can display different morphological profiles involving their cytoplasmic density and the clumping of nuclear chromatin, described as light, medium and dark (Mori and Leblond, 1970). These authors speculated that in the corpus callosum of young rats the different morphologies may reflect cells in different stages of maturation, with the larger light ones being the most actively dividing cells and the most mature being the small dark ones. There are no unique organelles within the oligodendrocyte cytoplasm, however they contain an abundance of ribosomes both free and associated with membranes. In contrast to astrocytes, oligodendrocytes lack GFAP containing filaments, however have an abundance of microtubules similar to those present in neurons.

At the light microscope level oligodendrocytes are morphologically distinct from astrocytes as they display a small perikarya with little cytoplasm. In contrast to astrocytes oligodendrocyte processes are not readily detected *in situ*. In vitro, oligodendrocytes can be easily identified by surface antigens which change through each stage of maturation. However, successful immunological markers for oligodendrocytes in tissue sections have been more difficult to develop. Oligodendrocytes have an abundance of transferrin which although is not unique to oligodendrocytes allows immunological detection of these cells at levels below the threshold for detection in other cell types. Anti-transferrin antibodies are therefore used routinely in conjunction with anti-GFAP status and morphology to identify oligodendrocytes *in situ* (Conner and Finne, 1986; Martin, et. al. 1991) (see Fig. 5).

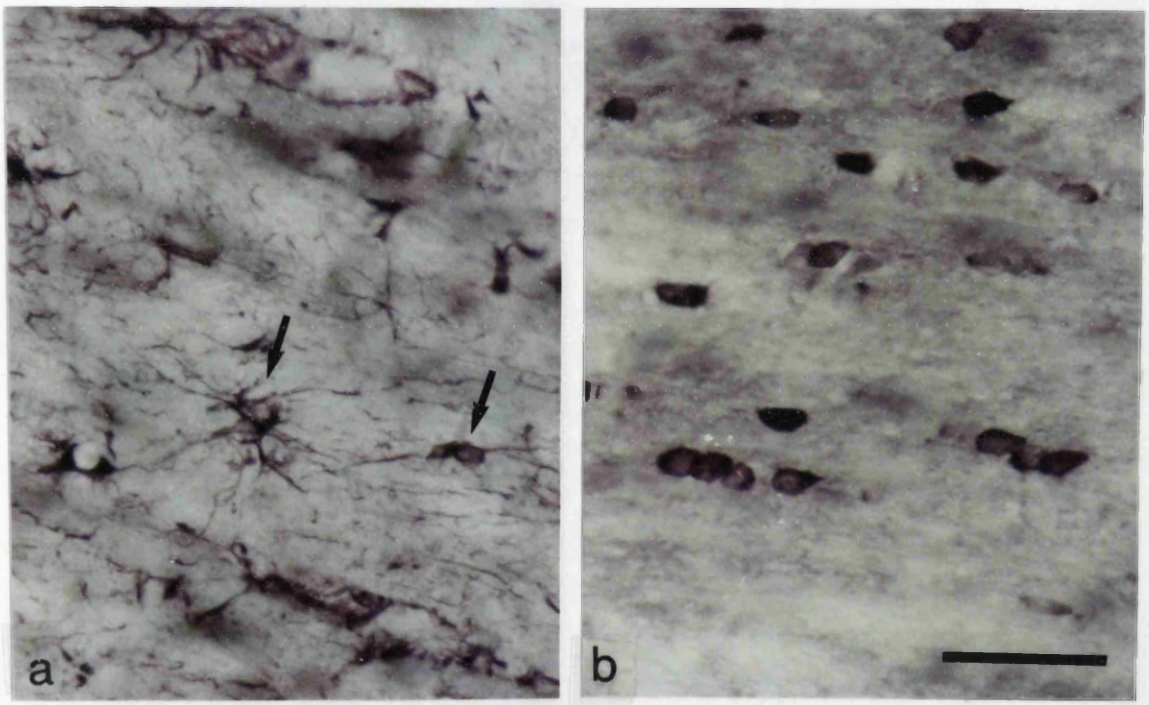



**Figure 5**. Rat corpus callosum showing astrocytes immunostained with GFAP (a) and oligodendrocytes stained with transferrin (b). *In situ* astrocytes and oligodendrocytes can be easily distinguished by both immunological and morphological criteria. Astrocytes contain abundant intermediate filaments which consist mainly of GFAP, which in the CNS is unique to astrocytes. Immunohistochemical staining of tissue with antibodies raised against GFAP therefore provides a cellular marker for astrocytes (a). Oligodendrocytes contain an abundance of transferrin, therefore antibodies directed against this protein can serve as a good cellular marker in the CNS (b). Staining with these markers distinguishes these cells immunologically and also displays the cellular morphology of these cells. The oligodendrocyte has a small soma with sparse cytoplasm and processes are not readily detectable *in situ*. In contrast however, astrocytes have a larger soma and display an elaborate array of cellular processes (arrows). Scale bar = 50μm.

### 1.5.5 Oligodendrocyte function

*Myelination*

Myelination is the most well documented function of oligodendrocytes and detailed discussion of this process is outwith the scope of this thesis, however see McLaurin and Yong, (1994) for review. During development, axons in the CNS become ensheathed with myelin produced by oligodendrocytes, each cell being capable of myelinating many segments of different axons (Bunge, et. al. 1962; Bunge, 1968; Mathews and Duncan, 1971). Myelin is a highly complex and specialised extension of the oligodendrocyte plasma membrane, with each cell capable of making approximately three times its weight in membrane each day during active myelination (Norton and Poduslo, 1973). Myelin is composed mainly of lipids, none of which are unique to oligodendrocytes, however cerebroside is the most characteristic of oligodendrocytes and is often used as a cellular marker (Raff, et. al. 1978). Lipid accounts for approximately 70% of myelin the rest of which consists of protein, the major ones being proteolipid protein (PLP) and myelin basic protein (MBP) which account for 50% and 30% of total myelin protein respectively (Lees and Brostoff, 1984). More minor protein components include myelin-associated oligodendrocyte basic protein, 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) and myelin associated glycoprotein.

As the oligodendrocyte process encircles its axon, the cytoplasm is eliminated forming a compacted multilamellar sheath. Each cell generating myelin only provides myelin for a segment of the axon which therefore results in a discontinuous sheath with areas of axon uncovered called the nodes of Ranvier. At the node of Ranvier there is a high concentration of ion channels and this is the only point where the axon comes into contact with the extracellular fluid. As the nerve impulse passes along the axon they "jump" from node to node. This feature permits signal transmission to occur at a greater velocity, frequency and efficiency than unmyelinated fibers. Demyelination during disease such as multiple sclerosis therefore results in impaired signal transmission, leading to devastating neurological symptoms.

***Inhibition of neurite outgrowth***

Using video time lapse cinematography, Schwab and colleagues have provided evidence for a role of oligodendrocytes and myelin in the inhibition of neurite outgrowth. Astrocytes were shown to be a good substrate for neurite extension while contact with oligodendrocytes or myelin resulted in growth cone collapse (Schwab, et. al. 1988). Biochemical analysis has revealed two groups of proteins of molecular weights 35kDa and 250kDa that are uniquely found tightly bound to membrane proteins of CNS

oligodendrocytes and have been implicated in the inhibitory action of oligodendrocytes to neurite extension (Schwab, et. al. 1993). These proteins are expressed late in development and may therefore play a role in guiding late developing CNS tracts in order to avoid abnormal sprouting and regulate axon number (Cadelli, et. al. 1992; Chiquet, 1989; Collelo and Schwab, 1994). The continued expression of these factors on oligodendrocytes following brain injury may therefore be a key feature in the failure of axonal regeneration. Biochemical analysis of these proteins has revealed two groups of proteins of molecular weights 35kDa and 250kDa that are uniquely found tightly bound to membrane proteins of CNS oligodendrocytes (Schwab, et. al. 1993).

***Neuronal-glial signalling***

The presence of glutamate receptor subtypes on oligodendrocytes and astrocytes (see section 1.5.7) raises the possibility that neurotransmitters released at the synapse or along axons can not only influence neurons, but also glial cells that are intimately associated with neuronal elements (for review see Finkbeiner, 1993). This raises the suggestion that signalling may occur between neurons and glia, via excitatory amino acids. In support of glutamate mediated signalling between neurons and glia, Dani, et. al (1992) showed that glial cells receive signals during neuronal activity in hippocampal organotypic cultures possibly mediated through glutamate. Excitatory amino acid receptor activation may result in the release of growth factor, neuropeptides, or neurotransmitters from glia (Parpura, et. al. 1994) and the release of such substances from glia may participate in activity-dependant plasticity in the adult brain (Kang and Schuman, 1995). Glutamate receptor activation has also been shown to increase *c-fos* and *c-jun* (immediate early genes) expression and inhibit proliferation of oligodendrocyte progenitors through non-NMDA receptor activation (Liu and Almazan, 1995; Pende, et. al. 1994). Repetitive axonal stimulation or treatment of the axons with glutamate in neonatal rat optic nerve also stimulated this response in oligodendrocytes (Mack, et. al. 1994). Immediate early genes encode transcription factors that regulate the expression of late genes which may result in permanent phenotypic alterations (Sheng and Greenberg, 1990).

Most studies to date have concentrated on the role of astrocytes in glia-neuronal signalling, for example $K^+$ buffering. During neuronal activity $K^+$ levels increase immediately surrounding the neuron, which may lead to prolonged depolarisation if not removed. Glutamate receptor activation in astrocytes during neuronal activity leads to increased intracellular $Ca^{++}$ within these cells resulting in $K^+$ uptake through the activity of the $Na^+/Ca^{++}$ exchanger and $Na^+/K^+$ pump (for review see Barres, et. al 1991). These $Ca^{++}$ signals can be propagated as waves through astrocyte gap junctions (Giaume, et. al. 1991). Recently Nedergaard (1994) showed that $Ca^{++}$ signals can pass from astrocytes to neurons

through gap junctions suggesting that neurons can signal to glia which in turn can signal back to neurons. Due to the presence of glutamate receptors on oligodendrocytes it is possible to speculate that these cells also have the capacity to respond to neuronal signals such as glutamate release. Glutamate receptor activation on these cells results in increased intracellular calcium suggesting that oligodendrocytes may also be capable of $K^+$ buffering.

### 1.5.6 The oligodendrocyte cytoskeleton

In contrast to astrocytes, mature oligodendrocytes *in vivo* do not contain intermediate filaments, they do however contain large amounts of microtubules. Similarly to those found in neurons (see section 1.3.1) these microtubules are 25 nm in diameter and consist of tubulin dimers. They are arranged randomly throughout the perikaryal cytoplasm although in the processes they are arranged parallel to one another as in axons and dendrites of neurons. Since myelination involves the extension of long processes requiring substantial changes in the shape of the oligodendrocyte and given what is known about neuronal cytoskeletal functions it seems likely that the cytoskeleton of oligodendrocytes may play an important role in process extension. In support of this theory, tubulin has been identified as a constituent of myelin isolated from rat brain (Gozes and Richter-Landsberg, 1978). This protein is synthesized prior to myelination and has therefore been suggested to be involved in the formation and maintenance of myelin shape. In addition, purified MBP can form complexes with tubulin and actin (Modesti and Barra, 1986). Using cell culture both MBP and CNP have been shown to associate with the cytoskeleton of oligodendrocytes. CNP and MBP are synthesized on free ribosomes (Colman, et. al. 1982; Gillespie, et. al. 1989) and have to be transported into the processes for myelin formation. It is possible to speculate therefore that microtubular networks within these cells are essential for the transportation of the machinery required for myelin formation into the processes. In immature cells with many processes, CNP appears to localise with filamentous actin which is more abundant than microtubules at this stage. In more mature oligodendrocytes, where the number of processes is decreased and the diameter thickened, filaments appear to be lost and microtubules predominate to which MBP is found to colocalize (Wilson and Brophy, 1989). It is possible to speculate that actin filaments are responsible for the direction of growth however microtubules are involved in the elongation and thickening of processes.

The microtubule associated proteins as discussed in sections 1.3.2 play an important role in microtubule function in neurons. In neurons these proteins appear to be developmentally regulated and are thought to be involved in the formation and extension of axons and dendrites. Although traditionally believed to be neuron specific, MAP2, MAP4, MAP5 and tau have recently been identified in oligodendrocyte cultures (Diaz-Nido and Avila, 1989;

Fischer, et. al. 1990; Lopresti, et. al. 1995; Ulloa, et. al. 1994b; Vouyiouklis and Brophy, 1995). MAP2c expression in neurons is present during development which regresses and is replaced by the higher molecular weight isoforms. In oligodendrocytes a novel MAP2c isoform containing four repeat sequences is present in progenitor cells. This expression of MAP2c is higher in pre-oligodendrocytes than progenitors but decreased again when reaching the final stages of maturation suggesting that MAP2c is required with the transitional pre-oligodendroglial stage (Vouyiouklis and Brophy, 1995). MAP2c is therefore unlikely to be involved in stabilizing myelin processes but important in initiating the extension of processes. The heterogeneous nature of MAP2c may represent various phosphorylation states of the protein (Vouyiouklis and Brophy, 1995). Similarly to neurons, MAP5 expression is developmentally regulated and its appearance coincides with the development of pre-oligodendrocytes from progenitors (Vouyiouklis and Brophy, 1995), when the cells start to develop a network of extensive processes. MAP5 found in oligodendrocytes colocalizes with tubulin (Fischer, et. al. 1990) and has a phosphorylation pattern distinct from that in neurons (Ulloa, et. al. 1994). In contrast to neurons, however MAP5 levels appear to remain elevated in mature oligodendrocytes. Together these results suggest a role for MAP5 in the stabilization of the cytoskeleton and extension of processes in mature oligodendrocytes. The presence of the microtubule-associated protein tau in oligodendrocytes remains a controversial issue. Many groups have failed to detect the presence of tau in non-neuronal cells using antibodies directed towards neuronal tau (Brion, et. al. 1988; Trojanowski, et. al. 1989; Vouyiouklis and Brophy, 1995). However, tau has been reported in oligodendrocytes of rat brain tissue before (Migheli, et. al. 1988) and after enzymatic dephosphorylation of the tissue (Papasozomenos and Binder, 1987). It should be noted however, that tau was not detected in intrafasicular oligodendrocytes following dephosphorylation of the tissue and was only detected in perineuronal oligodendrocytes (Papasozomenos and Binder, 1987). Lopresti, et. al. (1995) failed to detect tau within oligodendrocytes of rat brain tissue with Tau 1 however did detect tau in these cells using Tau 5, an antibody raised against glial tau. In addition Lopresti et. al. (1995) provided evidence for the presence of tau protein and tau mRNA in oligodendrocyte cultures and that tau appears to colocalize with MBP. This raises the possibility that tau may be involved in the transportation of MBP to the site of myelination.

### 1.5.7 The effect of ischaemic injury on oligodendrocytes

Research to date has mainly concentrated on the mechanisms underlying neuronal death in both acute and chronic degenerative disorders of the brain. However in light of the growing evidence showing the importance of glia in the maintenance of neuronal integrity it is essential to gain an understanding of events occuring within these cells under pathological conditions. The role of astrocytes in glutamate uptake and their proliferation following

cerebral ischaemia has been well documented (Hertz, et. al. 1992; Petito and Babbiak, 1982; Schousboe, et. al. 1988). In contrast however the reaction of oligodendrocytes to ischaemia is not well known.

Oligodendrocytes are relatively resistant to ischaemia (Brierly and Graham, 1984), but can be selectively damaged following cardiac arrest (Plum, et. al. 1962) or severe hypotension (Ginsberg, et. al. 1976). Using a model of transient cerebral ischaemia in the rat Petito (1986) showed that in addition to neurons and astrocytes, oligodendrocytes can also respond rapidly to ischaemic injury. Medium-light oligodendrocytes increased in size 3, 30 and 180 min following ischaemia. These cells showed increased microtubules and tubovascular profiles and accounted for approximately 50% of all glial cells within 2 h of injury, however the number present decreased to 6.5% by 3 h. In contrast, dark cells thought to represent the most mature oligodendrocytes, showed little change following cerebral ischaemia. Petito suggests that cell loss may represent a transition of medium-light oligodendrocytes to intermediate glia which have some characteristics of both oligodendrocytes and astrocytes. This may represent an "active" oligodendrocyte. Further evidence showing that oligodendrocytes are capable of responding rapidly to injury *in vivo* was provided by Ludwin, (1984) who showed that medium oligodendrocytes proliferate 24 h following TBI in the mouse. The density of responsive cells was greatest in the cortex immediately surrounding the wound and decreased in as the distance from the wound increased. Proliferating oligodendrocytes were also detected in the white matter immediately below the wound and across the midline of the corpus callosum similar to the spread of oedema in this model.

Recently rat neonatal oligodendrocytes and human oligodendroglioma cells have been shown to increase ferritin synthesis following 6 h of hypoxia *in vitro* (Qi and Dawson, 1992; 1994; 1995). This increase in ferritin synthesis was seen in conjunction with a decrease in MBP levels but no change in structural proteins such as actin. The increased synthesis of ferritin occurs at a post-transcriptional level, and has been suggested to be part of a protective response of oligodendrocytes to hypoxic injury (Qi, et. al. 1995). Ferritin is a natural iron chelator (Balla, et. al. 1992) and may therefore play an important role in the protection of oligodendrocytes and myelin from free-radical mediated damage. Iron is a catalyst for the production of $OH^{\cdot}$ from $H_2O_2$ therefore chelation of iron would protect the cells from the damaging effects of $OH^{\cdot}$. Exposure of the same cultures to hydrogen peroxide induced ferritin synthesis (Qi, et. al. 1995). Together these results show that oligodendrocytes can respond rapidly to hypoxic injury *in vitro* which involves free radical mediated mechanisms.

The studies described above suggest that oligodendrocytes may play a more active role in the pathogenesis of brain damage resulting from cerebral ischaemia than originally believed. The mechanisms underlying the changes described following ischaemic injury are however unknown. As described above glutamate toxicity and free radical mechanisms have been implicated in ischaemic injury, however whether these mechanisms are involved in the responses of oligodendrocytes to ischaemia is not known. Electrophysiological studies have shown that mature oligodendrocytes respond directly to glutamate exposure *in vitro* (Ballanyi and Kettenman, 1990; Butt and Tutton, 1992; Patneau, et. al. 1994; Puchalski, et. al. 1994). Molecular analysis of glutamate receptor subunit expression in progenitors, 01 and 04 positive oligodendrocytes revealed the expression of AMPA and kainate preferring subunit transcripts (Patneau, et. al. 1994). Neither mature oligodendrocytes nor their progenitors appear to express NMDA receptors (Patneau, et. al. 1994; for review Gallo and Russell, 1995). Electrophysiology studies showed that the currents elicited by glutamate receptor activation were coincident with cells expressing both AMPA and kainate preferring receptors. This was shown by their different affinities for these receptor agonists, AMPA and kainate and the sensitivity to the AMPA modulator cyclothiazide (Patneau, et. al. 1994). Cyclothiazide acts to block the rapid desensitisation of AMPA receptors following glutamate activation and produces marked potentiation of the response to kainate (Patneau, et. al. 1993; Yamada and Tang, 1993).

Glutamate induced injury has been reported to be mediated through non-NMDA receptors in oligodendrocytes in a $Ca^{++}$ dependant manner (Patneau, et. al. 1994; Puchalski, et. al. 1994; Yoshioka, et. al 1995). Contrasting studies however, report that glutamate induced injury in oligodendrocytes is mediated, not through glutamate receptor activation, but through the actions of free radicals. Similarly to the transport system present in neurons increased extracellular glutamate concentrations lead to the depletion of cystine and therefore glutathione within these cells rendering them vulnerable to oxidative stress (Oka, et. al. 1993; Yonezawa, et. al. 1996). In support of this, primary cultures of bovine oligodendrocytes were shown to be susceptible to oxygen radicals derived from glucose/glucose oxidase and hypoxanthine/xanthine oxidase reactions (Kim and Kim, 1991). In this study cytotoxicity was measured by tryptan blue exclusion and lactate dehydrogenase leakage, showing that oxygen radicals induce significant membrane damage to oligodendrocytes within 2 h of exposure.

Calcium influx is involved in neuronal degeneration and in glutamate receptor mediated toxicity described above. It has previously been shown that complement activated injury to oligodendrocytes characteristic of demyelinating diseases such as multiple sclerosis, is accompanied by a significant rise in intracellular calcium (Scolding, et. al. 1989).

Oligodendrocytes posses myelin-specific calcium dependant enzymes, including a basic protein kinase (Petrali and Sulakhe, 1982) and a neutral protease responsible for MBP hydrolysis (Banik, et. al. 1985). These enzymes may render oligodendrocytes more susceptible to changes in intracellular calcium than other glial cells (Scolding, et. al. 1990). Moreover, there is evidence that the effects of anoxia on white matter tracts may reflect calcium-mediated injury to the oligodendrocyte and myelin unit (Stys, et. al. 1990; 1992; Waxman, et. al. 1994). Calcium ionophores have been shown *in vitro* to cause cell body swelling, the appearance of cytoplasmic granules and the gradual disintegration of oligodendrocyte processes, leading to cell lysis (Scolding, et. al. 1992) and *in vivo* to cause demyelination of white matter tracts (Smith and Hall, 1994). In addition, MBP has been shown to be a substrate for the calcium activated protease calpain further implicating a role for calcium in the degeneration of the oligodendrocyte-myelin unit (Banik, et. al. 1994).

Together these studies provide evidence that oligodendrocytes can respond to glutamate, free radicals and $Ca^{++}$, all of which have the potential to cause degeneration of these cells. In light of this it is possible to envisage that during ischaemia and TBI, where the levels of all these substances are elevated, oligodendrocytes may become stimulated and/or undergo degeneration. Due to the importance of these cells in maintaining neuronal integrity oligodendroglial injury or death may have profound consequences for neuronal function and survival. Glutamate excitotoxicity is thought to be predominantly mediated through NMDA receptor activation. Although the use of animal models of acute brain injury have shown significant protection of neuronal elements by NMDA antagonists, compounds such as these will not protect oligodendrocytes since glutamate toxicity is mediated through non-NMDA receptors and/or free radical damage in these cells. It is possible to speculate that protection of neurons without a similar protection of glial cells may result in delayed neuronal degeneration rather than neuron protection since the degeneration of glial elements would ultimately result in neuronal death. An understanding of the mechanisms underlying glial responses to ischaemic brain injury is therefore pertinent to highlighting new areas of therapeutic intervention.

## 1.6. Aims of the thesis

Animal models of acute brain injury have been utilised to determine the mechanisms underlying neuronal degeneration in pathological circumstances. Glutamate toxicity and free radical mediated mechanisms are two such pathways which have been shown to be involved in neuronal degeneration following ischaemic brain injury. Assuming that similar mechanisms underly neuronal degeneration in both acute and chronic degenerative disorders these factors have also been implicated in the pathogenesis of chronic degenerative conditions. Although traditionally considered to be relatively resistant to ischaemic brain damage, oligodendrocytes have recently been shown to be susceptible to both glutamate and free radicals *in vitro*, suggesting that oligodendrocytes may also be susceptible to ischaemic brain damage. Breakdown of the neuronal cytoskeleton is thought to be one of the neurochemical events triggered by elevated extracellular concentrations of glutamate and free radicals resulting in irreversible neuronal death. Changes in the neuronal cytoskeleton resulting from acute brain injury have been well documented, although the mechanisms underlying these changes for the most part remain unclear. In contrast to the intensive investigations of neuronal responses to acute injury, the response of oligodendrocytes to ischaemia has been largely ignored.

The main aims of this thesis were therefore : 1) to investigate the distribution of the cytoskeletal proteins tau, MAP2 and MAP5 in oligodendrocytes in various rat models of acute brain injury and in human post-mortem brain tissue from patients who died following head injury or a stroke and to compare this to changes occurring in neurons and 2) to determine the mechanisms through which changes in oligodendrocyte and neuronal cytoskeletal proteins occur.

# CHAPTER 2 MATERIALS AND METHODS

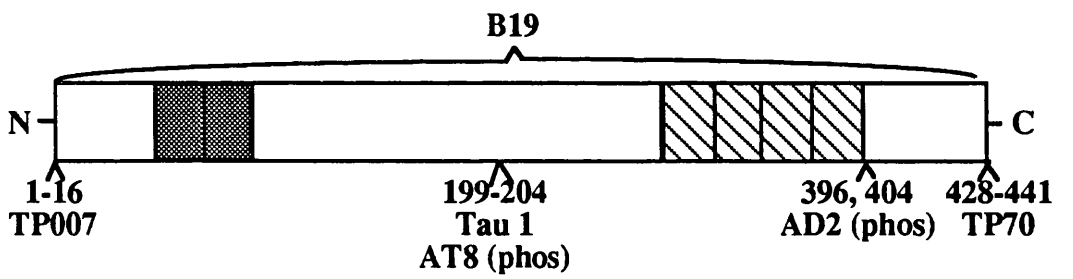
## PART 1 : ANALYTICAL END POINTS

A variety of different techniques were employed to assess tau protein in animal models of acute brain injury; human post-mortem brain tissue or oligodendrocyte cultures. These included : immunohistochemistry; western blotting and *in situ* hybridisation.

## 2.1 Immunohistochemistry

### 2.1.1 Description of antibodies

Tau was detected using six different antibodies : Tau 1; AT8; B19; TP70,TP007 and AD2 which have been previously characterised by others (Fig. 6). The monoclonal antibody Tau1 labels a dephosphorylated epitope between residues Ser 199 and 204 (Binder, et. al. 1985; Papasozomenos and Binder, 1987). AT8 is a monoclonal antibody which labels tau phosphorylated at residue 200 (Biernat, et. al. 1992). B19 is a polyclonal antibody raised against full length tau (Brion, et. al. 1991b). TP70 is a polyclonal antibody directed against a synthetic peptide corresponding to residues 428-441 at the C-terminal of the tau protein (Brion, et. al. 1993). TP007 is a polyclonal antibody raised to a synthetic peptide corresponding to the 16 most N-terminal residues of human tau protein (Davis, et. al. 1995). Tau1, B19, TP70 and TP007 were the kind gift of Professor B.H. Anderton




(Institute of Psychiatry, London).

**Figure 6.** Schematic representation of the epitopes to which the tau antibodies used in this study bind. Residue numbers correspond to those of the longest human isoform of tau containing 4 C- terminal repeats (hatched area) and 2 N-terminal inserts (shaded area). Tau1 and AT8 recognise tau dephosphorylated and dephosphorylated between residues 199-204 respectively. AD2 immunoreactivity is dependant on the phosphorylation of tau at residues 396 and 404. In contrast, detection of tau with B19, TP70 and TP007 is independent of the phosphorylation state of the protein.

AD2 is a phosphorylation dependant antibody raised against tau proteins found in AD. The epitope for this antibody includes Ser-396 (phosphorylated) and the participation of Ser-404 (phosphorylated) (BueeScherrer, et. al. 1996).

MAP2 was labelled using a monoclonal antibody which recognises MAP2a, b and c (clone HM-2; Sigma). MAP5 was labelled using an anti-MAP5 monoclonal antibody (clone AA6; Sigma).

Anti-transferrin was used as a cell marker for oligodendrocytes (Conner and Fine, 1986; Martin, et. al. 1991) and was detected using a polyclonal anti-transferrin (1:300) antibody from DAKO. GFAP was used as a cell marker for astrocytes (Martin, et. al. 1991) and was detected using a polyclonal anti-GFAP antibody (1:750) from DAKO.

### 2.1.2 Dephosphorylation of tissue prior to immunostaining

In some experiments, dephosphorylation of the tissue was performed prior to tau immunostaining using the method previously described by Papasozomenos & Binder (1987). Tissue sections were incubated with 130mg/ml of type VII-L alkaline phosphatase from bovine intestinal mucosa (Sigma) in 100mM Tris-HCl, pH8 for 2.5 h in a shaking water bath at 32°C. To inhibit proteolysis, 1mM phenylmethylsulfonyl fluoride, 10μg/ml pepstatin and 10μg/ml leupeptin were included in the incubation fluid. For controls, 100mM sodium pyrophosphate was included in the incubation solution as a competitive inhibitor of alkaline phosphatase. The dephosphorylation reaction was quenched by bathing the sections in ice-cold Tris saline (50mM Tris-HCl, 0.2M sodium chloride, pH7.6), and immunostained immediately as described below. Tau 1 immunoreactivity is localised within the axon in histologically normal tissue, however following dephosphorylation Tau 1 immunoreactivity was also detected within neuronal perikarya indicating that tau present in neuronal perikarya is phosphorylated at the Tau 1 epitope therefore preventing antibody binding (Fig. 7). Dephosphorylation of tissue prior to immunostaining was performed in situations where Tau 1 immunoreactivity was decreased, to determine whether this was due to phosphorylation of existing protein or due to protein loss.

### 2.1.3 Single label immunohistochemistry

The immunohistochemical procedures described below were performed on free floating rat brain sections, modification of this procedure for immunostaining of human post-mortem brain tissue and cell cultures are described in sections 2.5 & 2.7 respectively. Sections were rinsed in phosphate buffered saline (PBS) and incubated in PBS containing 0.2% Triton X-100 for 30 min. After rinsing with PBS the endogenous peroxidase activity

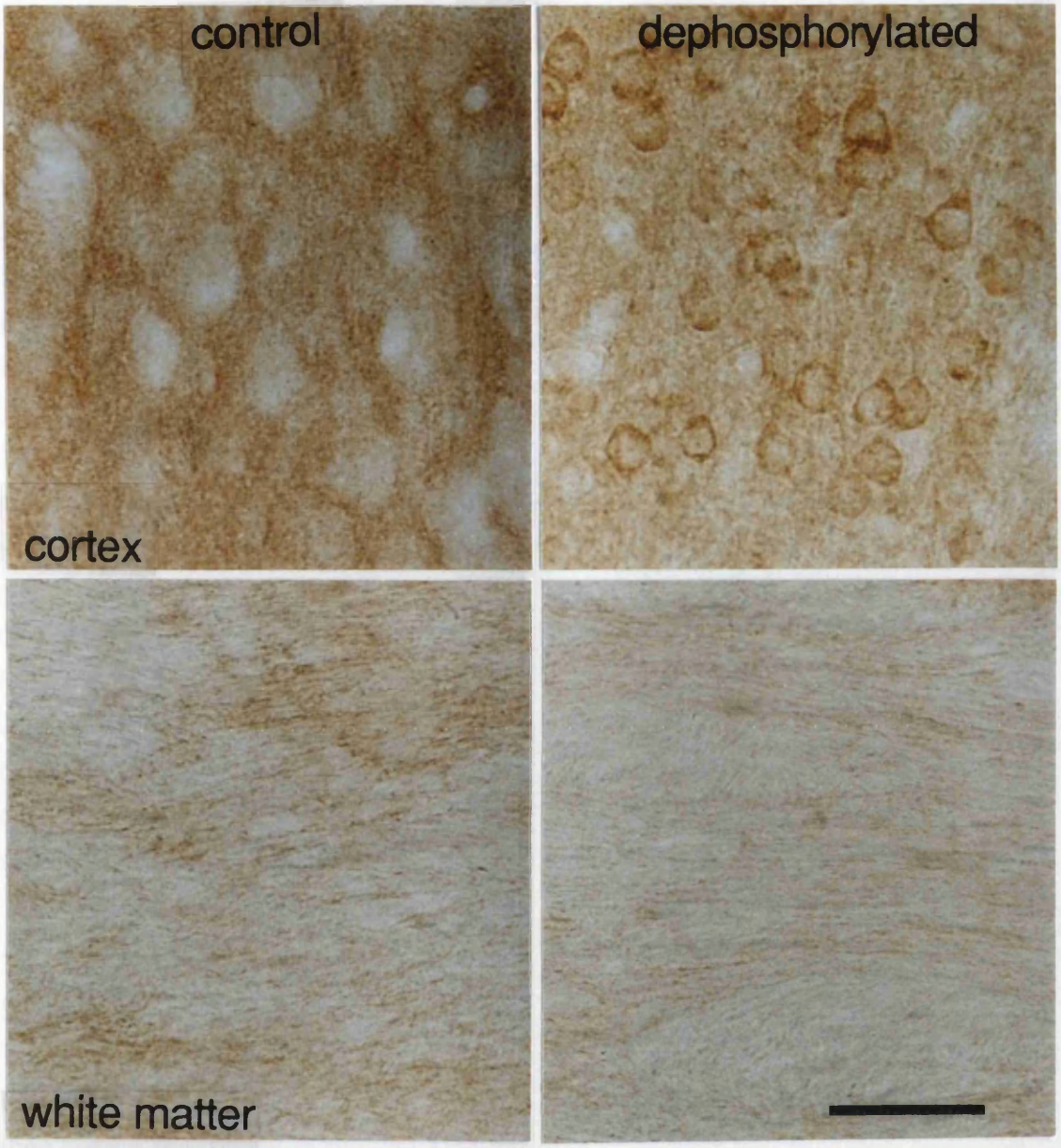



**Figure 7.** Photomicrographs showing the pattern of Tau 1 immunoreactivity in the parietal cortex and subcortical white matter of the rat before and after dephosphorylation. Tau 1 recognises tau protein dephosphorylated between residues 199-204 and therefore immunoreactivity is localised to axons in both grey matter and white matter. No neuronal perikarya or glial cells are detected in normal brain tissue. Following dephosphorylation of the tissue, in addition to axonal staining, Tau 1 immunoreactivity became apparent within neuronal perikarya of grey matter. In white matter however, immunoreactivity remained predominantly localised to axons, suggesting the absence of tau in glial cells rather than tau being masked by phosphorylation. Thus tau is compartmentalised within the neuron; phosphorylated at the Tau 1 epitope within neuronal perikarya but dephosphorylated at this epitope in the axonal compartment of both grey and white matter. Scale bar = 50μm.

was blocked by incubating in 3% hydrogen peroxide (Sigma) for 20 min. To block non-specific binding, sections were incubated in PBS containing 10% normal horse serum and 1% bovine serum albumin (BSA; Sigma) for monoclonal antibodies (Tau 1, AT8, MAP 2 and MAP5) or PBS containing 10% normal goat serum and 1% BSA for polyclonal antibodies (B19, TP70 & TP007) for 1 h. Dilution curves were carried out to determine the optimum dilution for each antibody i.e. that which produced the best signal to background ratio. Incubation with primary antibody in appropriate blocking solution (Tau 1, 1:1000; AT8, 1:50; MAP2, 1:750; MAP5, 1:1000; B19 1:750; TP70, 1:2000; TP007, 1:500) was overnight at 4°C. Following two rinses with PBS, monoclonal antibody binding was detected by incubating sections with a biotinylated horse anti-mouse IgG secondary antibody preadsorbed against rat serum (1:100; Vector Labs.) for 1 h, while polyclonal antibody binding was detected using a biotinylated goat anti-rabbit IgG secondary antibody (1:400; DAKO). All sections were then incubated with an ABC complex (Vector Labs.) for one hour and peroxidase visualised with 3'-3'-diaminobenzamine (10mg DAB tablet (Sigma); 0.024% hydrogen peroxide in 50mM Tris HCl pH7.4). Sections were then mounted onto poly-L-lysine coated slides, dehydrated through graded alcohols, cleared and coverslipped ready for microscopic examination. Negative controls were included in each experiment, in which the primary antibody was omitted from the procedure. Non-specific immunoreactivity was not detected in these sections (Fig. 8).

### 2.1.4 Double label immunohistochemistry (chromagen)

Sections were stained as described above for single label immunohistochemistry using Tau1, however antibody binding was visualised using the purple chromagen VIP (Vector Labs.). Following this procedure the sections were washed in water followed by PBS and then incubated with PBS containing 10% goat serum and 1% BSA for 1h. Incubation with the second primary antibody (GFAP or transferrin) was for 1h at room temperature in blocking solution. Sections were then rinsed in PBS (2x10 min) and incubated with species specific anti-rabbit biotinylated secondary antibody (1:400) for 1 h. All sections were then incubated with an ABC complex for one hour and peroxidase visualised with a grey chromagen SG (Vector Labs.). Negative controls were included in each experiment, where the primary antibody was omitted from the procedure. Non-specific immunoreactivity was not detected in these sections (Fig. 8).

### 2.1.5 Double label immunohistochemistry (fluorescence)

Double label experiments were also carried out using immunofluorescence techniques. Free floating sections were treated as described above however it is unnecessary to block endogenous peroxidase activity since this is not involved in the visualisation of antibody

binding using this technique. Non-specific binding was prevented by incubating sections in PBS containing 10% normal horse serum / 10% normal goat serum and 1% BSA for 1h. Primary antibodies were added together Tau 1(1:1000) + anti-GFAP (1:750) or Tau 1 + anti-transferrin (1:750) in blocking solution, overnight at 4°C. Sections were rinsed in PBS and incubated with a biotinylated horse anti-mouse secondary antibody (1:100 in PBS) for 30min (this binds to the Tau 1 antibody only). Following two rinses in PBS sections were incubated in PBS containing streptavidin-FITC conjugate (1:75, Sigma) and Texas Red labelled goat anti-rabbit IgG antibody (1:100, Vector Labs.). Sections were rinsed in PBS and water and mounted onto poly-L-lysine coated slides and coverslipped using citifluor anti-fading agent (Citifluor, Labs.). The FITC conjugate binds to the biotinylated secondary antibody and therefore detects Tau 1 antibody binding. FITC absorbs light of wavelength 495nm and emits at 525nm and herefore can be visualised as green light using a blue filter. The Texas red labelled goat anti-rabbit binds to the transferrin or GFAP polyclonal antibody (rabbit IgG) and absorbs light at 596nm and emits at 620nm and therefore can be visualised as red light using a green filter.

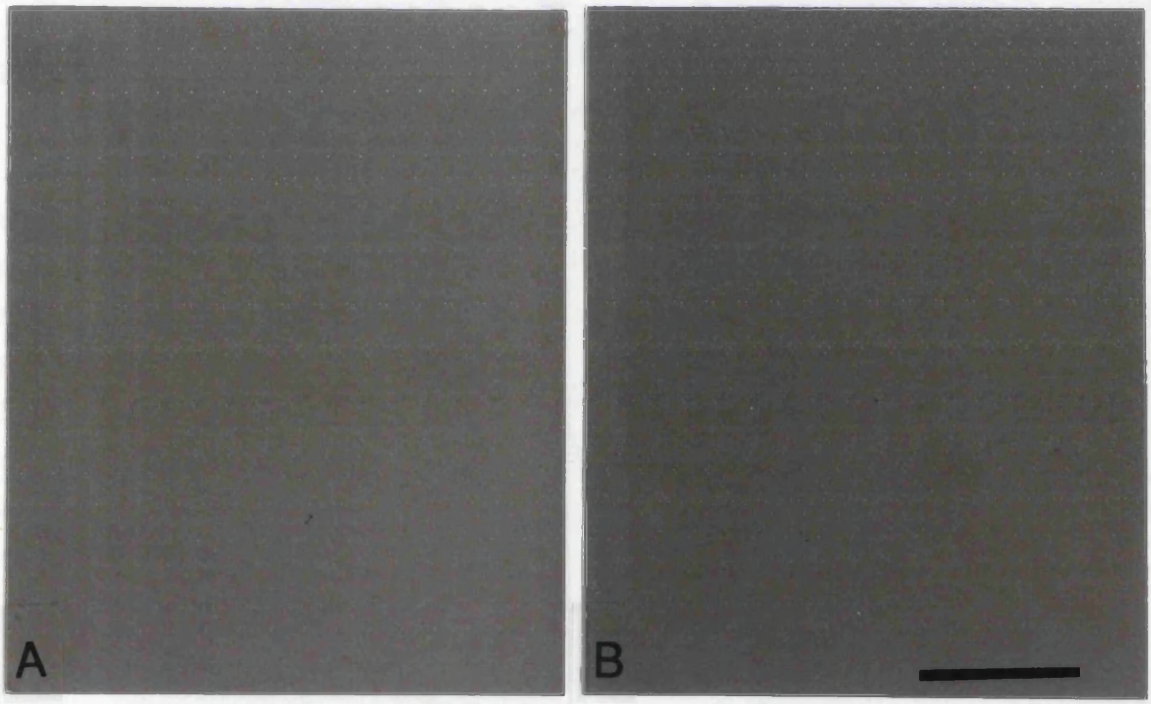



**Figure 8.** Typical examples of negative controls for single (A) and double (B) label immunohistochemistry using DAB, VIP or SG as chromagens. Negative controls in which the primary antibody was omitted were carried out for every immunohistochemical experiment : A) shows a rat brain section processed for single label immunohistochemistry using DAB as the chromagen as described in section (2.1.3); B) shows a rat brain section processed for double label immunohistochemistry using Vector VIP and SG as chromagens as described in section (2.1.4). Non-specific immunoreactivity was not detected in these tissue sections. Scale bar = 50μm.

## 2.2 Western blot analyses

### 2.2.1 Preparation of cell cultures

Cell cultures were removed from -70°C and thawed on ice prior to use (for details of cultures used see section 2.7). 250µl of sample buffer (2% SDS, 5% mercaptoethanol, 10% glycerol in 62.5mM Tris pH 6.8) was added to each culture flask and a suspension of lysed cells made using a rubber spatula. Once all cells were lysed and dissociated from the flask the suspension was transferred to a sterile eppendorf, heated to 100°C for 5 min, centrifuged at 13000rpm for 5 min and returned to ice. Bromophenol blue was added to each tube and a 30µl aliquot loaded onto the gel. Remaning homogenates were stored at -70°C until further use.

### 2.2.2 Preparation of whole brain homogenate

An adult rat was killed by an overdose of halothane, decapitated and the brain was rapidly dissected and frozen in liquid nitrogen. Whole brain homogenates were prepared by homogenising the brain sample in 5mls of sample buffer and centrifuged at 13000rpm at 4°C for 30 min. The supernatant was retained and heated at 100°C for 5 min, spun at 13000rpm for 5 min, the supernatant harvested and placed on ice. For protein qauntification, brain proteins were precipitated with tri-chloroacetic acids as follows. An appropriate volume of brain homogenate was made up to 1ml with distilled water in an eppendorf tube, and sodium deoxy cholate was added to a concentration of 1%. The samples were vortexed and ice-cold tri-chloracetic acid was added to a final concentration of 10%. The samples were vortexed and placed on ice for 30 min to precipitate the protein which was pelleted by spinning briefly in a microcentrifuge. The protein pellet was resuspended in a small volume of 1M Tris/HCl pH 8 and a sample removed for protein analysis as described by Lowry et. al. (1951), using bovine serum albumin as a standard. The remaining brain samples were then diluted in 5x sample buffer.

### 2.2.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The denatured proteins were separated by discontinuous slab gel electrophoresis (Laemmli, 1970). The resolving gel, consisted of 12% acrylamide, 0.32% bis acrylamide, 0.38M Tris/HCl pH8.8, 0.1% SDS and was polymerised using 0.1% ammonium persulphate and 0.04% TEMED. The gel was cast between 2 glass plates in an ATTA gel setter to give a gel approximately 13 cm in length. While polymerising the top surface of the gel was covered with isopropanol. Once polymerised the isopropanol was removed and the stacking gel, consisting of 4.8% acrylamide, 0.13% bis acrylamide, 0.1% SDS, 0.14M Tris/HCl pH 6.8, 0.1% ammonium persulphate and 0.04% TEMED, poured above

the resolving gel and a 14 well comb inserted. Once solidified the gel was placed in an electrophoresis rig, the comb removed and the rig filled with electrophoresis buffer (190mM glycine, 25mM Tris and 0.1% SDS). 30µl of each of the cell homogenates and 20µg of brain homogenate were loaded onto the gel alongside pre-stained molecular weight markers (14-220kDa; Amersham). Proteins were electrophoresed at 200mV for 2-3 h until the dye front had reached the bottom of the gel and all molecular weight markers could be seen. Proteins were then transferrrd to PVDF membrane (Millipore) by means of semi-dry electroblotting.

### 2.2.4 Semi - dry electroblotting

After electrophoresis, the resolving gel was separated from the stacking gel and twelve sheets of 3mm filter paper (Watmann) and one sheet of PVDF membrane were cut to fit the gel. The filter paper was soaked in semi-dry transfer buffer (60mM Tris, 50mM glycine, 1.6mM SDS, 30% methanol) for 15 min and 6 sheets placed on the electroblotter, ensuring there were no air bubbles. The PVDF membrane was soaked in methanol and placed on top of gel, and the gel sandwiched with the remaining six sheets of filter paper. Transfer was carried out at 200mA for 45 min. Efficient transfer was confirmed by the appearance of the pre-stained molecular weight standards on the PVDF membrane sheet, the positions of these were highlighted with pencil. Following transfer the PVDF membrane was soaked in methanol and cut ready for Western blotting. The gel was placed in Coomassie stain (10% glacial acetic acid; 50% methanol; 0.25% Coomassie Brilliant Blue R) and agitated overnight at 4°C. The following day the gel was placed in destain (7% acetic acid; 25% methanol) during western blot procedure, to confirm equal protein loading of cell samples.

### 2.2.5 Western blot (Enhanced chemiluminescence-ECL$^{TM}$)

Non-specific binding was prevented by incubating the PVDF blots in blocking solution (3% non-fat dried milk in Tris buffered saline pH8 containing and Tween-20 (TBS-T : 10mM Tris; 150mM NaCl; 0.05% Tween-20)) for 1h at room temperature. Blots were rinsed in TBS-T (1 x 10 min, 2 x 5 min) and incubated with primary antibody (Tau 1, 1: 500 or TP70, 1: 3000) in blocker overnight at 4°C. Following rinses in TBS-T (1 x 15 min, 2 x 5 min) blots were incubated with HRP labelled sheep anti-mouse IgG (1:5000; Amersham), or donkey anti-rabbit IgG (1:2000; Amersham) in blocker for Tau 1 or TP70 respectively, at room temperature for 1h. Blots were rinsed in TBS-T (6 x 10 min), incubated in ECL reagent (equal volumes of each detection reagent; Amersham) for 1 min, blotted dry with filter paper, wrapped in Saranwrap and exposed to blue light sensitive autoradiography film (Fuji) for 1-10 min depending on the amount of antigen present. Films were developed using an automatic processor. The light produced by ECL peaks

after 5-20 min and has a half life of 60 min therefore exposure to film must be within 20 min of the reaction for maximum signal detection.

## 2.3 In situ hybridisation

### 2.3.1 Core materials and solutions

Di-ethyl pyrocarbonate (DEPC) minimises RNase activity, therefore all solutions were made using DEPC treated sterile distilled water (SDW). This was prepared by treating distilled water with 0.01% DEPC overnight and sterilising by autoclaving at 15lb/in$^2$ for 15 min. Glasswear (once cleaned by soaking in 6% sulphuric acid and 6% potassium dichromate overnight) and plastics were soaked in DEPC SDW prior to sterilisation by baking at 180°C or autoclaving at 15lb/in$^2$ for 15 min respectively. 3-Aminopropyltriethoxy-silane (APES) coated slides were prepared using DEPC treated glass slides. Slides were soaked in 5% Decon 90 (Decon Labs. Ltd.) overnight, washed well, wrapped in foil and oven dried. Once dry slides were soaked in 0.25% APES (Sigma) in methylated spirit for 2 min in a fume hood. Slides were then rinsed in DEPC SDW for 2 min and oven dried in foil and stored at room temperature.

### 2.3.2 Preparation of tau DNA for riboprobe production

*Amplification of tau cDNA*

cDNA encoding full length adult rat tau (Kosik, et. al. 1989a) was kindly gifted by Dr. K. Kosik. Two copies of this adult rat cDNA were cloned into a pGEM3 vector head to tail at the EcoRI site. Competent cells, kindly prepared by Dr. P. Dickinson, were transformed with plasmid to amplify the tau cDNA. Briefly, transformations were performed in sterile 40ml polypropylene tubes pre-chilled on ice. 25ng of tau cDNA was aliquoted and made up to a final volume of 100µl with Tris EDTA buffer (TE) pH8 (10mM Tris HCl pH8 / 1mM EDTA pH8). An aliquot of competent cells was thawed and a 100µl aliquot added to the tau DNA, swirled gently and incubated on ice for 30 min. The competent culture was heat shocked at 42°C for 45 s and rapidly transferred to ice for 2 min. 800µl of SOC medium (see appendix for preparation) was added to the DNA and incubated at 37°C at 100rpm for 1hr in an orbital incubator. 2x 200µl aliquots were then plated out onto Luria-Bertrani agar indicator plates (see appendix for preparation) using a sterile bent glass rod. Once all liquid had been absorbed, the plates were inverted and incubated at 37°C overnight.

Single white colonies were removed from the indicator plates and the plasmid DNA isolated and purified from bacterial cells using a modified technique based on the alkaline lysis procedure (Birnboim and Doly, 1979). Single colonies of cells were incubated overnight at 37°C in Terrific Broth (see appendix for preparation) in an orbital incubator at 100rpm. 4.5ml aliquots of cells were spun for 1 min, the supernatant removed and the pellet resuspended in 200µl of GTE buffer (50mM glucose, 25mM Tris, 10mM EDTA, pH8). The cell suspension was then mixed with 300ml 0.2M NaOH/1% SDS (SDS denatures bacterial proteins and NaOH denatures DNA) and incubated on ice for 5 min. The solution was then neutralised by mixing with 300µl of 3M potassium acetate pH4.8 on ice. This causes plasmid DNA to reanneal, while the chromosomal DNA and bacterial proteins are precipitated with the SDS and form a complex with the potassium. This is removed by centrifuging at 13000rpm for 10 min at room temperature and removing the supernatant to a clean tube. The solution was then incubated with RNase A (DNase free) at 37°C for 20 min to remove any RNA present. The supernatant was then extracted by adding 400µl of chloroform, mixing for 30 s, centrifuging for 1 min and removing the aqueous phase into a clean tube. This step was then and the two aqueous phase added together. The DNA was then precipitated from the solution by adding an equal volume of 100% isopropanol and centrifuging for 10 min at 13000rpm at room temperature. The supernatant was removed and the pellet washed with 500µl 70% ethanol by spinning at 13000rpm for 15 min, the ethanol was then removed and the pellet vacuum dried. The pellet was dissolved in 32µl SDW and DNA precipitated by adding 8µl of 4M NaCl and then 40µl of filter sterilised 13% $PEG_{8000}$ overnight at -20°C. Plasmid DNA was pelleted by centrifuging at 13000rpm for 15 min at 4°C, the supernatant removed and the pellet washed in 500µl of 70% ethanol by spinning at 13000rpm for 15 min at 4°C. After vacuum drying the pellet was resuspended in 20µl SDW and the quantity of DNA measured by using a RNA/DNA calculator (Pharmacia Biotech).

***Linearisation of plasmid DNA***

Plasmid DNA was incubated with appropriate restriction enzymes to linearise the plasmid. EcoR1 restriction was used to confrim that the tau cDNA was cloned in at this site on the vector. Restriction enzyme Sma 1 was used to confirm the orientation of the tau cDNA in the plasmid and the template for the generation of the anti-sense tau riboprobe. Xho 1 was used to provide the template for the generation of the sense ribopobe (Fig. 9 and 10). Restriction reactions consisted of 5µg plasmid DNA, 20U restriction enzyme and the appropriate reaction buffer, incubated for 2-3 h at 30°C for Sma 1 or 37°C for Xho 1 and EcoR1. 1% agarose gels were prepared using ultra pure electrophoresis grade agarose (Gibco BRL). Agarose was melted in the presence of Tris acetate EDTA (TAE) buffer with a final concentration of 0.04M Tris acetate, 0.001M EDTA and 0.5µg/ml ethidium

bromide. The melted agarose was cooled slightly and poured into a mini gel rig, a comb inserted and allowed to set. The solidified gel was then immersed in TAE buffer. 500ng of linearised product was loaded with 6x TAE gel loading buffer (6x TAE, 30% glycerol (Sigma), 0.25% bromophenol blue (BDH) and 0.25% xylene cyanole FF (Sigma)) and electrophoresed at 50mV for 1.5 h to confirm linearisation of plasmid DNA (see Fig. 9). Gels were viewed using a "Fotoprep I" ultraviolet transilluminator (Fotodyne Inc.) and photographed using a Polaroid MP4 land camera (Polaroid), a T2201 transilluminator (Sigma), a Wratten 22A filter (Kodak) an Polaroid 667 (ASA 3000) film.

Following conformation of linearisation, the digestion product from each reaction was ethanol precipitated by adding 1/10 x volume 3M sodium acetate (pH 5.2) and 3 x volume of ethanol (-20°C) overnight at -20°C. The solution was then centrifuged at 13000 rpm at 4°C for 30min, the supernatant removed using a finely pulled glass pipette and the pellet washed with 500µl of 70% ethanol/SDW by spinning at 13000rpm for 15 min. The supernatant was removed, the pellet air-dried and resuspended in SDW at 500ng/µl and stored at -20°C until use.

### 2.3.3 Preparation of $^{35}$S labelled riboprobes

Sense and anti-sense $^{35}$S riboprobes were generated using a SP6/T7 transcription kit (Boehringer Mannheim). Labelling reactions contained 2ng linearised plasmid DNA, 0.5mM ATP, 0.5mM GTP, 0.5mM UTP, 50µCi $^{35}$S-CTP (specific activity 37TBq/mM; <1000Ci/mM; Amersham), 20U RNase inhibitor, 20U SP6 or T7 RNA polymerase and transcription buffer and were carried out in a DEPC treated sterile eppendorfs. The mixture was incubated at 37°C for 30 min, a further 20U of polymerase was then added and the incubation repeated. 20U RNase free DNase was added to this mixture and incubated at 37°C for 15 min to remove the DNA template. To deproteinise the sample an equal volume of phenol chloroform was added and the sample, vortexed for 10 s, centrifuged at 13000rpm for 3 min and the aqueous layer (containing nucleic acid) removed into a DEPC treated eppendorf. The solvent was then back extracted by adding an equal volume of DEPC SDW. The sample was centrifuged at 13000rpm for 3 min and the aqueous phase harvested and added to other aqueous phase. The RNA was precipitated using 1/10 volume 7.5M ammonium acetate and 2.5 x volume of ethanol (-20°C) for 3 h at -20°C. A 5µl aliquot was removed prior to centrifugation and placed in 5ml "Ecoscint" in a scintillation vial (total amount of isotope present). Following centrifugation at 13000rpm for 30min at 4°C, 5µl of the supernatant was removed and placed in 5ml "Ecoscint" (amount of isotope unincorporated). The remaining supernatant was discarded and the pellet resuspended in 100µl DEPC.SDW, 5µl of resuspended pellet was removed and placed in "Ecoscint" (amount of incorporated isotope). The vials were

counted on a Beckman LS 1801 scintillation counter and the percentage of incorporated isotope calculated. RNA was then re-precipitated overnight at -20°C with 1/10 x volume 3M sodium acetate (pH5.2) and 3 x volume of ethanol (-20°C). The preparation was centrifuged at 13000rpm at 4°C for 30min the pellet washed with 70% ethanol/DEPC SDW, air dried and resuspended at 1ng/µl/kb. The probes were stored at -20°C and used within 6-8weeks.

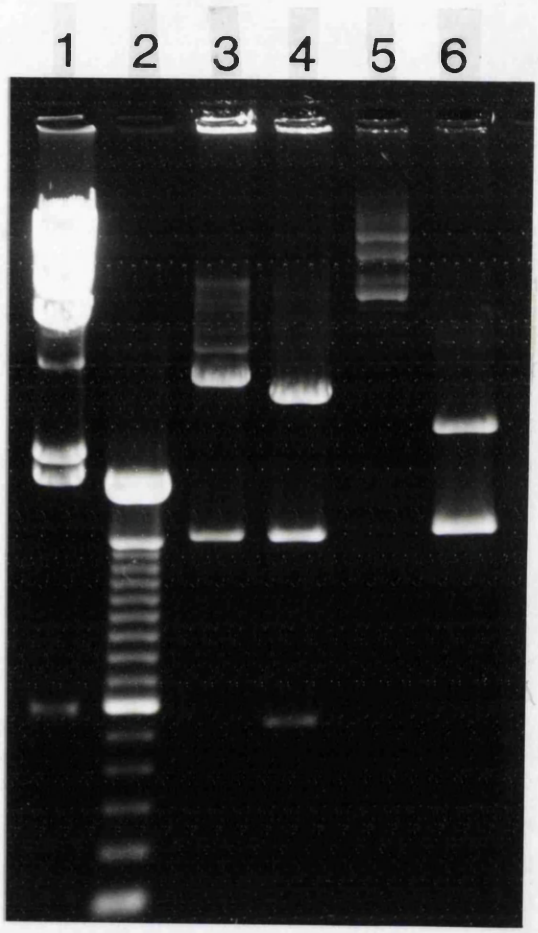
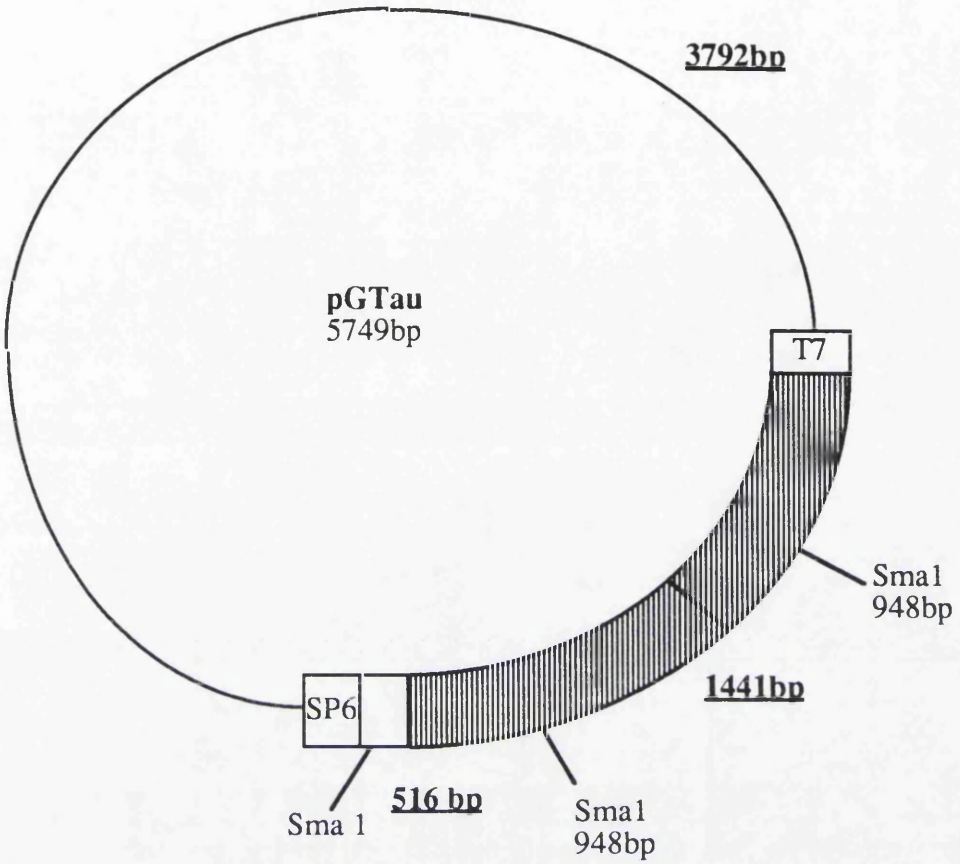



**Figure 9.** 1% agarose gel showing plasmid digests used for tau $^{35}S$ riboprobe preparation. Lane 1; lamda Hind III fragments (Gibco, BRL). Lane 2 ; 100bp ladder (Gibco, BRL). Lane 3; Xho1 digestion products (4308 - linearised plasmid and 1441bp - tau cDNA), used for antisense riboprobe preparation. Lane 4; Sma1 digestion products, bands were detected at approximately 3792 (linearised plasmid), 1441 (tau cDNA) and 516bp (small tau fragment), confirming that the tau inserts were positioned head to tail 5'-3'5'-3' as expected (see Fig. 10). Sma 1 was used to linearise cDNA for the preparation of the tau sense riboprobe. Lane 5; undigested plasmid containing tau inserts (5794bp). Lane 6; EcoR1 digestion products show linearised plasmid (3792bp) and tau DNA (1441bp).

a)




b)

| Orientation of tau fragments | Fragments resulting from Sma1 digest |
|---|---|
| 5'-3'5'-3' | 3792, 1441, 516 |
| 5'-3'3'-5' | 3792, 986, 971 |
| 3'-5'3'-5' | 3337, 1441, 971 |
| 3'5'5'-3' | 3337, 1996, 516 |

**Figure 10**. Linearisation of plasmid DNA with Sma1. a) A schematic representation of the location of the two tau cDNA fragments within the pGEM3 plasmid and the site of linearisation of the plasmid with the restriction enzyme Sma 1. b) Shows the fragment sizes resulting from digestion of the plasmid containing tau cDNA (pGTau) with Sma1 depending on the orientation of the tau inserts. Linearisation of pGTau with Sma1 resulted in fragments approximately 4000, 1400 and 500bp (see Fig. 9) confirming that tau cDNA was inserted within the plasmid 5'-3'5'-3'.

### 2.3.4 In situ hybridisation procedure

***Pre-treatment of tissue***

Pre-treatment of the tissue involved several steps that increased the availability of RNA to the riboprobe, while maintaining tissue morphology. Frozen rat brain sections were mounted onto APES coated slides and allowed to dry. Sections were rinsed in PBS and fixed for 20 min in 4% paraformaldehyde in PBS. After rinsing in PBS, sections were placed in a solution of 0.1M triethanolamine and 0.25% acetic anhydride for 5 min. A further 0.25% acetic anhydride was then added for a further 5 min, this decreases non-specific binding of the probe to the tissue through electrostatic interactions. Sections were then place in PBS (5 min), 0.85% saline (5 min), methylated spirits (5 min) and dehydrated in absolute alcohol (2 x 5 min).

***Hybridisation***

Hybridisation buffer (50% formamide, 10% dextran sulphate, 1 x Denhardt's, 20mM Tris HCl pH8, 0.3M NaCl, 5mM EDTA, 10mM $NaPO_4$ pH8 and 0.5mg/ml yeast tRNA) was heated to 50°C to reduce viscosity. Hybridisation mixture, consisting of hybridisation buffer, 1mM dithiothreitol (DTT) and appropriate amount of probe (1:10 dilution) was heated to 80°C for 2 min to denature the RNA and then placed on ice. 8µl of diluted probe was placed at the edge of each section and covered with a siliconised coverslips (clean coverslips soaked in Repelcot™ (BDH) for 20 min, rinsed and baked at 130°C for 90 min). Slides were placed horizontally in a slide box containing tissue paper soaked in 50% formamide and 5 x SSC (0.75M sodium chloride, 0.075M sodium citrate) to prevent dehydration of the sections. The box was sealed with tape and placed in two vacuum sealed bags and incubated at 50°C overnight in a waterbath.

***Post hybridisation treatment***

Stringency washes remove unbound or poorly hybridised probe therefore reducing non-specific labelling of tissue. Sections were placed in : 5 x SSC/10mM DTT at 50°C for 30 min to remove coverslips; 2 x SSC in 50% formamide/10mM DTT at 65°C for 20 min; 500mM NaCl , 5mM EDTA , 10mM Tris HCl pH7.5 at 37°C for 10 min (x3); 0.02mg/ml RNase A, 500mM NaCl , 5mM EDTA , 10mM Tris HCl pH7.5 at 37°C for 30 min to remove single stranded RNA; 500mM NaCl , 5mM EDTA , 10mM Tris HCl pH7.5 at 37°C for 15 min; 2 x SSC in 50% formamide/10mM DTT at 65°C for 20 min; 2 x SSC at room temperature for 15 min and finally 0.1 x SSC at room temperature for 15 min.

*Dehydration*

Sections were dehydrated through graded alcohols - 30%, 60%, 80%, 95% containing 0.3M ammonium acetate, and finally absolute alcohol. The presence of ammonium acetate prevents denaturation of the hybridisation. Sections were then allowed to air dry and exposed to Cronex (DuPont) medical screen film inside a radiographic cassette at room temperature overnight. Sections were then dipped in Ilford K5 emulsion (1:1 dilution with distilled water containing 1% glycerol) at 42°C. Slides were air dried for 4-6 h and stored at 4°C in light tight boxes containing a sachet of silica gel, for the required exposure time (determined by intensity of film image), between 1 and 7 days. This procedure was carried out by Prof. I.R. Griffiths. Sections were washed in water, allowed to air dry, counter-stained with haematoxylin and permanently mounted using DPX mountant for microscopic analysis.

# PART II - PREPARATION OF TISSUE

Tau was examined by the methods described in Part I in brain tissue from either rat models of acute brain injury or human post-mortem brain tissue and in oligodendrocyte cultures.

## 2.4 Intracortical perfusion of monosodium glutamate *in vivo*

A model of glutamate toxicity *in vivo* was developed in this laboratory by Bullock and collegues (Fujisawa, et. al. 1993a; 1993b; 1996). This model was used to determine the effects of high extracellular glutamate concentrations on tau immunoreactivity *in vivo*.

### 2.4.1 Surgical preparation of animals

Twenty-three adult male Sprague-Dawley rats, each weighing between 322-424g were used in this study. Animals were initially anaesthetised with a mixture of 5% halothane, 70% nitrous oxide and 30% oxygen. A tracheostomy was performed to allow artificial ventilation and anaesthesia subsequently maintained between 1 and 1.25% halothane. Cannulation of a femoral artery and vein allowed the monitoring of blood pressure and arterial blood gases. Blood gas analyses was performed immediately after arterial cannulation, prior to glutamate perfusion and at 1 h intervals thereafter until the end of the experiment. Animals were maintained normocapnic ($pCO_2$ 38-42mmHg), normoxic ($pO_2$ > 100mmHg) and normothermic (37°C ± 1) throughout the experimental period by adjusting the volume of gases delivered to the animal and controlling rectal temperature using a heat lamp (Table 1).

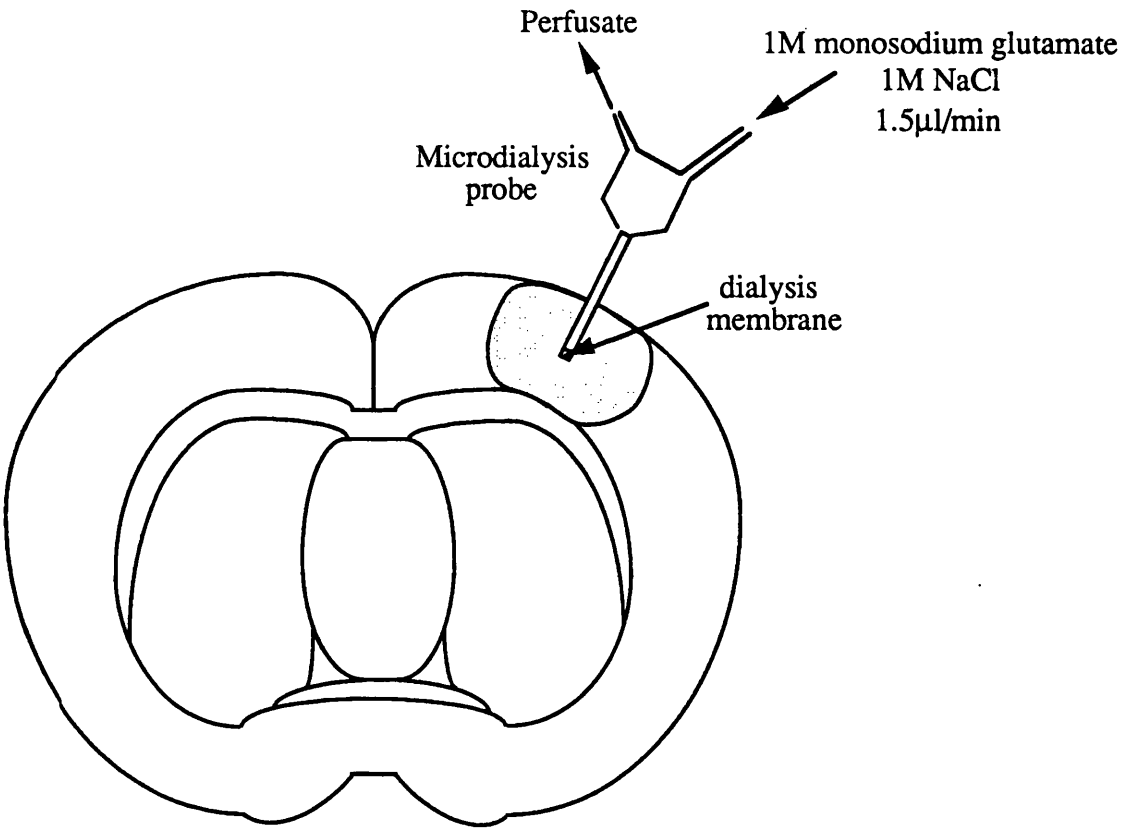
**TABLE 1 Physiological Variables - Glutamate toxicity study**

| PHYSIOLOGICAL PARAMETER | TREATMENT GROUPS Glutamate (n=8) | NaCl (n=10) | CSF (n=5) |
|---|---|---|---|
| **Temperature $^{o}C$** | | | |
| at probe placement | 36.8 ± 0.1 | 36.8 ± 0.1 | 36.8 ± 0.1 |
| 4 h after probe placement | 36.9 ± 0.0 | 37.0 ± 0.1 | 36.8 ± 0.1 |
| **Blood Pressure (mmHg)** | | | |
| at probe placement | 91 ± 1 | 90 ± 2 | 93 ± 1 |
| 4 h after probe placement | 92 ± 1 | 92 ± 1 | 93 ± 1 |
| **Arterial pH** | | | |
| at probe placement | 7.44 ± 0.01 | 7.43 ± 0.01 | 7.41 ± 0.02 |
| 4 h after probe placement | 7.44 ± 0.01 | 7.42 ± 0.01 | 7.40 ± 0.01 |
| **$pCO_2$ (mmHg)** | | | |
| at probe placement | 40 ± 1 | 40 ± 1 | 40 ± 1 |
| 4 h after probe placement | 39 ± 1 | 39 ±0 | 39 ± 1 |
| **$pO_2$ (mmHg)** | | | |
| at probe placement | 182 ± 5 | 177 ± 6 | 179 ± 5 |
| 4 h after probe placement | 188 ± 5 | 180 ± 3 | 183 ± 5 |

Animals were maintained normocapnic ($pCO_2$ 38-42mmHg), normoxic ($pO_2$ > 100mmHg) and normothermic (37°C ± 1)throughout the experimental period. Values are mean ± SEM. No differences in these physiological variables were seen between experimental groups.

### 2.4.2 Microdialysis probe implantation

Animals were turned prone and mounted onto a Kopf stereotaxic frame (Clark Electromedical, London, UK.). Using an operating microscope, the left parietal skull was exposed and a burr hole made with a saline cooled dentist drill (stereotactic co-ordinates anterior -1.0mm, lateral +4.0mm relative to bregma). The microdialysis probe (CMA/12 membrane length 1mm, outside diameter 0.5 mm, Biotech Instruments Ltd.) was angled at $15^0$ to the saggital plane and lowered 1mm into the cortex through the burr hole after the dura-arachnoid had been carefully incised using a fine dental needle (for schematic representation see Fig. 11). Once in position, the probe was perfused with CSF for 30 min prior to the perfusion of 1M monosodium glutamate or 1M NaCl.




Schematic representation of the area of tissue damage resulting from the perfusion of 1M monosodium glutamate

**Figure 11.** Schematic representation of microdialysis probe placement in the parietal cortex. 1M monosodium glutamate or 1M NaCl was the perfused at 1.5µl/min for 90 min using a microinjection pump. Intracortical perfusion of 1M monosodium glutamate resulted in a sizeable lesion which extended in anterior and posterior directions and was characterised by an area of pallor as shown by H&E staining (see Figure 15).

### 2.4.3 Glutamate and sodium chloride perfusion

1M monosodium glutamate (pH7.4) was prepared in artificial cerebrospinal fluid (CSF) (NaCl 135mM, KCl 1mM, $KH_2PO_4$ 2mM, $CaCl_2$ 1.2mM, $MgCl_2$ 1mM, pH7.4). 1M glutamate was selected for this study on the basis of a prior investigation of dose dependency and lesion size (Landolt, et. al. 1993). The concentration of glutamate being delivered to the CNS is less than that within the fibre, being approximately 100mM due to the dynamics of the dialysis membrane. The vehicle control solution consisted of 1M sodium chloride in artificial CSF (pH7.4). Mechanical damage induced by probe placement was assessed by the perfusion of artificial CSF (pH7.4) alone. After placement in the cortex, the probe was perfused with the appropriate solution at a flow rate of 1.5µl/min for 90 min using a Carnegie Medicine CMA 100 infusion pump (Biotech Instruments, Luton, UK). After 90 min of perfusion, the probe was removed from the cortex and the scalp wound closely sutured. Two-and-a-half hours after the removal of the probe (i.e. 4 h from the start of perfusion), the animals were killed by transcardiac perfusion as described below.

### 2.4.4 Transcardiac perfusion

At the end of the experimental procedure anaesthesia was deepened using a mixture of 2% halothane, 70% nitrous oxide and 30% oxygen, the heart exposed and a needle inserted through the left ventricle to the aorta. Animals were exsanguanated by perfusing 0.9% heparinised saline at 120mmHg (approximately 150mls) then fixed by perfusing 150-200mls of ice cold 4% paraformaldehyde until the animal was rigid.

### 2.4.5 Processing of brain tissue for histology and immunohistochemistry

Following transcardiac perfusion, brains were removed and post-fixed in 4% paraformaldehyde at 4°C for 48 h and cryoprotected in 30% sucrose in 50mM phosphate buffer at 4°C for 2-3 days. The brains were then frozen in isopentane chilled on dry-ice to -42°C for 2 min and 30µm coronal sections cut on a cryostat. Sections were stored in multiwell plates containing cryoprotectant (30% glycerol / 30% ethylene glycol in 50mM sodium phosphate buffer) at -20°C until use. Sections for H&E staining were washed in 50mM PBS, mounted onto poly-L-lysine coated slides, allowed to dry and stained using standard methods. Free floating sections were rinsed in PBS and immunostained as described in section 2.1.

### 2.4.6 Processing of tissue for electron microscopy

Four additional animals each weighing 338g & 380g were used to produce tissue for electron microscopy. Two animals received intracortical perfusion of 1M monosodium glutamate and 2 received 1M NaCl as described above. At the end of the experimental procedure, the animals were killed by transcardiac perfusion of 150 ml, 0.9% saline followed by 150-200 ml of strong fix (4% paraformaldehyde; 5% gluteraldehyde; 0.05% calcium chloride in 0.08M sodium cacodylate buffer, pH7.2). Once removed, brains were post-fixed in strong fix for 24 h. A coronal section was cut through the resulting cortical lesion using a scalpel blade and a triangular section cut through the cortical lesion to the underlying corpus callosum. This section was embedded in araldite and processed on a Lynx tissue processor.

In collaboration with Prof. I.R. Griffiths, ultra thin sections were cut at 70nm on a Riechart-Jung Ultracut E ultratome and mounted onto copper grids. Sections were then stained by covering grids with saturated solution of uranyl acetate in 50% ethanol for 5-15 min at room temperature and then rinsed in 50% and 75% ethanol, distilled water (x2) and air-dried. Grids were then stained with Reynold's lead citrate for 5-10 min inside a NaOH moisturised chamber and washed 3 times in 1M NaOH and 5 times with distilled water.

## 2.5 Human post-mortem brain tissue

### 2.5.1 Sources of post-mortem brain tssue

*Head injury*

Formalin-fixed post-mortem brain tissue was obtained from 11 patients dying within <1 and 23 h following severe head injury (Table 2). The age of the patients ranged from 12 to 58 years and the post-mortem delay (i.e. the time between death and tissue fixation) ranged from 24 - 72 h. The corpus callosum was the brain area of choice in this study, since this area of the brain is commonly affected by diffuse axonal injury following head injury (Adams and Graham, 1989). Head injury in these patients occurred during a road traffic accident. The accumulation of APP immunoreactivity within axons was used as a marker for the early stages of axonal bulb formation and all patients used in this study had an APP score of at least 1 (McKenzie, et. al. 1995). In patients surviving for more than 2 h following head injury axonal swellings were detected with antibodies directed towards APP, 23 h following head injury many axonal bulbs were detected. APP immunostaining and classification of axonal injury was carried out by Prof. D.I. Graham in the Dept. of Neuropathology, University of Glasgow.

*Stroke*

Formalin fixed post-mortem brain tissue was obtained from 4 patients dying between 2 days and several weeks following a stroke (Table 2). The age of these patients ranged from 70 - 95 years. Post-mortem delay ranged from <24 - 48 h, with one patient outstanding at 6 days, however, no significant differences were detected within this tissue compared to other cases and was therefore included in this study. The corpus callosum, cingulate cortex and thalamus was selected for this study as these were areas affected by ischaemic brain injury. All strokes were occlusive, with no evidence of haemorrhage.

*Controls*

Formalin fixed post-mortem tissue from 7 patients with no known history of neurological or psychiatric disorders and no significant neuropathological abnormalities served as controls in this study (Table 2). The distribution of MAPs has been shown to be altered by post-mortem delay in the rat (Irving and Dewar, in press). This highlights the importance of using control tissue closely matched for post-mortem delay when extrapolating information from post-mortem tissue of brain injured patients. The control patients in this study were therefore selected according to both age and post-mortem delay. The age of control cases ranged from 12 - 59 years for comparison with head injured patients and 71 - 84 years for comparison to stroke patients. Post-mortem delay in the control cases ranged from 19 - 72 h.

### 2.5.2 Histology and immunohistochemistry

At post-mortem the whole brain was removed and immersed in 4% formalin for 48 h. Dissected blocks were then embedded in paraffin wax, 12μm sections cut on a microtome and mounted onto microscope slides. Prior to histological or immunohistochemical staining paraffin wax was removed by heating the sections at 60°C in an oven for 60 min and then placing in histoclear for 10 min. Sections for H&E staining were rehydrated and stained with H&E by standard methods. Sections for immunohistochemical staining were immersed in: 100% alcohol (2 x 10 min); methanol containing 0.5% hydrogen peroxide for 30 min to inhibit endogenous peroxidase activity and water for 10 min. At this stage microwave antigen retrieval was carried out on sections to be stained with Tau 1 (see below). Following two rinses in PBS all sections were incubated with PBS containing 0.2% Triton X-100. Sections were then rinsed in PBS (2 x 10 min) and immunostained as described in section 2.1.3. In addition to those antibodies described in section 2.1.3, human brain tissue sections were also stained using AD2, an antibody raised against PHF tau. The concentration curve to determine the optimum concentration of AD2 was therefore carried out using formalin-fixed paraffin embedded post-mortem tissue from a patient diagnosed with AD. The concentration found produce the best signal was 1:2000

and this dilution was therefore used throughout the investigations carried out in this thesis. As a positive control, an Alzheimer brain section was included in every immunohistochemical experiment (see Fig. 12). Following antigen detection the sections were rinsed and allowed to dry. Once dry, sections were dehydrated using graded alcohols and coverslipped using DPX mountant ready for microscopic analysis.

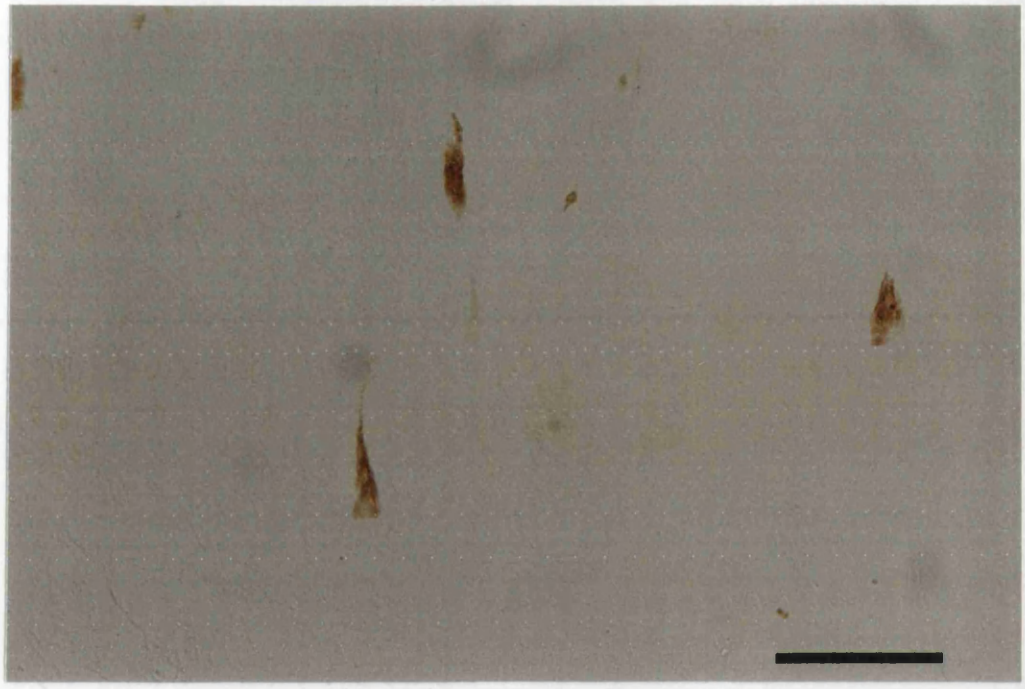


**Figure 12.** Photomicrograph showing NFTs immunostained with AD2 in the hippocampus of a patient diagnosed with AD. AD2 is a monoclonal antibody raised against PHF tau (see section 2.1.1) and therefore fails to detect normal tau, immunoreactivity is therefore specifically localised to NFTs in AD tissue. Staining of Alzheimer tissue was included in each immunohistochemistry experiment involving AD2, as a positive control. Scale bar = 50μm.

### 2.5.3 Antigen retrieval

Microwave pre-treatment was carried out on tissue being used for Tau 1 immunohistochemistry as this antibody does not react well with formalin fixed paraffin embedded tissue. 10mM citric acid pH6 or distilled water was heated for 5 min in a 65watt microwave at 70% power. The sections were then placed in the warmed solutions and heated for a further 5 min at 70% power. The solution volumes, lowered through heating, were topped up using distilled water and the tissue heated for a further 5 min. The sections were then removed from the microwave and allowed to stand in their solution for 20 min, rinsed in PBS and immunostained as described above. Citric acid gave a better antigen retrieval than water and so experiments were carried out using this method.

**TABLE 2 Case details**

| Cases | Age (yrs) | Survival | PM delay |
|---|---|---|---|
| Head injury | 20 | <1 h | 48 h |
| Head injury | 20 | <1 h | 48 h |
| Head injury | 18 | 1.5 h | N/K |
| Head injury | 39 | 2 h | 48 h |
| Head injury | 36 | 6 h | 60 h |
| Head injury | 14 | 7 h | 48 h |
| Head injury | 23 | 8 h | 48 h |
| Head injury | 58 | 10 h | 24 h |
| Head injury | 25 | 17 h | 24 h |
| Head injury | 46 | 19 h | 72 h |
| Head injury | 12 | 23 h | 24 h |
| Stroke | 84 | 2 days | 24 h |
| Stroke | 77 | 5 days | < 24 h |
| Stroke | 95 | 12 days | 6 days |
| Stroke | 70 | > 21 days | 48 h |
| Control | 12 | N/A | 36 h |
| Control | 18 | N/A | 24 h |
| Control | 25 | N/A | 31 h |
| Control | 34 | N/A | 72 h |
| Control | 59 | N/A | 19 h |
| Control | 71 | N/A | 24 h |
| Control | 84 | N/A | 69 h |

Post-mortem brain tissue was obtained from 11 patients dying following severe head injury and 4 patients dying following stroke. Survival time of patients following severe head injury ranged from <1-23 h, while that of stroke patients was 2->21 days. Seven patients with no history of neurological deficit served as controls. Control tissue was matched for age and post-mortem delay. The corpus callosum of head injured patients was examined as this area is commonly affected in head injury. For stroke patients, the cingulate cortex and thalamus was utilised as these were the areas affected by ischaemic brain damage. Both the corpus callosum and cingulate cortex were examined in the control brain tissue. N/A: not appropriate; N/K: not known.

## 2.6 Permanent focal cerebral ischaemia

The MCAO model of permanent focal cerebral ischaemia in the rat was used (Tamura, et. al. 1981). In this model both glutamate toxicity and free radical mediated mechanisms have been shown to be involved in the mechanisms underlying neuronal degeneration which occurs following decreased cerebral blood flow within the territory of the MCA (see section 1.2.3).

### 2.6.1 Surgical preparation of animals

Thirty adult male Fischer-344 rats, each weighing between 250-300g were used in this study. Animals were initially prepared as described in section 2.4.1. Arterial blood gas and glucose analyses was performed prior to and immediately following middle cerebral artery occlusion and prior to transcardiac perfusion. Animals were maintained normocapnic, normoxic and normothermic throughout the experimental period by adjusting the volume of gases delivered to the animal and controlling temperature using a heat lamp (Tables 3, 4 & 5).

### 2.6.2 Induction of cerebral ischaemia

The left middle cerebral artery (MCA) was exposed using a subtemporal approach based on the method originally described by Tamura et. al. (1981). This surgical procedure was performed by Dr. K. Yatsushiro. The animals were placed on their left side and a 2cm vertical incision made between the left orbit and the external auditory canal. The temporalis muscle was dissected from the cranium and retracted to expose the infero-temporal fossa. The zygomatic arch was left intact throughout the procedure. Under a high magnification operating microscope, a small craniectomy was made using a saline cooled dentist drill, at the junction between the medial wall and the roof of the infero-temporal fossa. The underlying dura was opened by an incision using a fine needle and retracted to reveal the main branch of the MCA. The exposed MCA was occluded by bipolar electrocoagulation from the area where it crossed the inferior cerebral vein to proximal to the origin of the lenticulostriate branch. All side branches including the lenticulostriate were coagulated and the artery transected. The muscle and skin was then sutured and the animals maintained under anaesthesia for appropriate intervals. Separate groups of animals were used for three distinct studies : 1) to determine the time of the induction of tau-positive oligodendrocytes following the onset of focal cerebral ischaemia; 2) to investigate the effect of drug intervention on the induction of tau-positive oligodendrocytes and 3) to determine whether increased tau-immunoreactivity in oligodendrocytes following focal cerebral ischaemia reflects increased *de novo* synthesis of the protein.

## TABLE 3 Physiological variables - Time course study

| Physiological Parameter | Time after MCAO | | |
|---|---|---|---|
| | 20 min | 40 min | 80 min |
| **MABP (mmHg)** | | | |
| Pre | 93 ± 4 | 88 ± 7 | 102 ± 6 |
| Post | 88 ± 4 | 90 ± 5 | 85 ± 5 |
| **Rectal Temperature (°C)** | | | |
| Pre | 37.0 ± 0.2 | 37.2 ± 0.1 | 36.8 ± 0.3 |
| Post | 36.8 ± 0.1 | 37.0 ± 0.0 | 37.0 ± 0.3 |
| **Arterial pH** | | | |
| Pre | 7.45 ± 0.01 | 7.42 ± 0.01 | 7.39 ± 0.02 |
| Post | 7.44 ± 0.01 | 7.44 ± 0.03 | 7.40 ± 0.01 |
| **$pCO_2$ (mmHg)** | | | |
| Pre | 38 ± 1 | 40 ± 1 | 44 ± 1 |
| Post | 36 ± 2 | 37 ± 1 | 40 ± 0 |
| **$pO_2$ (mmHg)** | | | |
| Pre | 156 ± 8 | 151 ± 10 | 175 ± 12 |
| Post | 155 ± 4 | 179 ± 14 | 160 ± 17 |
| **Plasma Glucose (mM)** | | | |
| Pre | 8.6 ± 0.8 | 9.2 ± 0.6 | 9.4 ± 0.3 |
| Post | 7.3 ± 0.9 | 7.8 ± 0.6 | 9.1 ± 0.3 |

Mean arterial blood pressure (MABP) and physiological parameters were recorded both immediately after femoral vessel cannulation (pre) and after MCAO (post). Groups of rats were killed : 20 min (n=3); 40 min (n=4) and 80 min following MCAO. There were no significant differences in the physiological parameters detected throughout the experimental period between experimental groups.

## TABLE 4 Physiological variables - Drug intervention study

| | Drug Pretreatment | | |
|---|---|---|---|
| **Physiological Parameter** | **MK-801** | **NBQX** | **PBN** |
| **MABP (mmHg)** | | | |
| Pre | 84 ± 7 | 91 ± 1 | 88 ± 5 |
| Post | 82 ± 1 | 89 ± 5 | 85 ± 4 |
| **Rectal Temperature (°C)** | | | |
| Pre | 37.1 ± 0.2 | 37.1 ± 0.2 | 36.8 ± 0.1 |
| Post | 37.2 ± 0.2 | 37.0 ± 0.1 | 37.4 ± 0.3 |
| **Arterial pH** | | | |
| Pre | 7.45 ± 0.02 | 7.42 ± 0.02 | 7.40 ± 0.02 |
| Post | 7.38 ± 0.02 | 7.39 ± 0.02 | 7.40 ± 0.01 |
| **$pCO_2$ (mmHg)** | | | |
| Pre | 39 ± 1 | 40 ± 1 | 39 ± 1 |
| Post | 39 ± 1 | 39 ± 2 | 38 ± 1 |
| **$pO_2$ (mmHg)** | | | |
| Pre | 166 ± 7 | 167 ± 13 | 171 ± 7 |
| Post | 160 ± 9 | 164 ± 12 | 175 ± 5 |
| **Plasma Glucose (mM)** | | | |
| Pre | 8.5 ± 0.4 | 10.0 ± 0.6 | 9.5 ± 0.8 |
| Post | 8.3 ± 0.3 | 11.8 ± 0.9 | 9.1 ± 0.5 |

Mean arterial blood pressure (MABP) and physiological parameters were recorded both immediately after femoral vessel cannulation (pre) and after MCAO (post). Rats received drug pretreatment 30 min prior to MCAO and were killed 40 min after MCAO. The following pre-treatments were examined: MK-801 (0.5 mg/kg, i.v.), n=5; NBQX (30 mg/kg, i.v.; 30 min prior to and 30 min after MCAO), n=5; PBN (100 mg/kg, i.p.), n=5. There were no significant differences detected throughout the experimental period between experimental groups, with the exeption of increased plasma glucose levels following NBQX administration.

## TABLE 5 Physiological variables - In situ hybridisation study

| Physiological Parameter | Time after MCAO | | |
|---|---|---|---|
| | 40 min | 120 min | 240 min |
| **MABP (mmHg)** | | | |
| Pre | 80 ± 0 | 90 ± 5 | 83 ± 3 |
| Post | 75 ± 0 | 87 ± 7 | 75 ± 0 |
| **Rectal Temperature (°C)** | | | |
| Pre | 37.2 ± 0.3 | 36.9 ± 0.3 | 36.8 ± 0.2 |
| Post | 36.9 ± 0.1 | 36.6 ± 0.2 | 36.8 ± 0.1 |
| **Arterial pH** | | | |
| Pre | 7.39 ± 0.04 | 7.37 ± 0.05 | 7.44 ± 0.03 |
| Post | 7.40 ± 0.01 | 7.39 ± 0.03 | 7.36 ± 0.04 |
| **$pCO_2$ (mmHg)** | | | |
| Pre | 44 ± 1 | 40 ± 1 | 37 ± 1 |
| Post | 38 ± 2 | 42 ± 1 | 37 ± 1 |
| **$pO_2$ (mmHg)** | | | |
| Pre | 145 ± 14 | 148 ± 11 | 168 ± 5 |
| Post | 145 ± 6 | 144 ± 3 | 156 ± 11 |

Mean arterial blood pressure (MABP) and physiological parameters were recorded both immediately after femoral vessel cannulation (pre) and prior to kill (post). Animals remained normocapnic, normoxic and normothermic during the experimental period. Groups of rats were killed : 40 min (n=2); 120 min (n=2) and 240 min (n=2) after MCAO.

### 2.6.3 Immunohistochemical studies

To determine the earliest time at which tau-positive oligodendrocytes could be detected following permanent focal cerebral ischaemia, animals were killed by transcardiac perfusion of 4% paraformaldehyde, at 20 min (n=3); 40 min (n=4) or 80 min (n=3) after MCAO. In order to investigate the effect of drug intervention on the induction of tau immunoreactivity in oligodendrocytes 40 min following MCAO rats were treated with : MK801 (0.5mg/kg, i.v.)(n=5); NBQX (30mg/kg, i.v.)(n=4) or PBN (100mg/kg i.p)(n=5). All drugs were administered 30 min prior to MCA occlusion, NBQX was administered again 30 min after MCA occlusion. MK801 was prepared as a 1mg/ml solution in 0.9% saline and PBN as a 25mg/ml solution in distilled water. NBQX was prepared as a 15mg/ml solution by dissolving 15mg of compound in 20µl 1M NaOH and adding 9.98ml of 5.5% glucose (made in distilled water). The doses for each drug used in this study has previously been shown to reduce infarct volume following focal cerebral ischaemia in the rat (Cao and Phillis, 1994; Gill, et. al. 1992; Park, et. al. 1988). For the drug intervention studies animals were killed, by transcardiac perfusion of 4% paraformaldehyde, 40 min after MCAO.

Following transcardiac perfusion, brains were removed and processed for histology and immunohistochemistry as described in section 2.4.5. Free floating sections were immunostained as described in section 2.1.

### 2.6.4 Quantification of tau positive oligodendrocytes following cerebral ischaemia

For each animal from both the time course study and the drug intervention study, the total number of tau-positive oligodendrocytes in the entire subcortical white matter, extending from that underlying the perirhinal cortex to that underlying cingulate cortex was counted in one section from each of the two brain levels shown in Fig. 13. Sections were viewed at x40 magnification, blind to the drug treatments of each animal. The area of subcortical white matter in each section counted was measured using an MCID image analyser and the number of tau-positive oligodendrocytes / $mm^2$ calculated for the white matter of each brain level. From this the average number of tau-positive oligodendrocytes / $mm^2$ present in the 2 sections counted was calculated for each individual animal. The mean number of tau-positive oligodendrocytes / $mm^2$ in each treatment group was calculated and data expressed as mean ± SEM. The same quantitative analysis was performed in both hemispheres, ipsilateral and contralateral to the occluded MCA. To determine the reproducibility of this quantitative approach, the number of tau-positive oligodendrocytes was determined and the area of corpus callosum measured in one animal from each treatment group 1 week later (see Fig. 14). The coefficient of variance for repeated measurements 1 week apart was 7%. For comparison of drug treated groups statistical

analyses consisted of an ANOVA followed by Students one-tailed t-test with appropriate Boneforroni correction. The *a priori* hypothesis which was tested was that drug-treatment would reduce the number of tau-positive oligodendrocytes present following 40 min MCAO as compared to untreated animals.

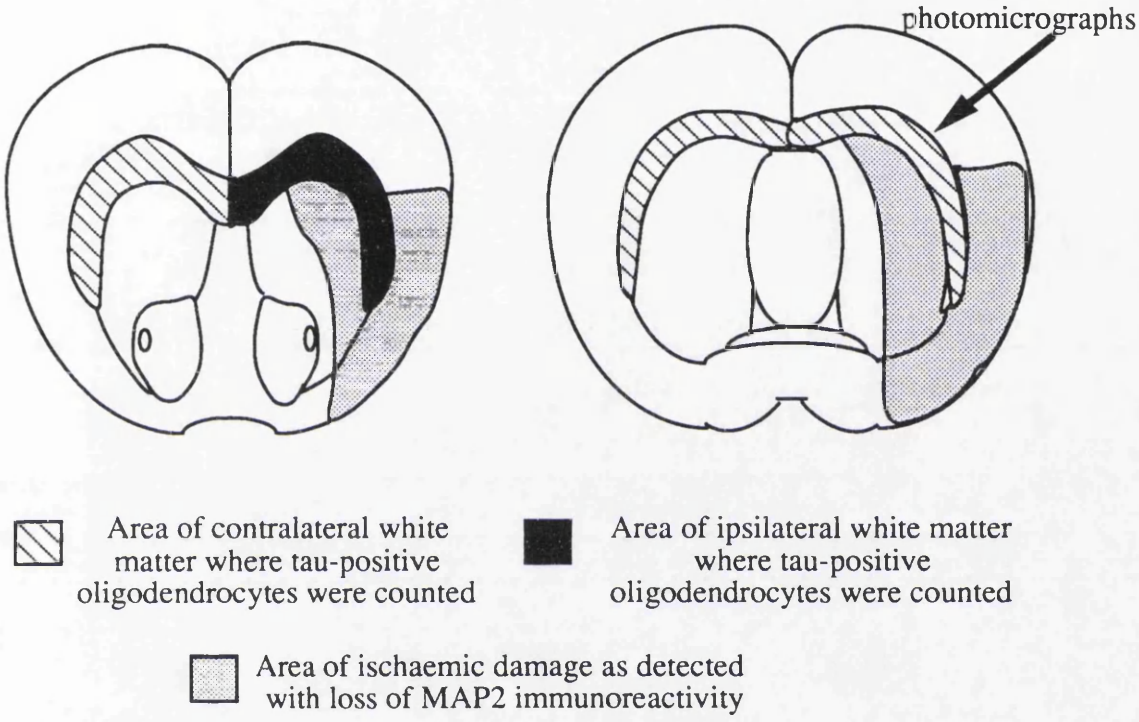



**Figure 13**. Schematic representation of the sections in which the number of tau-positive oligodendrocytes/$mm^2$ was determined in subcortical white matter. The sections corresponded to those approximately 1.2 mm and 0.7 mm to Bregma in the atlas of Paxinos and Watson. The arrow indicates the region of subcortical white matter from which photographs shown in Fig. 36 were taken.

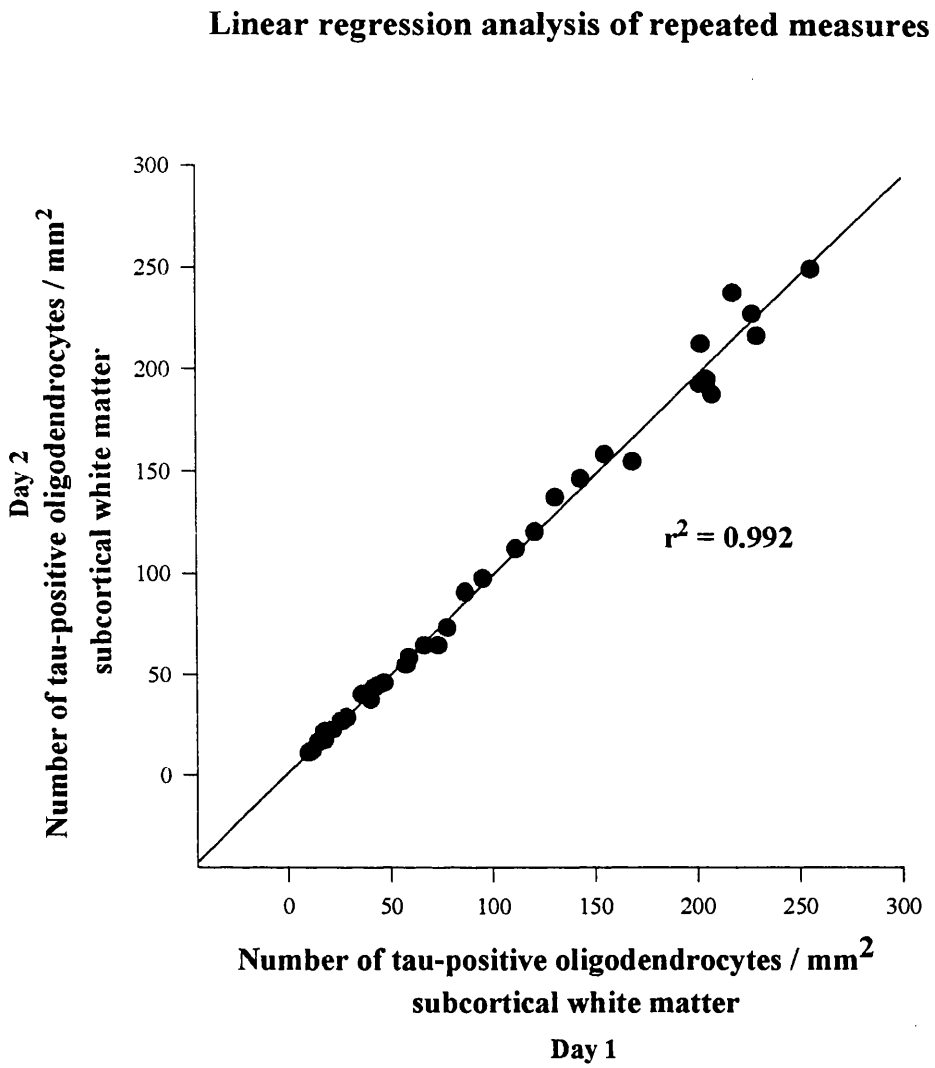



**Figure 14** Relationship between the number of oligodendrocytes / $mm^2$ subcortical white matter calculated on day 1 and day 2 (1 week later). The number of oligodendrocytes / $mm^2$ was calculated at two brain levels for each animal in the study as described above. This analysis was repeated for 1 animal from each treatment group 1 week later, the correlation coefficient for repeated measures was 7%. The line of linear regression is shown in the graph and the correlation coefficient ($r^2$) is presented.

### 2.6.5 In situ hybridisation studies

In situ hybridisation studies were carried out to determine whether increased tau immunoreactivity in oligodendrocytes following permanent focal cerebral ischaemia represents increased *de novo* synthesis of the protein. Animals were killed by decapitation : 40 min (n=2); 120 min (n=2) or 240 min (n=2) following the induction of cerebral ischaemia. One animal was anaesthetised and decapitated without any surgical intervention. Following decapitation, the brains were rapidly removed and submerged in a small volume of tissue mountant contained within a small cup made with aluminum foil. This was then lowered into isopentane chilled in liquid nitrgen until the tissue mountant solidified. 15μm sections were then cut using a cryostat, mounted on to APES coated slides and processed for tau *in situ* hybridisation using $^{35}S$ riboprobes as described in section 2.3.

## 2.7. Oligodendrocyte cultures

Pure oligodendrocyte cultures, were derived from an O-2A cell line kindly provided by Dr. S.C. Barnett (Department of Neurology, University of Glasgow). This cell line has recently been described by Barnett and Crouch, (1995) and preparation of cultures for immunohistochemistry and immunoblotting experiments was kindly carried out by Dr. S.C. Barnett.

### 2.7.1 Preparation of oligodendrocyte cultures

Cell line clones were maintained in 25cm$^2$ flasks (NUNC) at -70°C until use. Cells were detached from the flasks by washing in 2.5mg/ml trypsin (bovine pancreas, Sigma) for a few minutes at room temperature. Cells were then pipetted into a centrifuge tube and trypsin activity inhibited using an equal volume of Soya bean trypsin inhibitor: 0.52mg/ml soya bean trypsin inhibitor; 0.4mg/ml bovine pancreas DNase; 3mg/ml BSA fraction V (Sigma). The suspension was spun and the cells resuspended in modified Sato medium (Bottenstein and Sato, 1990) containing 5ng/ml platelet derived growth factor (PDGF) and 5ng/ml basic fibroblast growth factor (bFGF) - PF medium (Barnett and Crouch, 1995). The presence of these growth factors allows self-renewal of O-2A progenitor cells while preventing their differentiation (Bogler et. al. 1990). Cells were counted using a haemocytometer and approximately 7,000 placed on poly-L-lysine coated coverslips in 24 well plates for immunohistochemical experiments. For immunoblotting experiments an equal number of cells were placed on the surface of individual 25cm$^2$ NUNC flasks. All cells were incubated at 37°C in a $CO_2$ incubator until they had adhered to the coverslips or flasks respectively. PF medium was added and cells were incubated at 37°C in a $CO_2$ incubator until the cells had reached 80% confluency. PF medium was then replaced with Sato medium to allow differentiation of the cells into oligodendrocytes. Every second day

half the medium was removed and replaced with fresh Sato's medium. Following 4-5 days in culture oligodendrocyte cultures for immunocytochemical analysis were exposed to cytotoxic injury, as described below, and processed for tau immunocytochemistry. Batch cultures for immunoblotting experiments were washed in cold PBS and frozen on dry ice. Cultures were stored at -70°C until use for western blot analyses.

### 2.7.2 Exposure of oligodendrocytes to glutamate and sodium chloride

In a laminar flow hood, using sterile techniques, oligodendrocyte cultures prepared on coverslips as described above, were exposed to 10μM, 100μM or 1mM monosodium glutamate or sodium chloride (made up in Sato medium) for 4 h at 37°C in a $CO_2$ incubator. Controls were maintained in Sato medium during this time. Some cultures were dephosphorylated prior to immunostaining, this was carried out in the multiwell plates as described in section 2.1.3.

### 2.7.3 Immunocytochemistry

A control culture from each experimental run was stained with 01 antibody to confirm the presence of mature oligodendrocytes within the cultures. On removal from the incubator cells were rinsed with Hanks balanced salt solution (HBSS; 3 x 10 min) and 01 added (1 : 3 in HBSS) or 30 min at room temperature. Following 2 x 10 min washes with HBSS rhodamine labelled IgG was added (1 : 3) in HBSS for 30 min. Cells were then fixed and stained for tau as described below.

Following incubation, the cells were removed from the incubator washed in Hanks Hepes, fixed with ice cold 4% paraformaldehyde for 15 min, washed with PBS (2 x 5 min) and permeabilised in 0.2% Triton X-100 for 30 min. Following two washes with PBS (2 x 5 min) endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in PBS, and the cells immunostained for tau as described in section 2.1.3 using 3 different antibodies : Tau 1, 1:1250; TP70, 1:3000; TP007 1:750. Following immunostaining, cells were dehydrated in 70%, 90% and 100% alcohol (10 min each), immersed in histoclear for 10-15 min and permanently mounted onto poly-L-lysine coated slides using DPX, for microscopic analysis.

# CHAPTER 3 RESULTS

## 3.1 Intracortical perfusion of glutamate in vivo

Glutamate excitotoxicity has been implicated in the pathogenesis of a variety of neurodegenerative conditions in which NFT pathology is observed. However, whether increased extracellular concentrations of glutamate are involved in the mechanisms underlying tangle formation is unknown. This study was designed to investigate the effects of high extracellular concentrations of glutamate on the distribution of tau within neurons to determine if glutamate induces any changes in tau similar to those occurring in NFT formation. In vivo microdialysis was used to perfuse 1M monosodium glutamate or the control solution, 1M NaCl into the rat parietal cortex at 1.5µl/min for 90 min. Four h from the start of perfusion the animals were killed and the brain processed for histology and immunohistochemistry.

### 3.1.1 Histology - light microscope

Intracortical perfusion of 1M monosodium glutamate resulted in a sizeable lesion which extended in anterior and posterior directions and was characterised by an area of pallor as shown by H&E staining. In agreement with previous studies (Fujisawa, et. al. 1993a; 1993b; 1996; Landolt, et. al. 1993) the histopathological features of the lesion included a loss of H&E stained neurons, triangulation of the nucleus and shrinkage of neurons and neuropil. There was a sharp boundary between the area of the lesion and histologically normal tissue observed in the H&E stained sections (Fig. 15). In contrast, intracortical perfusion of the control solution, 1M NaCl, resulted in a smaller area of pallor on H&E stained sections compared to that resulting from glutamate perfusion. The boundary between the area of pallor and histologically normal tissue in the NaCl perfused brains was less well delineated than in glutamate perfused animals (Fig. 15). Within the core of the NaCl induced lesion, the number of H&E stained neurons appeared to be reduced compared to histologically normal tissue, while towards the edge of the lesion nuclei had undergone triangulation. The perfusion of artificial CSF did not cause significant neuronal degeneration (Fig. 16) and failed to show any significant alteration to the normal distribution of immunostaining obtained for all the antibodies used in this study, therefore only the results obtained with glutamate and NaCl perfusion are presented.

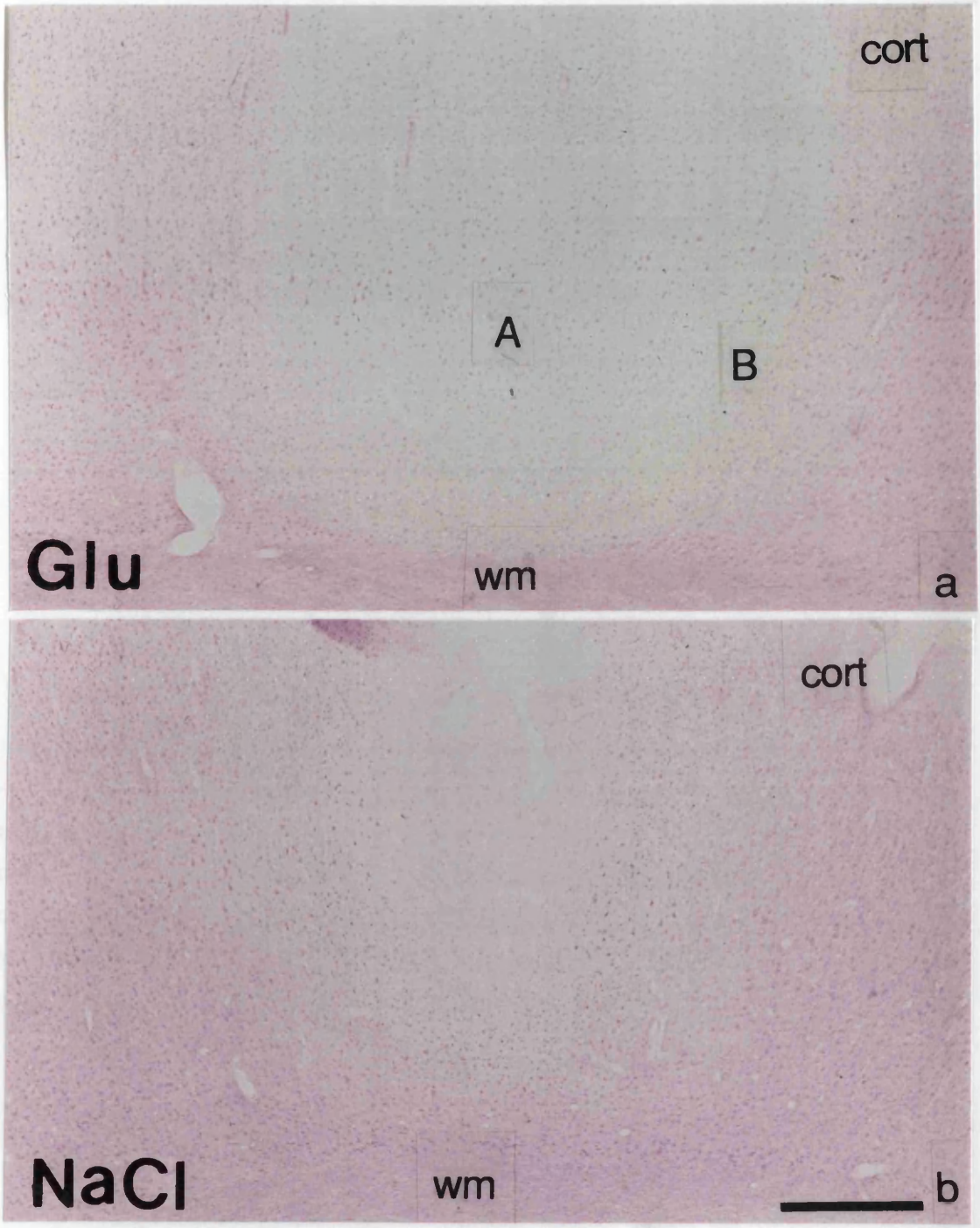



**Figure 15**. H&E stained coronal sections showing the area of pallor characterising the lesioned area produced by the intracortical perfusion of 1M monosodium glutamate (a) or 1M NaCl (b). Note the sharp boundary between lesioned and histologically normal tissue following 1M monosodium glutamate perfusion (a). This boundary was less well delineated after 1M NaCl perfusion (b). Both photographs are the same magnification; bar = 250μm. A - necrotic core; B - boundary region of the lesion; cort - parietal cortex; wm - subcortical white matter.

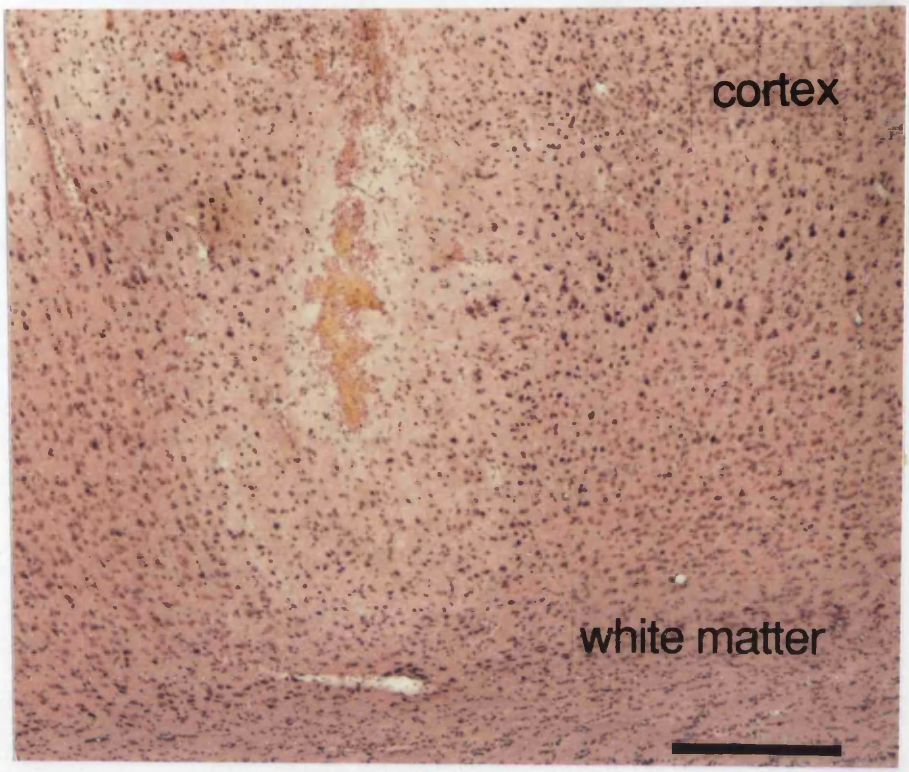



**Figure 16.** H&E stained coronal sections showing the area of damage resulting from the intracortical perfusion of artificial CSF. In comparison to intracortical perfusion of 1M monosodium glutamate or 1M NaCl, the perfusion of artificial CSF did not induce significant neuronal damage. Only mechanical damage caused by microdialysis probe placement was evident. Scale bar = 400µm.

In addition to histological damage present in the cortical grey matter, areas of pallor were detected in the subcortical white matter immediately underlying glutamate induced lesions, as compared to histologically normal tissue (Fig. 17). In contrast the white matter underlying NaCl induced cortical lesions appeared to be histologically normal as compared to that of the contralateral hemisphere. This suggests that the neuropil of the subcortical white matter is damaged by the perfusion of glutamate but not NaCl. Examination of H&E stained sections using light microscopy failed to detect significant histological changes in oligodendrocytes within the subcortical white matter following intracortical perfusion of glutamate or NaCl (Fig. 17), although ultrastructural changes in oligodendrocytes were detected using electron microscopy (see section 3.1.4).

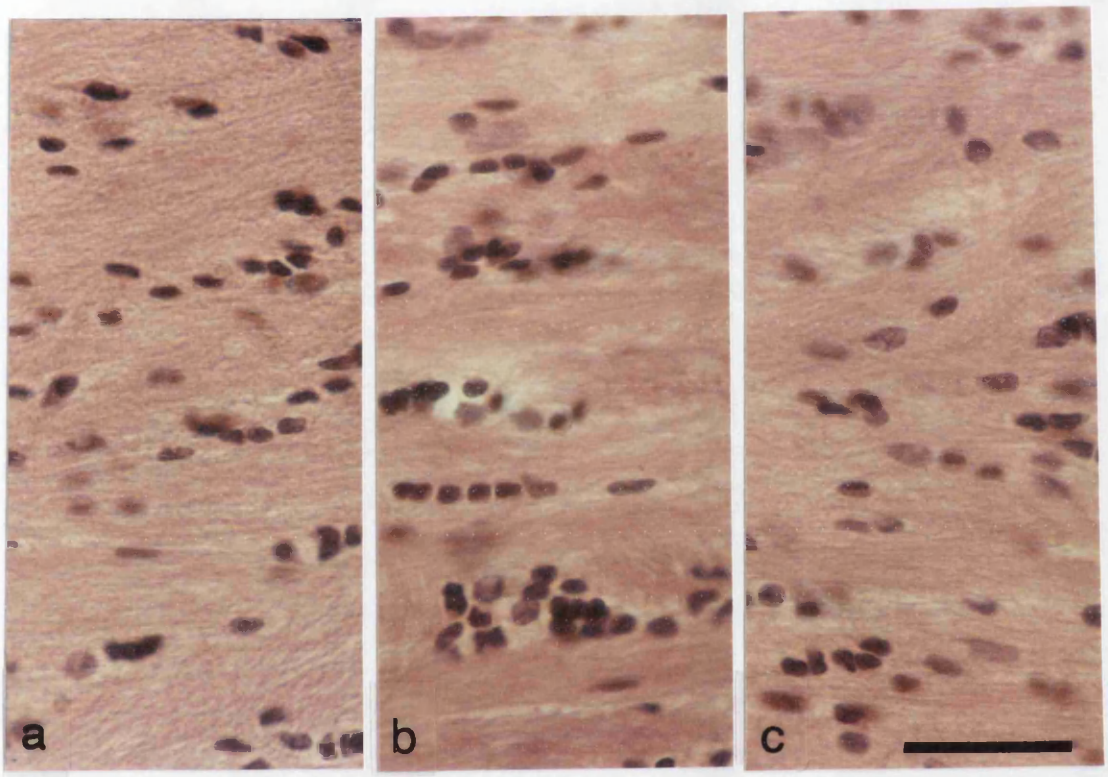



**Figure 17.** H&E stained coronal sections showing the subcortical white matter immediately underlying cortical lesions induced by the perfusion of 1M monosodium glutamate (b) or 1M NaCl (c). Areas where H&E staining was decreased in the neuropil were detected within the subcortical white matter immediately underlying the lesion induced by intracortical perfusion of 1M monosodium glutamate (a). Reduced staining of the neuropil, probably due to the swelling of axons, dendrites and astrocytic processes, suggests that in addition to the cortex, the subcortical white matter is also affected by the intracortical perfusion of glutamate. In contrast, H&E staining of the subcortical white matter immediately underlying the lesion induced by the intracortical perfusion of 1M NaCl (c) was not significantly different from that of the contralateral subcortical white matter (a). No significant alteration in glial cell morphology was detected in the subcortical white matter immediately underlying 1M monosodium glutamate or 1M NaCl induced cortical lesions. Scale bar = 50μm.

### 3.1.2 The effect of intracortical glutamate perfusion on the distribution of tau, MAP2 and MAP5 immunoreactivity in neurons

*Tau*

The Tau 1 antibody detects tau dephosphorylated between residues 199-202 and therefore in normal rat brain immunoreactivity was detected predominantly within axons where tau is dephosphorylated at this epitope. In contrast Tau 1 immunoreactivity was not detected in neuronal perikarya where tau is phosphorylated at this epitope. This pattern of Tau 1 immunoreactivity in histologically normal tissue is similar to that described previously (Binder, et. al. 1985; Papasozomenos and Binder, 1987). Following intracortical glutamate perfusion this pattern of Tau 1 immunoreactivity was altered (Fig. 18). At the core of the lesion, Tau 1 immunoreactivity was increased within neuronal perikarya and decreased in the neuropil compared to histologically normal tissue. Furthermore, it should be noted that perikaryal staining induced by intracortical glutamate perfusion was much more intense than that of histologically normal tissue incubated with alkaline phosphatase. In contrast to glutamate, NaCl perfusion did not increase Tau 1 immunoreactivity within neuronal perikarya, however neuropil staining was reduced (Fig.18). Alkaline phosphatase pre-treatment failed to alter the pattern of Tau 1 immunostaining present within the neurons following glutamate or NaCl perfusion (Fig. 19), suggesting that decreased Tau 1 staining was not due to phosphorylation of the protein at this epitope. In contrast to Tau 1, AT8 detects tau phosphorylated at residue 200 and therefore predominantly detects tau within neuronal perikarya in histologically normal tissue (Fig. 18). Following intracortical perfusion of either glutamate (Fig. 18) or NaCl (Fig. 18), AT8 immunoreactivity was decreased in neuronal perikarya, confirming that tau present within neuronal perikarya following glutamate perfusion is dephosphorylated at this epitope.

The distribution of B19 (directed towards full-length tau), TP70 (directed towards C-terminal of tau) and TP007 (directed towards N-terminal of tau) in histologically normal tissue is shown in Fig. 20. These antibodies are phosphorylation independent and therefore detect tau present in both neuronal perikarya and axons. In contrast to Tau 1; B19, TP70 and TP007 immunoreactivity was decreased within neuronal perikarya following glutamate perfusion (Fig. 20) compared to histologically normal tissue. Similarly to Tau 1, neuropil staining with B19 and TP007 was reduced following both glutamate and NaCl perfusion, however TP70 remained relatively unchanged. B19, TP70 and TP007 staining of neuronal perikarya was reduced following NaCl perfusion (Fig. 20).

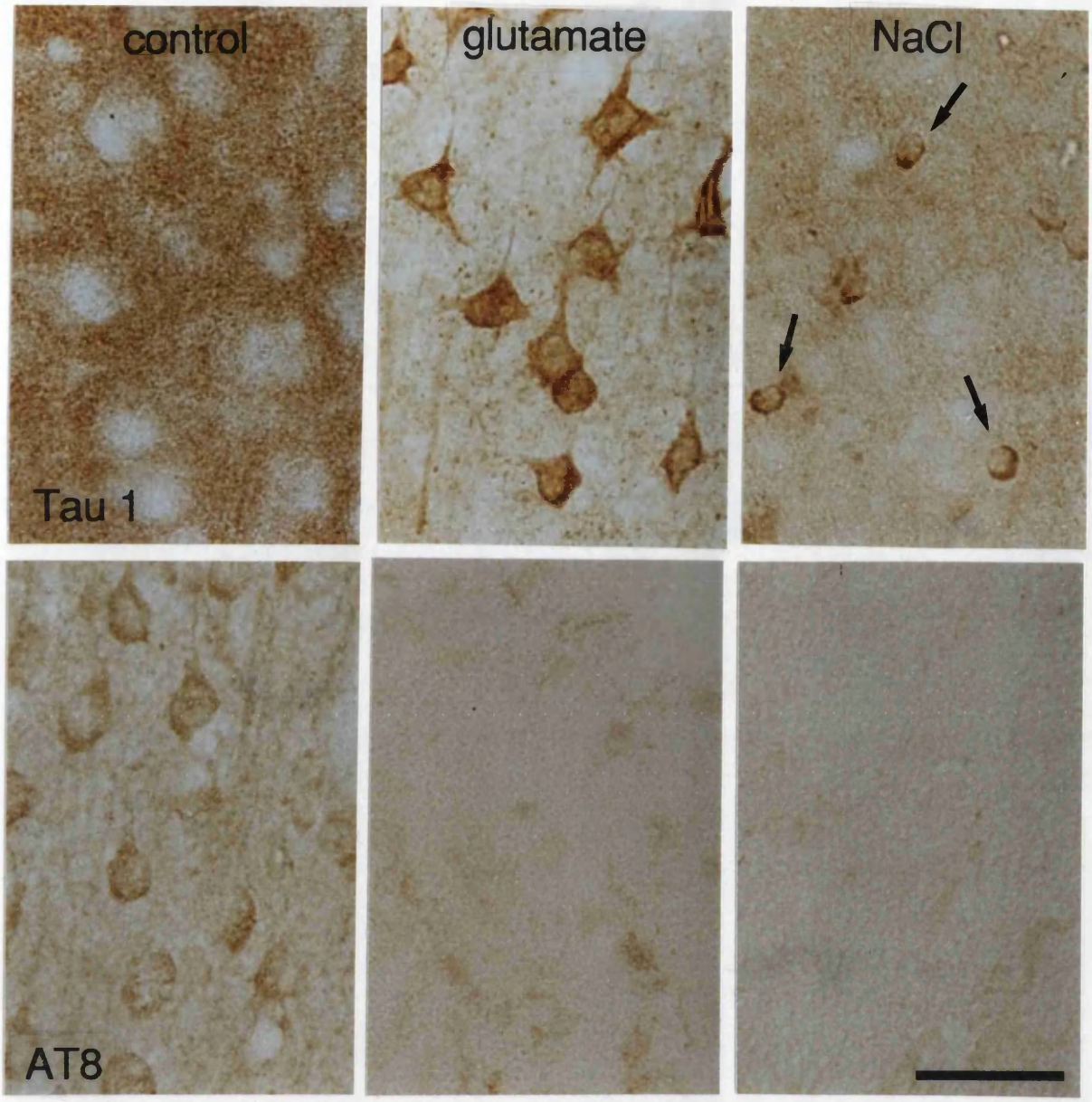



**Figure 18.** Distribution of Tau 1 and AT8 immunoreactivity in rat cerebral cortex. Tau 1 immunoreactivity, most abundant in axons in histologically normal tissue, was increased in neuronal perikarya at the core of the lesion produced by the intracortical perfusion of 1M monosodium glutamate. In contrast, AT8 immunoreactivity, most abundant within neuronal perikarya in histologically normal tissue, decreased following glutamate perfusion. Tau 1 immunoreactivity was decreased in axons following glutamate or NaCl perfusion compared to histologically normal tissue. AT8 immunoreactivity was not detected following glutamate or NaCl perfusion. In addition to the alterations in neuronal immunoreactivity, increased Tau 1 immunoreactivity was detected in small cells with the morphological appearance of glia within the cortical lesion induced by 1M NaCl perfusion (arrows) (see section 3.1.3). Scale bar = 50μm

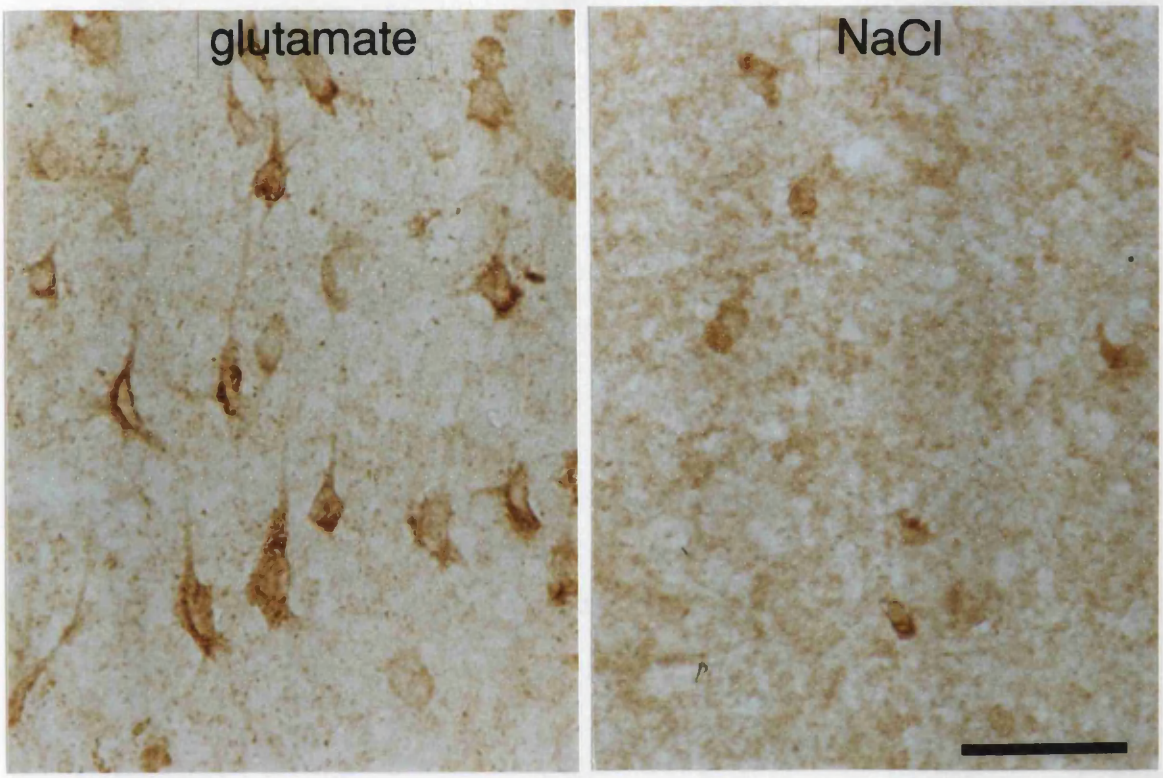



**Figure 19.** The effect of alkaline phosphatase pre-treatment on the distribution of Tau 1 immunoreactivity in neurons and glia following intracortical perfusion of 1M monosodium glutamate or 1M NaCl. Following intracortical perfusion of 1M monosodium glutamate, increased Tau 1 immunoreactivity was detected in neuronal perikarya while staining was decreased in the axons. Intracortical perfusion of 1M NaCl resulted in increased Tau 1 immunoreactivity within small cells with the morphological appearance of glia. This pattern of Tau 1 immunoreactivity described within the cortex following glutamate or NaCl perfusion was not altered by dephosphorylation of the tissue prior to immunostaining. This data confirms that decreased Tau 1 immunoreactivity within the neuropil detected following the perfusion of glutamate or NaCl was not due to phosphorylation of tau at the Tau 1 epitope. Scale bar = 50μm.

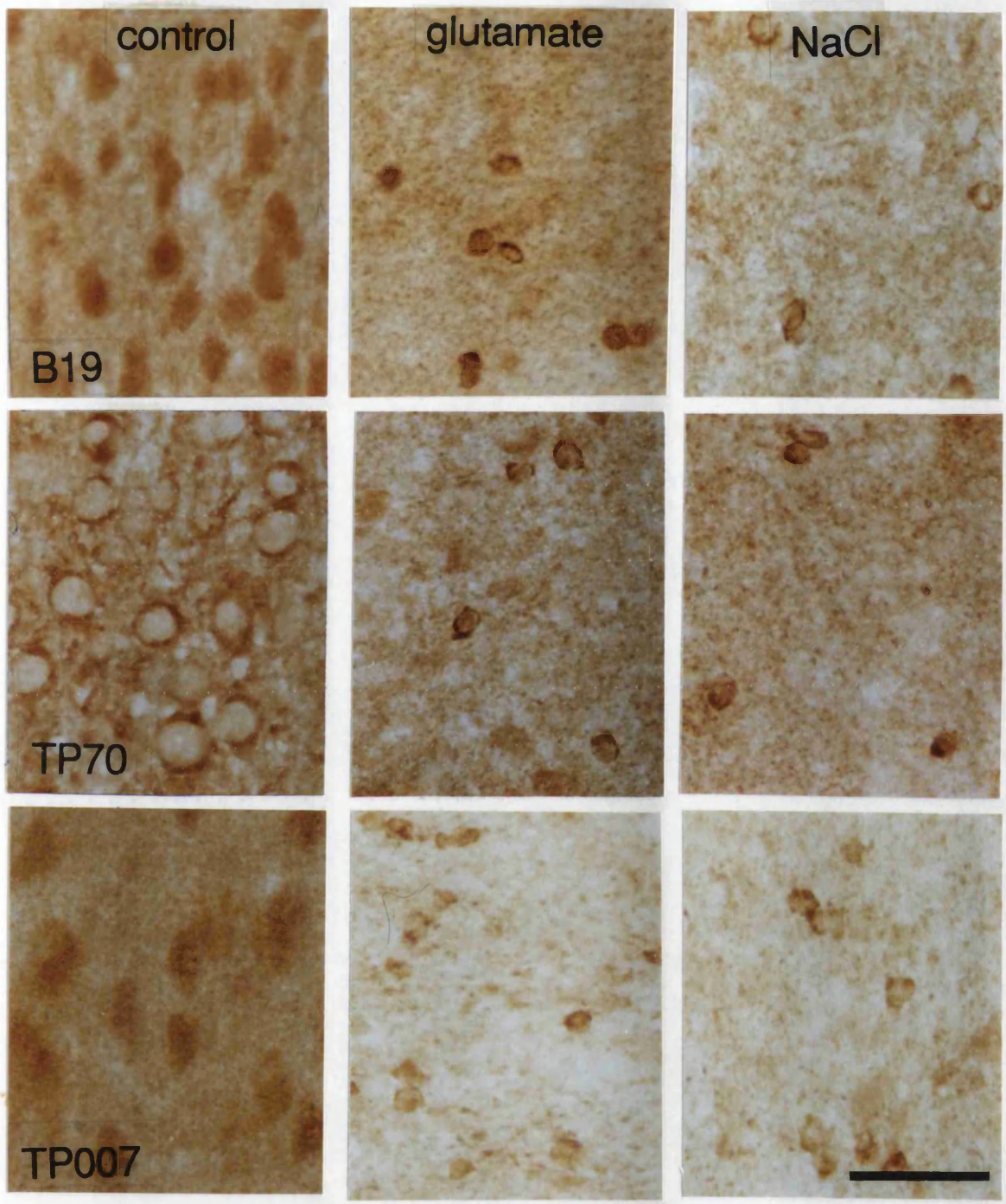



**Figure 20.** Distribution of B19, TP007 and TP70 immunoreactivity in rat cerebral cortex. B19, TP70 and TP007 detect tau in both axons and neuronal perikarya of histologically normal tissue. Following intracortical perfusion of 1M monosodium glutamate or 1M NaCl, B19, TP70 and TP007 immunoreactivity was decreased in neuronal perikarya. Similarly, B19 and TP007 staining of axons was reduced following both glutamate and NaCl perfusion however TP70 immunoreactivity remained relatively unchanged. Increased tau immunoreactivity was detected in small cells with the morphological appearance of glia within the cortical lesion resulting from the perfusion of 1M monosodium glutamate or 1M NaCl (see section 3.1.3). Scale bar = 50μm.

*MAP2 and MAP5*

To compare the changes in tau immunoreactivity with that of other MAPs, adjacent sections to those stained with tau, were labelled using antibodies directed towards MAP2 and MAP5. MAP2 immunoreactivity was detected mainly in dendrites and neuronal perikarya in histologically normal tissue (Fig. 21) as described previously (Cáceres, et. al. 1984; Huber and Matus, 1984). At the core of the lesion produced by the intracortical perfusion of 1M monosodium glutamate neuronal perikaryal staining with the MAP2 antibody was increased while dendritic staining was reduced (Fig. 21). In contrast to glutamate, 1M NaCl perfusion did not increase neuronal perikaryal immunostaining, instead both dendritic and perikaryal MAP2 immunoreactivity was reduced (Fig. 21). MAP5 immunoreactivity was detected in both perikarya and neuropil in histologically normal tissue (Fig. 21). Similarly to MAP2, MAP5 immunoreactivity increased within neuronal perikarya and decreased in neuropil within the core of the lesion produced by glutamate perfusion (Fig. 21). NaCl perfusion resulted in decreased MAP5 immunostaining in both perikarya and neuropil (Fig. 21). Thus the effects of intracortical perfusion of glutamate or NaCl on the immunostaining of MAPs within neurons were not specific for tau, but also included MAP2 and MAP5.

### 3.1.3 MAPs in oligodendrocytes following intracortical glutamate perfusion

In addition to changes in the distribution of tau, MAP2 and MAP5 immunoreactivity detected in neurons, tau immunoreactivity was detected in small cells with the morphological appearance of oligodendrocytes following intracortical glutamate or NaCl perfusion. This was an unexpected finding since tau is thought to be a neuron specific protein. However increased tau immunoreactivity was detected in oligodendrocytes within the cortical lesion and the subcortical white matter immediately underlying this area, both in glutamate and NaCl perfused animals. In light of this novel and unexpected finding of increased tau staining in glial cells, it was decided to persue this finding. Unravelling this response became the focus of future studies.

*Tau*

Within the subcortical white matter contralateral to the glutamate or NaCl induced cortical lesion, tau, as detected with Tau 1, TP70, TP007 and B19 was localised predominantly within axons (Fig. 22 & 23). Following intracortical perfusion of 1M monosodium glutamate or 1M NaCl, tau immunoreactivity was observed in small cells with the morphological appearance of glia. These tau-positive glial cells were found scattered throughout the cortical lesion (Fig. 18 & 20) but also abundantly within the subcortical white matter immediately underlying this area (Fig. 22 & 23). Following intracortical perfusion of 1M monosodium glutamate or 1M NaCl, tau positive glial cells were detected using B19, TP70 and TP007 suggesting the presence of full-length protein.

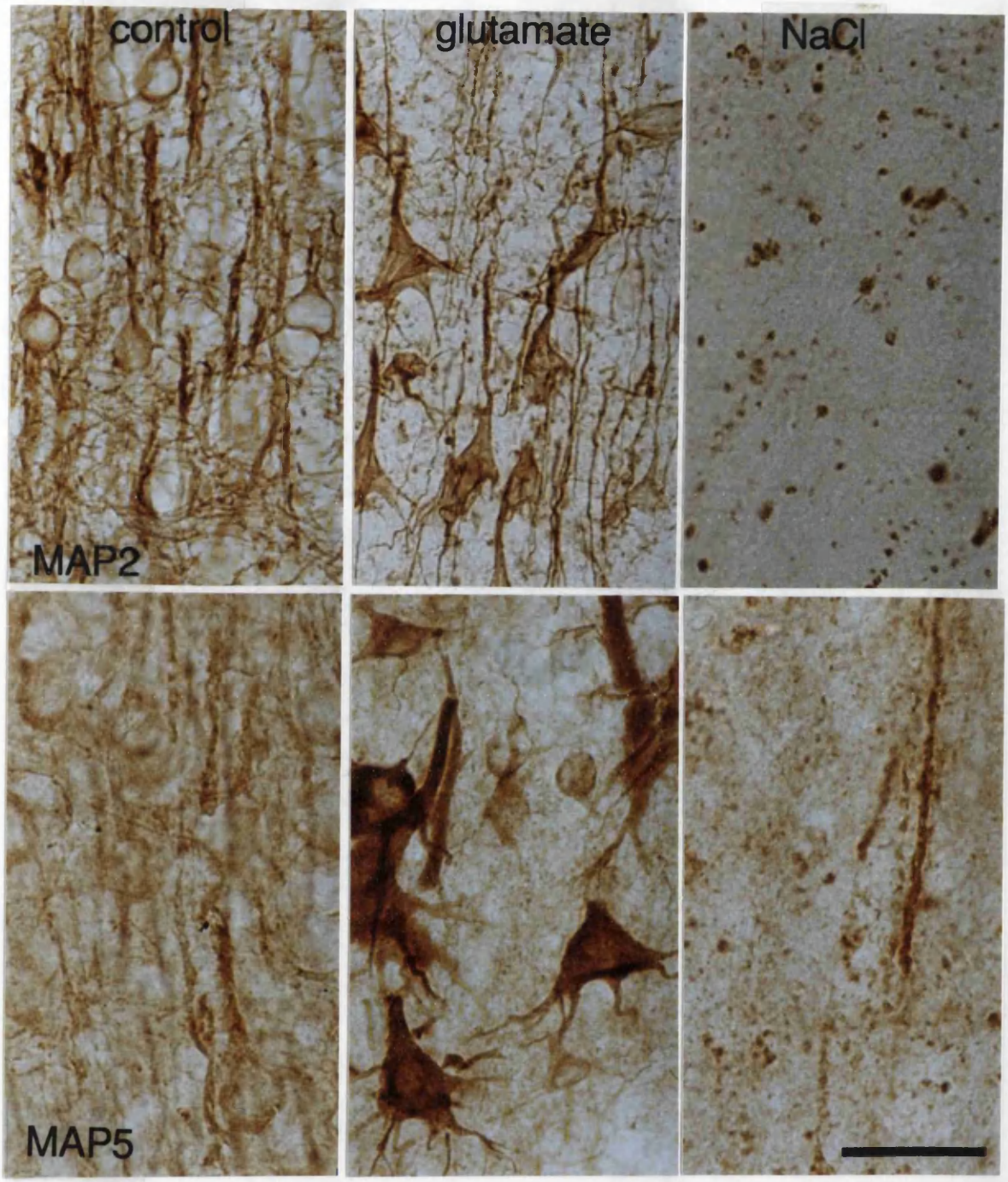



**Figure 21**. Distribution of MAP2 and MAP5 immunoreactivity in neurons following intracortical perfusion of 1M monosodium glutamate or 1M NaCl. MAP2 and MAP5 immunoreactivity was detected within neuronal perikarya and neuropil of histologically normal tissue. Following intracortical glutamate perfusion, both MAP2 and MAP5 immunoreactivity increased in perikarya and decreased in neuropil within the resulting cortical lesion. By contrast, MAP2 and MAP5 immunoreactivity was decreased in both neuronal perikarya and neuropil within the cortical lesion induced by the perfusion of 1M NaCl. Scale bar = 50μm.

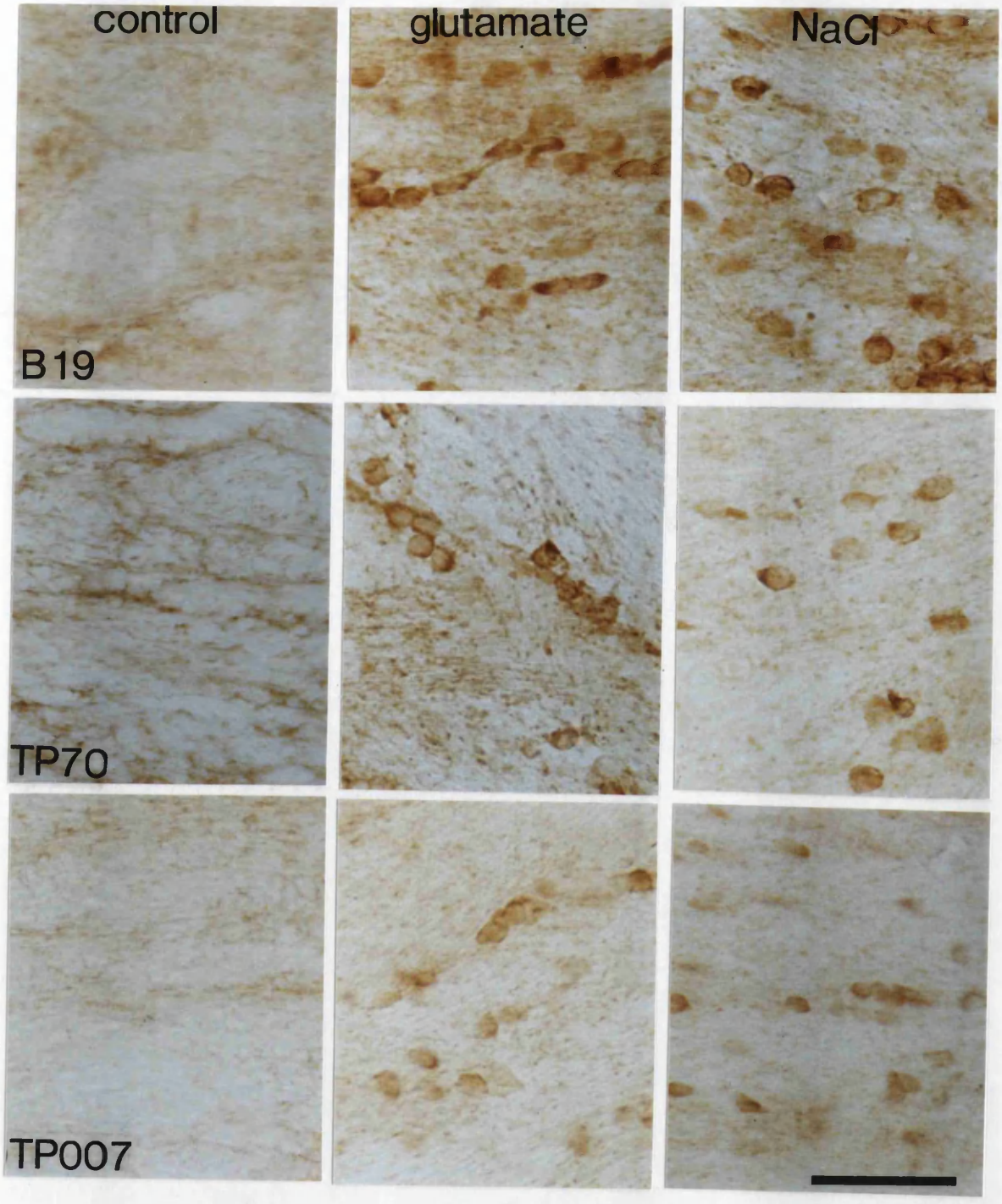



**Figure 22**. Tau immunoreactivity in glial cells of the subcortical white matter following intracortical perfusion 1M monosodium glutamate or 1M NaCl in the rat. B19, TP70 and TP007 immunoreactivity was localised within the axons of the contralateral subcortical white matter. However, following intracortical perfusion of 1M monosodium glutamate or 1M NaCl, increased tau immunoreactivity was detected within cells with the morphological appearance of glia both within the resulting cortical lesion and in the subcortical white matter immediately underlying this area. These glial cells were detected using B19, TP70 and TP007, suggesting the presence of full length tau protein. Scale bar = 50μm.

Tau-positive glia were also detected with the Tau 1 antibody, both within the cortical lesion induced by the perfusion of 1M monosodium glutamate or 1M NaCl and in the subcortical white matter immediately underlying this area. This suggests that tau present in these cells is dephosphorylated at the Tau 1 epitope. In support of this finding, the phosphorylation dependant antibody AT8 failed to detect these glial cells (Fig. 23), confirming that tau present within these cells is dephosphorylated at the Tau 1 epitope. Tau immunoreactivity was not detected within glial cells in histologically normal tissue with any of the tau antibodies used in this study with (Fig. 24) or without alkaline phosphatase pretreatment (Figs. 22 & 23).

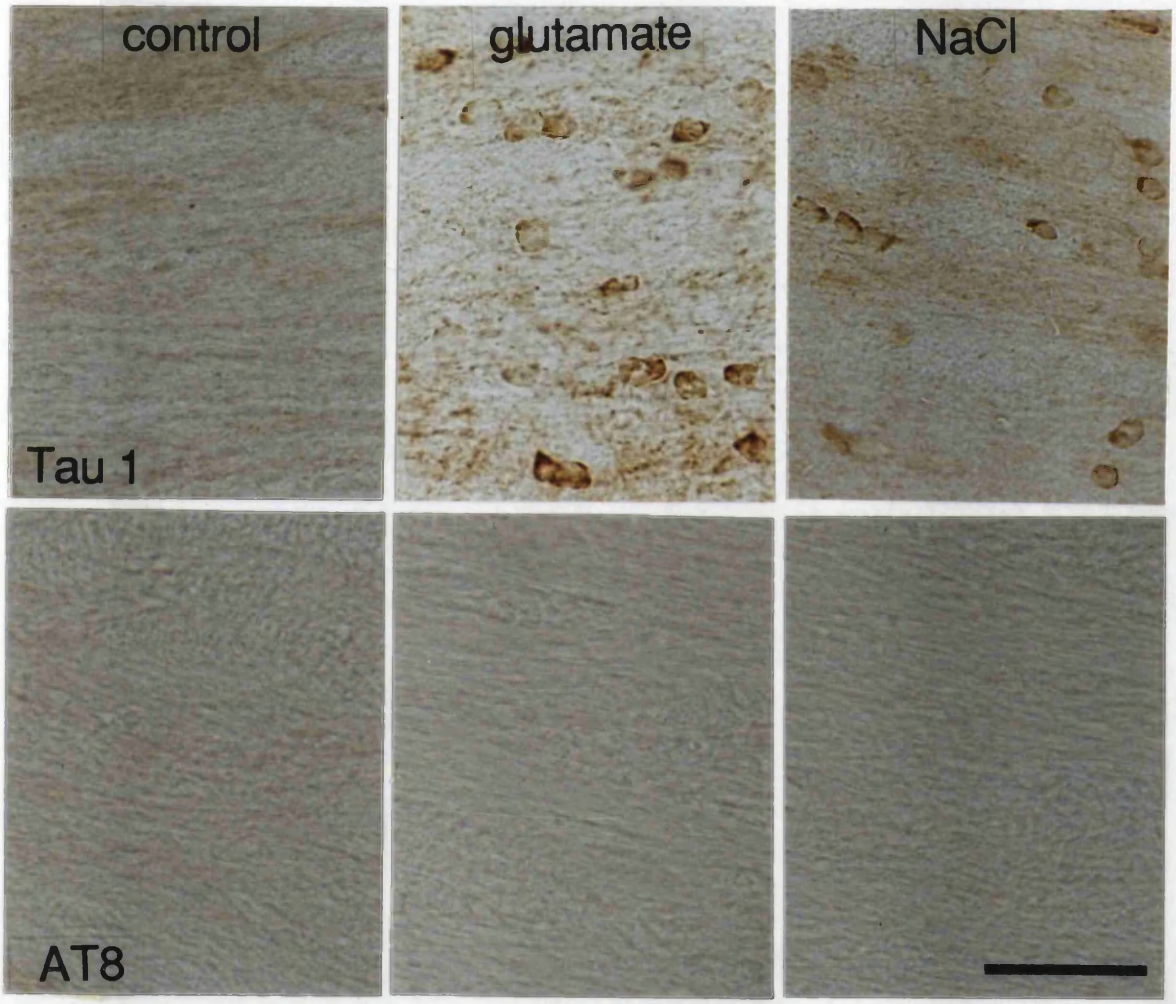



**Figure 23.** Distribution of Tau 1 and AT8 immunoreactivity in the subcortical white matter following intracortical perfusion of 1M monosodium glutamate or 1M NaCl. Tau 1 immunoreactivity was predominantly localised to axons within the contralateral subcortical white matter. However following the intracortical perfusion of either 1M monosodium glutamate or 1M NaCl, Tau 1 immunoreactivity was detected in glial cells both within the resulting cortical lesion and in the subcortical white matter immediately underlying this area. AT8 failed to detect glial cells prior to, or following intracortical perfusion of 1M monosodium glutamate or 1M NaCl. Scale bar = 50μm.

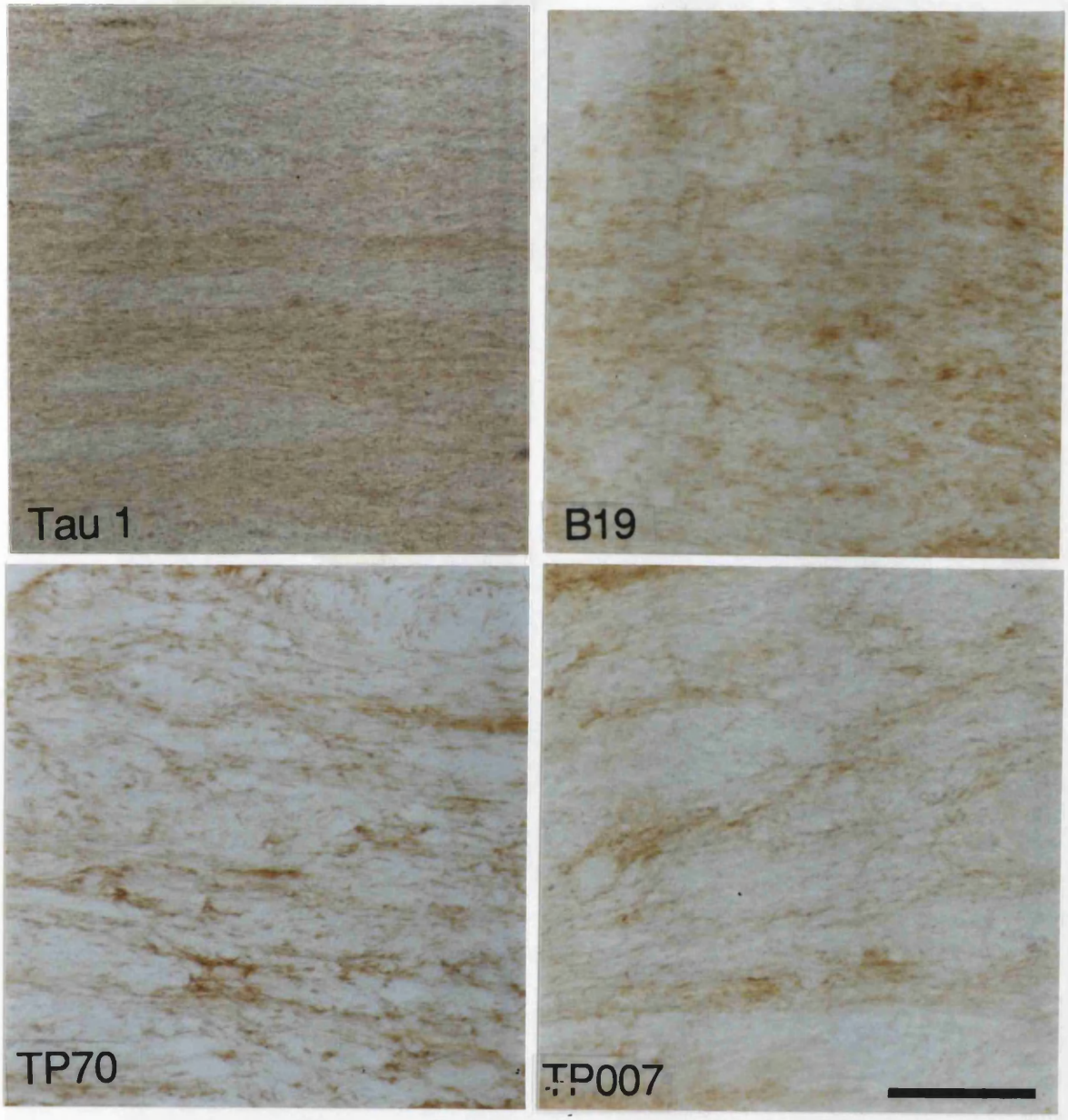



**Figure 24.** Tau immunoreactivity in the subcortical white matter following alkaline phosphatase pre-treatment of histologically normal rat brain tissue using Tau 1, B19, TP70 or TP007. Tau 1, B19, TP70 and TP007 immunoreactivity is located predominantly within axons of the subcortical white matter in histologically normal tissue. Alkaline phosphatase pre-treatment of rat brain tissue prior to immunostaining failed to alter this pattern of immunoreactivity as detected with all 4 tau antibodies. Glial cells were not detected using Tau 1, B19, TP70 or TP007 before, or after dephosphorylation of the tissue. This data suggests that the epitopes of tau recognised by these antibodies are not masked by phosphorylation within glial cells present in control tissue. Furthermore, the presence of tau immunoreactivity in glial cells, as detected with all 4 antibodies, following intracortical perfusion of 1M monosodium glutamate or 1M NaCl cannot be explained simply by the dephosphorylation of existing tau protein within these cells. Scale bar = 50μm.

Tau-positive cells displayed a small perikaryon and lacked visible processes consistent with the morphological appearance of oligodendrocytes in situ. Within the subcortical white matter tau-positive cells were aligned in rows, a feature characteristic of intrafascicular oligodendrocytes. Moreover, tau-positive oligodendrocytes, detected following intracortical perfusion of glutamate or NaCl, stained negative for GFAP and positive for transferrin therefore showing that these tau-positive cells were immunologically similar to oligodendrocytes (Fig. 25). Together this data confirms that tau-positive glial cells detected in response to intracortical perfusion of 1M monosodium glutamate or 1M NaCl were oligodendrocytes.

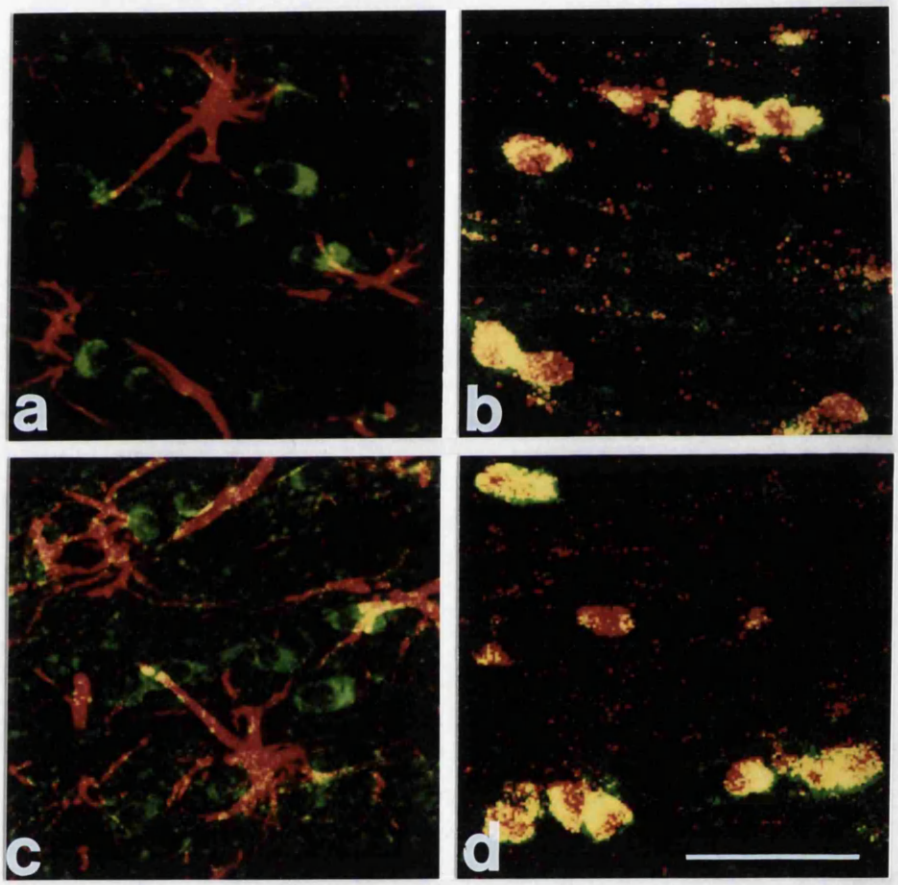



**Figure 25.** Confocal images showing Tau 1, GFAP and transferrin immunoreactivity in the subcortical white matter immediately underlying cortical lesions induced by the perfusion of 1M monosodium glutamate (a,c) or 1M NaCl (b,d). (a&b) GFAP shown in red and Tau 1 shown in green. Tau-positive cells were GFAP negative and morphologically distinct from astrocytes which displayed an elaborate array of processes. (c&d) Transferrin shown in red and Tau 1 shown in green. Yellow staining represents areas of overlapping immunoreactivity, showing that tau-positive cells are also positive for transferrin. Tau-positive cells were therefore immunologically and morphologically similar to oligodendrocytes. Confocal images were kindly provided by Prof. I.R. Griffiths. Scale bar = 50μm.

*MAP 2 and MAP5*

Neither MAP2 nor MAP5 immunoreactivity was detected in glial cells of the hemisphere contralateral to the occluded MCA. In contrast to tau, MAP2 and MAP5 immunoreactivity was not detected in oligodendrocytes following intracortical perfusion of 1M monosodium glutamate or 1M NaCl neither in the resulting cortical lesion nor in the subcortical white matter underlying this area (Fig. 26). In contrast to neurons, where tau, MAP2 and MAP5 underwent marked alteration in response to intracortical perfusion of both glutamate and NaCl, changes in MAPs within oligodendrocytes following injury appears to be specific for tau.

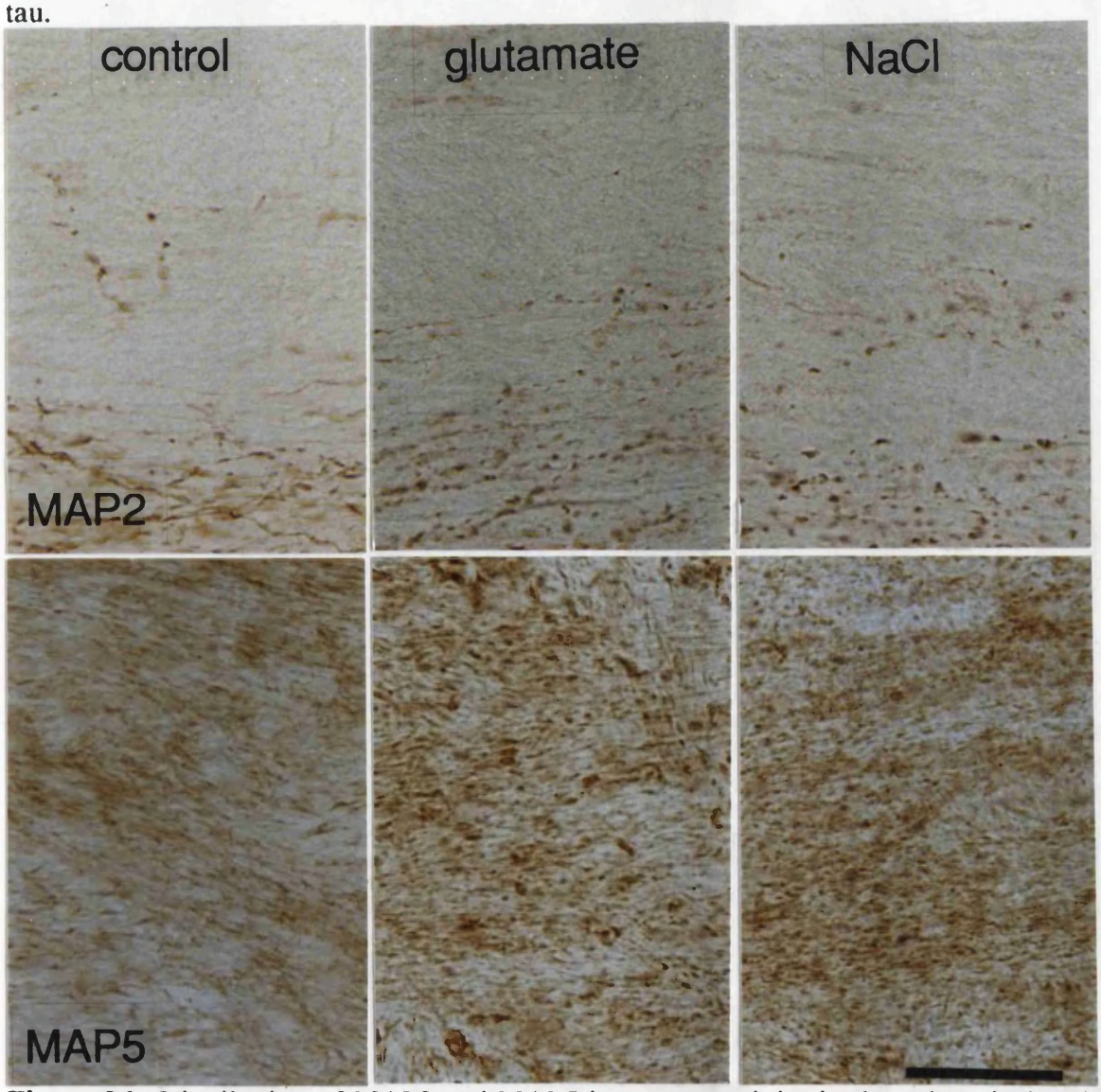



**Figure 26.** Distribution of MAP2 and MAP5 immunoreactivity in the subcortical white matter following intracortical perfusion of 1M monosodium glutamate or 1M NaCl. MAP2 and MAP5 immunoreactivity was localised predominantly within axons and dendrites of the contralateral subcortical white matter. In contrast to tau, neither MAP2 nor MAP5 immunoreactivity was detected in glial cells of the ipsilateral subcortical white matter following either 1M monosodium glutamate or 1M NaCl perfusion. Scale bar = 50μm.

### 3.1.4 Electron microscopy

Structural changes in glial cells present within the subcortical white matter immediately underlying the glutamate or NaCl induced lesion could not be detected by light microscopy. In order to determine if such changes could be detected at the ultrastructural level, the brains from animals which received intracortical perfusion of either 1M monosodium glutamate (n=2) or 1M NaCl (n=2) were processed for electron microscopy. The white matter of the corpus callosum immediately underlying cortical lesions induced by glutamate or NaCl perfusion, was markedly oedematous. Astrocytic swelling was marked, involving both nuclei and cytoplasm, while oligodendrocytes were involved to a lesser degree. Oligodendrocytes of the contralateral corpus callosum, had an electron dense nucleus and abundant microtubules (Fig. 27). Following the intracortical perfusion of 1M monosodium glutamate, the most obvious ultrastructural changes were that the oligodendrocyte nucleus became swollen and less electron dense as did the cytoplasm, as compared to oligodendrocytes in the contralateral hemisphere (Fig. 27). Oligodendrocyte processes were swollen however all cytoplasmic organelles were recognisable (Fig. 27). The ultrastructural changes in oligodendrocytes reported following intracortical perfusion of 1M monosodium glutamate were also seen following 1M NaCl perfusion, there were no significant differences detected between the two treatment groups. The swelling of oligodendrocytes and their processes confirmed that these cells, present within the subcortical white matter, were affected by the intracortical perfusion of glutamate or NaCl. Electron microscopy was performed by Prof. I.R. Griffiths.

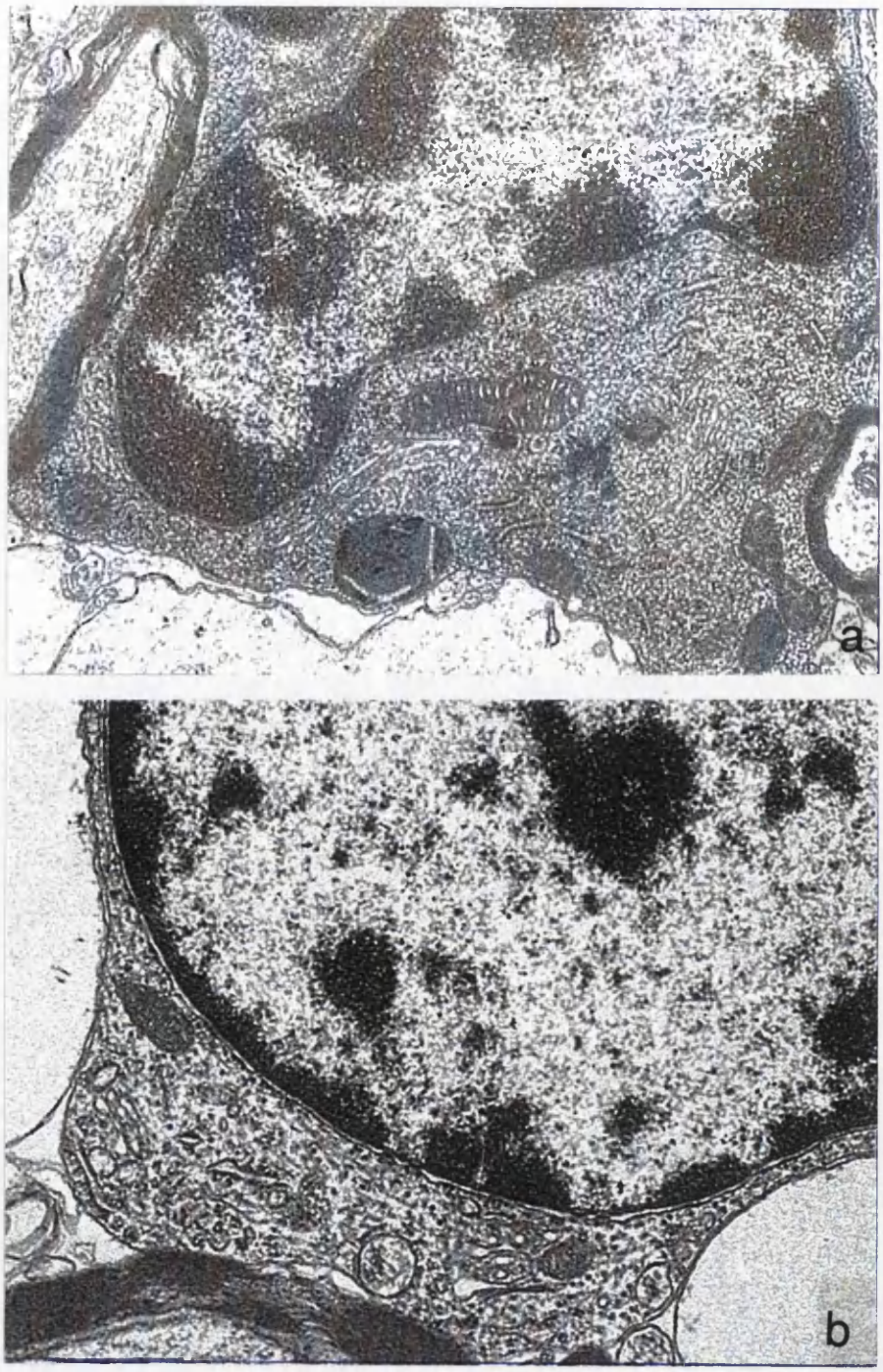



**Figure 27**. Electron micrograph showing oligodendrocyte morphology in the contralateral (a) and ipsilateral (b) subcortical white matter following intracortical perfusion of 1M monosodium glutamate. Oligodendrocytes present in the contralateral subcortical white matter had an electron dense nucleus and cytoplasm which contained abundant microtubules. Following intracortical perfusion of 1M monosodium glutamate (b) or 1M NaCl, the oligodendrocyte nucleus and cytoplasm became swollen and less electron dense, compared to those oligodendrocytes present in the contralateral hemisphere. Magnification factor = x 20,000.

## 3.2 Human post-mortem brain studies

Increased tau immunoreactivity was detected in oligodendrocytes following glutamate toxicity in rat brain. Previously, extracellular concentrations of glutamate were shown to be increased immediately following both traumatic brain injury and stroke in the rat (Butcher, et. al. 1990, Bullock, et. al. 1990). Therefore in the next study, human post-mortem brain tissue from patients dying following stroke or head injury was examined for the presence of tau-positive oligodendrocytes, in order to determine whether or not the presence of tau-positive oligodendrocytes following excitotoxic brain injury was a phenomenum peculiar to the rat brain.

### 3.2.1 Tau positive glial cells following severe head injury

For this study, cases of head injury were selected in which there was evidence of diffuse axonal injury within the corpus callosum. The accumulation of APP immunoreactivity within axons was used as a marker for the early stages of axonal bulb formation and all patients used in this study had an APP score of at least 1 (McKenzie, et. al. 1995). APP immunostaining and classification of axonal injury was carried out by Prof. D.I. Graham in the Dept. of Neuropathology, University of Glasgow. In the corpus callosum of control cases, tau immunoreactivity as detected with TP70, TP007 and Tau 1 was localised predominantly within axons (Fig. 28 & 29). However, in the corpus callosum from patients dying following head injury, in addition to its presence in axons, tau immunoreactivity was also detected within cells with the morphological appearance of glia (Fig. 28 & 29). The alignment of these glial cells in rows within the corpus callosum suggests that these tau-positive cells were oligodendrocytes. These tau-positive glia were detected using Tau 1, TP70 and TP007 suggesting the presence of full-length protein which was dephosphorylated at the Tau 1 epitope in these cells. It should be noted however that the number of oligodendrocytes detected with Tau 1 was less than that detected by TP70 and TP007 in all cases examined. The Tau 1 epitope is normally phosphorylated in NFTs, therefore in order to confirm that tau present in glial cells reported here was dephosphorylated, rather than hyperphosphorylated as seen in NFTs, sections were stained with the AD2 antibody. AD2 is an antibody raised against tau proteins found in Alzheimer's disease which recognises tau phosphorylated at Ser-396 and Ser-404 (BueeScherrer, et. al. 1996). The lack of staining of glial cells in the corpus callosum of head injured patients with AD2 suggests that in contrast to that of PHF tau, tau present in these cells is not phosphorylated at the AD2 epitopes (Fig. 28). The possibility that tau may be phosphorylated at different residues, similar to that in of PHF tau however, cannot be ruled out. Tau was detected within a few glia in the corpus callosum of patients surviving <1 h following head injury and the number of these tau-positive cells appeared to increase with survival time after injury, although quantitative analyses were not undertaken (Fig. 28 &

29). In patients surviving for 6 h or more, areas of pallor within the corpus callosum were detected in H&E stained sections outlining areas of tissue affected by injury (Fig. 29). In these patients tau-positive glia were localised within and immediately surrounding the area of tissue delineated by reduced H&E staining. In tissue distant from these affected areas where there was no histological evidence of brain injury, tau-positive glia were not detected. Tau-positive glia were detected in all brain injured patients examined in this study however, it should be noted that only a proportion of the glial population became tau-positive within damaged areas especially at early times following head injury.

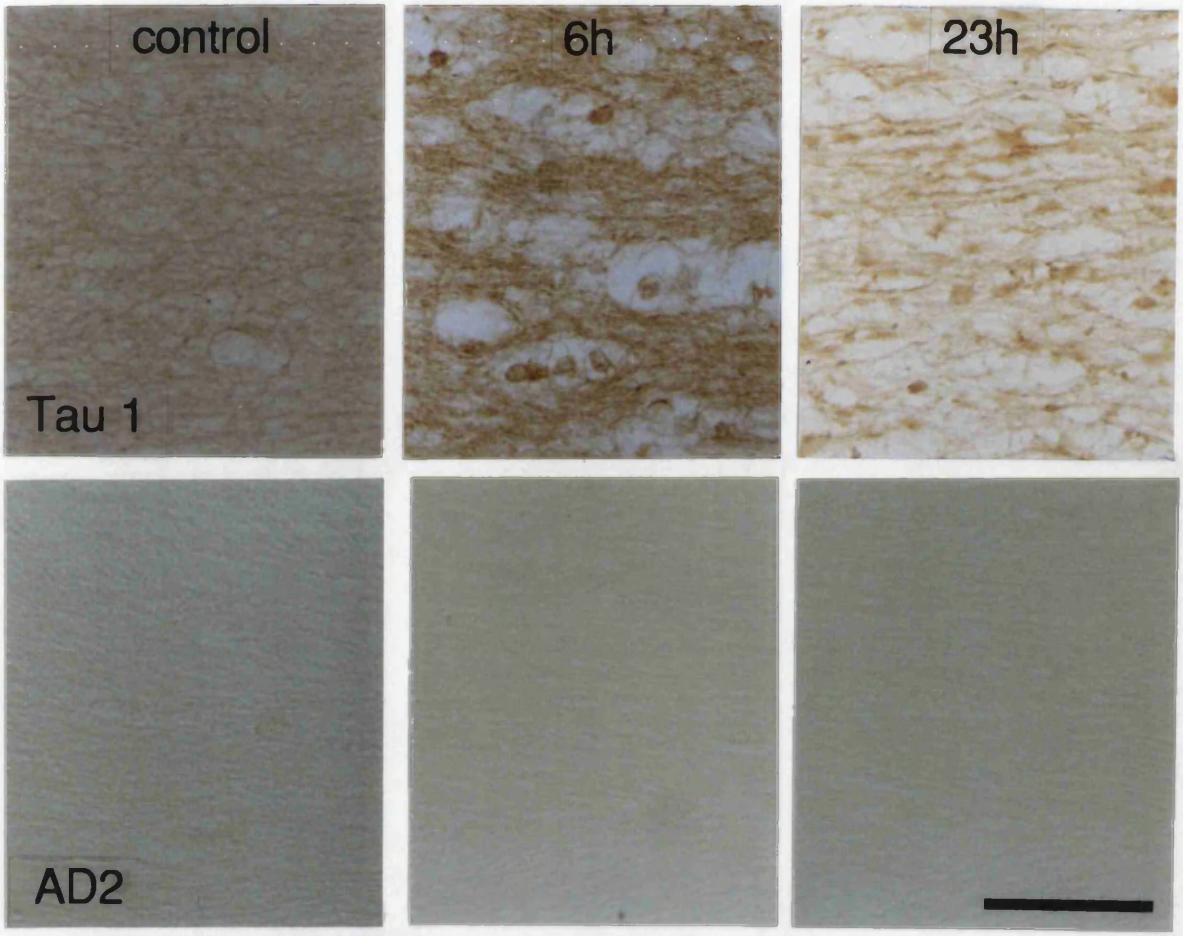



**Figure 28.** Tau 1 and AD2 immunoreactivity within the corpus callosum of control patients and 6 h and 23 h following head injury. In control tissue, Tau 1 immunoreactivity was localised predominantly within the axonal compartment where tau is dephosphorylated at this epitope. AD2 recognises PHF tau and therefore fails to detect tau in histologically normal tissue. 6 h following head injury Tau 1 immunoreactivity was increased in small cells with the morphological appearance of oligodendroglia, however the number of these cells detected was less than that detected by TP70 and TP007 (Fig. 29). 23 h following head injury Tau 1 positive oligodendrocytes were difficult to detect. AD2 immunoreactivity was not detected in glial cells of histologically normal or head injured tissue. Scale bar = 50μm.

In one patient surviving for 23 h following severe head injury, TP70 and TP007 immunoreactivity was intense in the cytoplasm of glial cells to one side of the cell (Fig. 29), within the corpus callosum where H&E staining was markedly decreased (Fig. 30). The morphological appearance of these tau-positive glial cells was similar to that reported previously for glial inclusions in neurodegenerative conditions such as MSA (Iwatsubo, et. al. 1994; Kato, et. al. 1991; Yamada, et. al. 1992). In this single case, glial cells were not detected with the Tau 1 antibody (Fig. 28), although they were stained with TP70 and TP007, suggesting that tau may be phosphorylated at the Tau 1 epitope. AD2 failed to detect these tau-positive cells showing that in contrast to PHF tau, tau present in these oligodendrocytes was not phosphorylated at the AD2 epitope (Fig. 28).

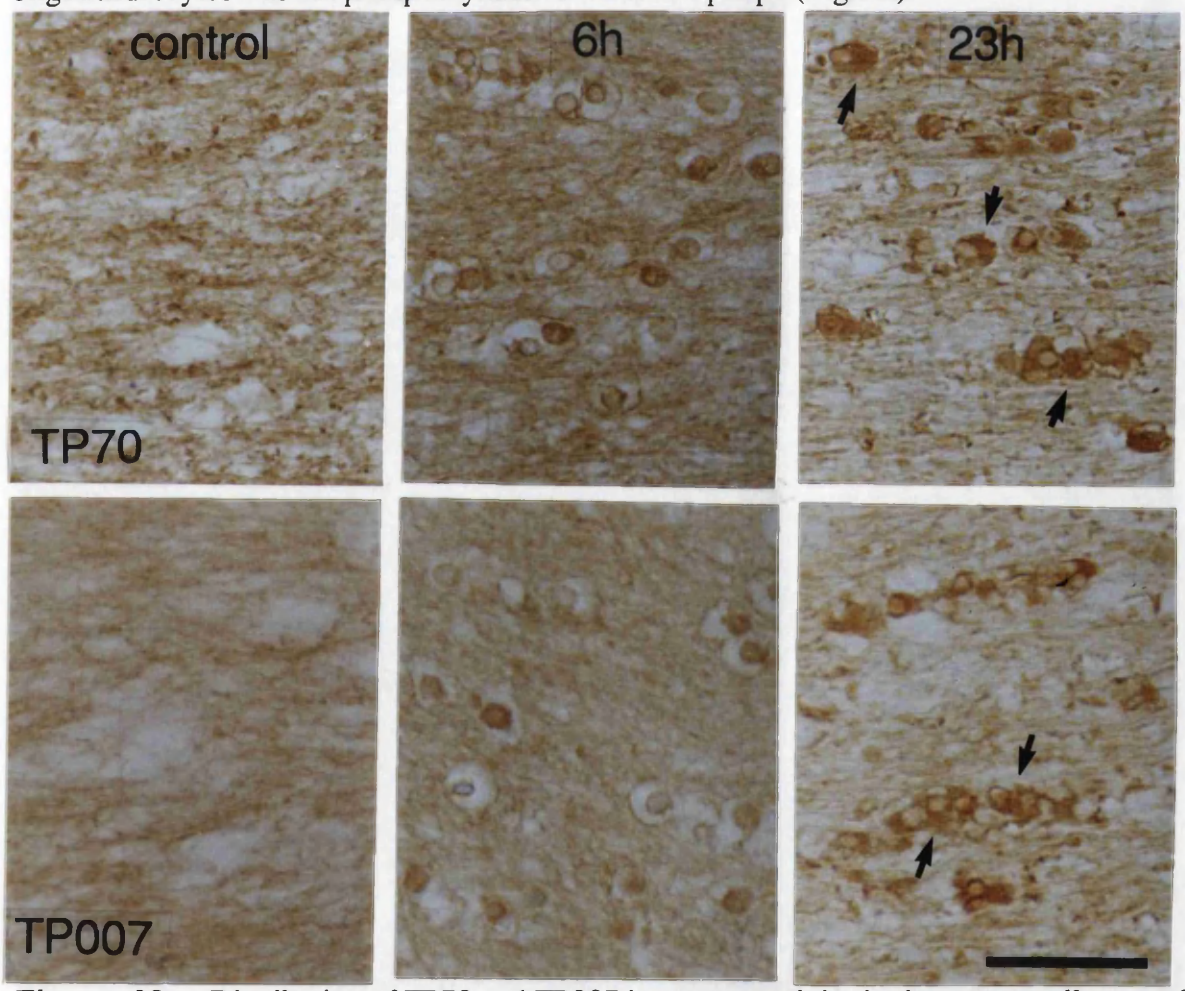



**Figure 29.** Distribution of TP70 and TP007 immunoreactivity in the corpus callosum of control and head injured patients 6 and 23 h following injury. In control tissue, immunoreactivity is localised predominantly within the axonal compartment. Following head injury however TP70 and TP007 immunoreactivity was increased in small cells with the morphological appearance of oligodendroglia. In the patient surviving for 23 h following head injury, TP70 and TP007, immunoreactivity was intense within the cytoplasm of some oligodendrocytes, showing a morphology similar to that of glial inclusions (arrows). Scale bar = 50μm.

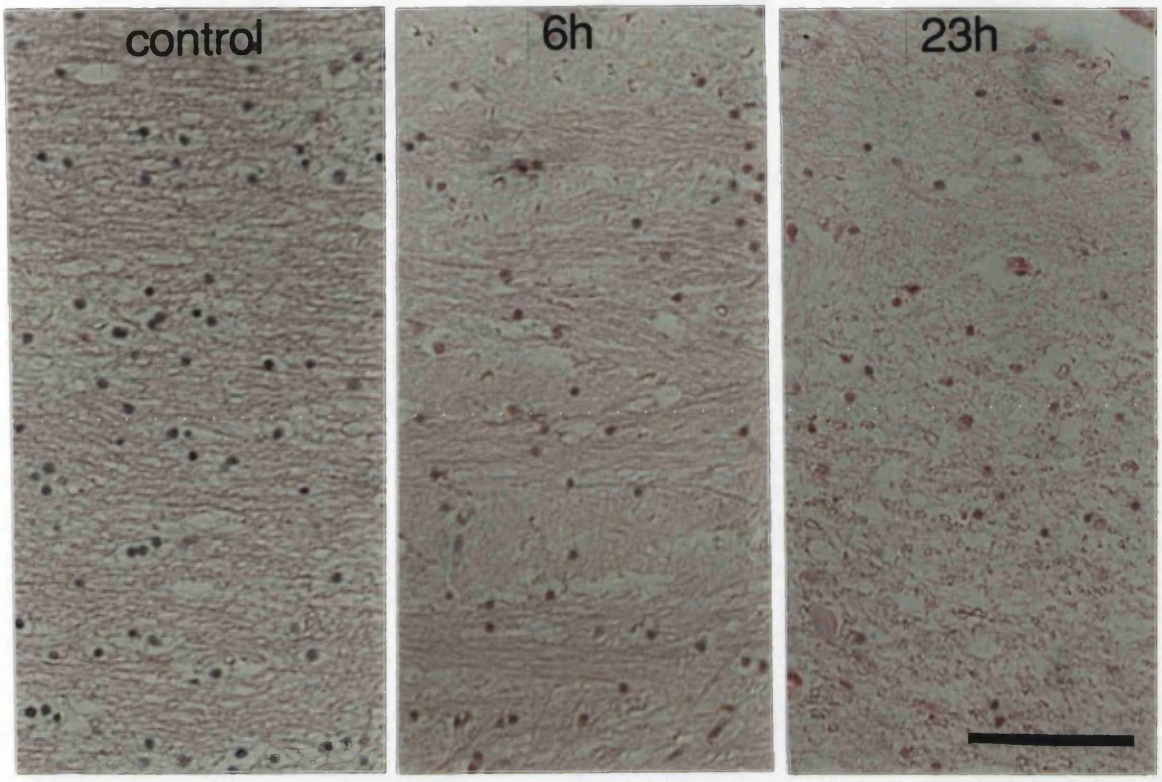



**Figure 30.** Corpus callosum of control and head injured patients stained with H&E. In patients surviving for 6 h or more after head injury, areas where H&E staining was decreased within the neuropil outlined the regions of the corpus callosum affected by brain injury. 6 and 23 h following head injury the number of glial cells present in the corpus callosum decreased as compared to histologically normal tissue. In addition nuclei appeared eosinophilic. 23 h following head injury H&E staining was markedly reduced within the neuropil as compared to that of control tissue. Scale bar = 100μm.

### 3.2.2 Tau-positive glia following stroke

Post-mortem brain tissue was obtained from patients who died following occlusive stroke. Brain areas examined encompassed the cingulate cortex and thalamus, depending on the location of the ischaemic injury. In brain tissue obtained from all four patients who died following a stroke, tau-positive glia were detected within and immediately surrounding areas of tissue in which there was histological evidence of ischaemic brain damage (Fig. 31). Tau-positive glia were detected at the core of the infarct, however were most abundant in the grey matter immediately surrounding the infarcted tissue (Fig. 32 & 33) and in the white matter lying in close proximity to the infarcted tissue. The detection of tau-positive glia with TP70 and TP007 suggests the presence of full-length protein in these cells (Fig. 32). Detection of glial cells with the Tau 1 antibody shows that, similarly to that occurring following head injury, tau is dephosphorylated at the Tau 1 epitope (Fig.33). AD2 failed to

to detect tau present within glial cells showing that in contrast to PHF tau, tau present in these cells is not phosphorylated at the AD2 epitope (Fig. 33).

In the patient surviving for several weeks following stroke, many glia within the infarcted tissue contained intense tau staining within the cell cytoplasm to one side of the cell as detected with TP70 and TP007 (Fig. 32). The morphological appearance of these tau-positive glial cells was similar to that reported previously for glial inclusions in neurodegenerative conditions such as MSA (Iwatsubo, et. al. 1994; Kato, et. al. 1991; Yamada, et. al. 1992). This pattern of tau immunoreactivity was not detected with the Tau 1 antibody (Fig. 33) suggesting that tau present in these cells may be phosphorylated at the Tau 1 epitope. Similarly to oligodendrocytes detected 23 h following head injury, AD2 failed to detect tau accumulations within the oligodendrocyte cytoplasm showing that in contrast to PHF tau, tau present within these inclusions was not phosphorylated at the AD2 epitope (Fig. 33). Tau present in glial inclusions can be detected with antibodies raised against PHF tau which is phosphorylated at many sites including the Tau 1 epitope, the possibility that tau present in these cells is phosphorylated at other residues of the tau protein similar to those occurring in glial inclusions cannot be ruled out.

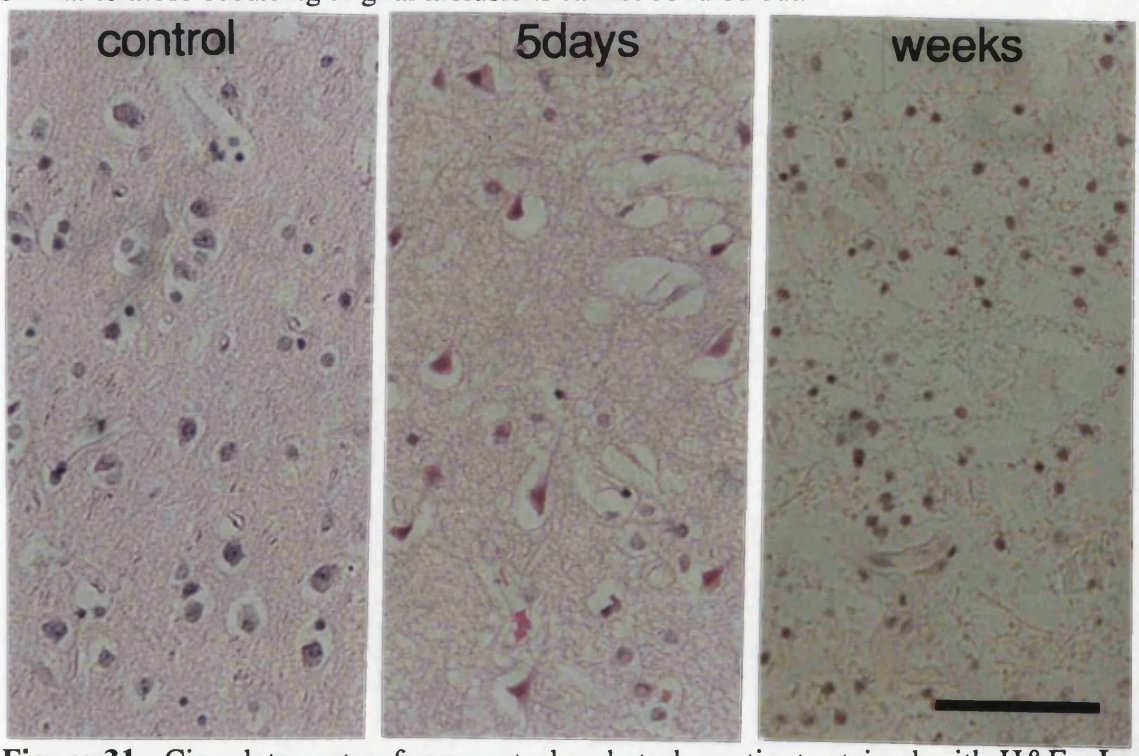



**Figure 31.** Cingulate cortex from control and stroke patients stained with H&E. In all cases areas where H&E staining was decreased outlined the regions of tissue affected by ischaemic brain injury. 5 days and following stroke, neurons showed triangulation of the nucleus and eosinophilic cytoplasm, in addition neuropil staining was decreased. In the patient surviving for several weeks following stroke, infarcted tissue was characterised by decreased H&E staining, of all cellular elements. In all cases neuropil staining was reduced within areas of tissue affected by ischaemic brain injury. Scale bar = 100μm.

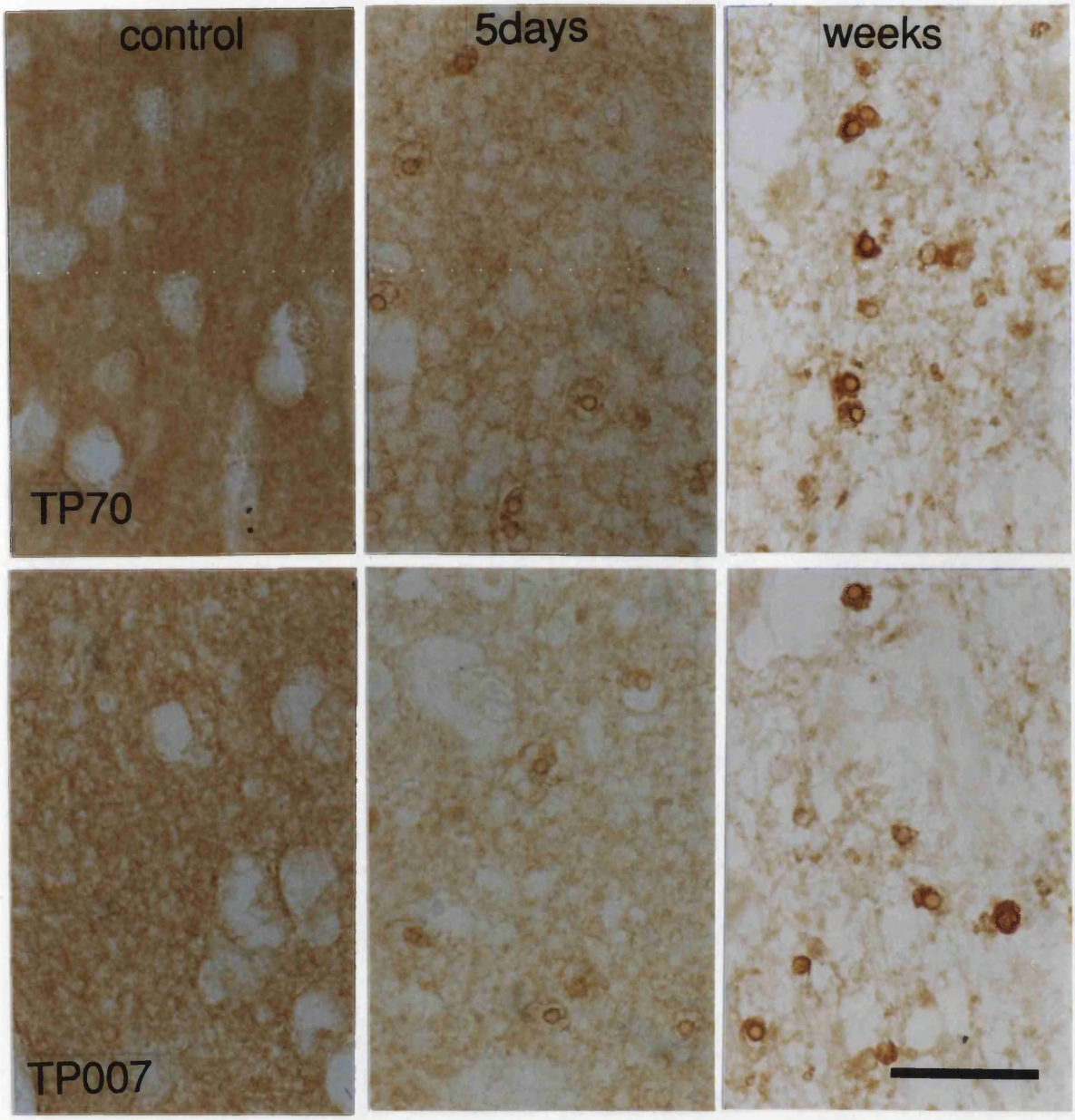



**Figure 32.** Distribution of TP70 and TP007 immunoreactivity in the cingulate cortex from control and stroke patients. In post-mortem brain tissue obtained from control patients, TP70 and TP007 immunoreactivity was localised predominantly within the axonal compartment. However, following stroke tau immunoreactivity was increased within oligodendrocytes in, and immediately surrounding the region of infarct. Figure shows tau-positive oligodendrocytes in the cingulate cortex 5 days and several weeks following stroke. In the patient surviving several weeks following a stroke, tau-positive glia displaying morphological features similar to that of glial inclusions, were detected with TP70 and TP007 (arrows) within the region of infarct. Scale bar = 50μm.

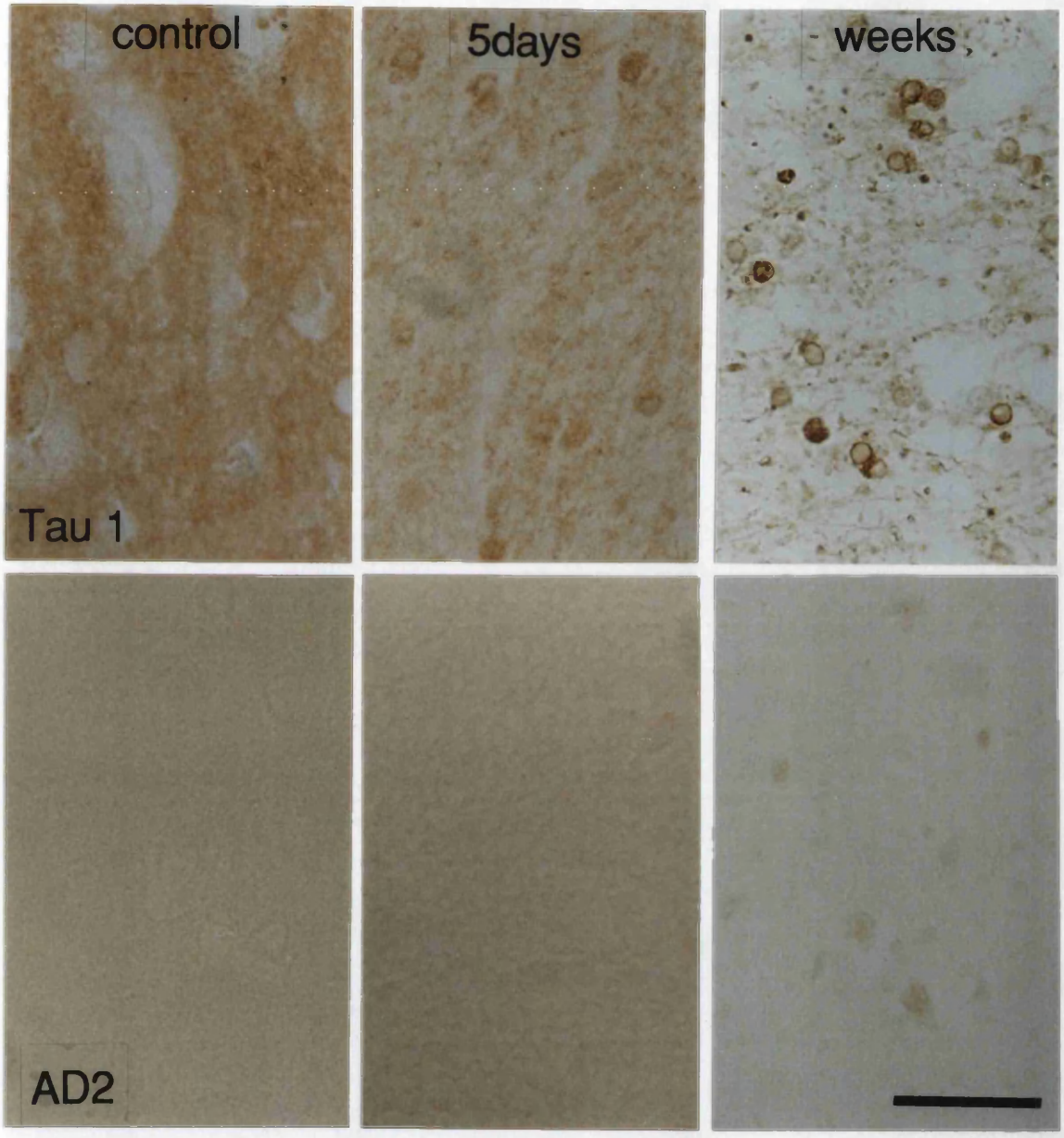



**Figure 33.** Distribution of Tau 1 and AD2 immunoreactivity in the cingulate cortex from control and stroke patients. In post-mortem brain tissue obtained from control patients, Tau 1 immunoreactivity was localised predominantly within the axonal compartment. However, following stroke Tau 1 immunoreactivity was increased within oligodendrocytes in, and immediately surrounding the region of infarct. Figure shows tau-positive oligodendrocytes in the cingulate cortex 5 days and several weeks following stroke. AD2 failed to detect tau-positive oligodendrocytes in both control and stroke tissue Scale bar = 50μm.

### 3.2.3 Identification of tau-positive glia

Sections double immunostained with GFAP, as a marker for astrocytes, and Tau 1 showed that tau-positive glial cells present in the corpus callosum following both head injury and stroke did not stain with a GFAP antibody and were morphologically distinct from astrocytes (Fig. 34). In contrast to the large cell body and elaborate array of processes displayed by astrocytes, tau-positive glial cells had small perikarya, limited cytoplasm and lacked visible cellular processes. This morphology is consistent to that described for oligodendrocytes. In addition, tau-positive glial cells also stained positive for transferrin, a specific marker for oligodendrocytes (Fig. 34) confirming that tau-positive glial cells were both immunologically and morphologically similar to oligodendrocytes.

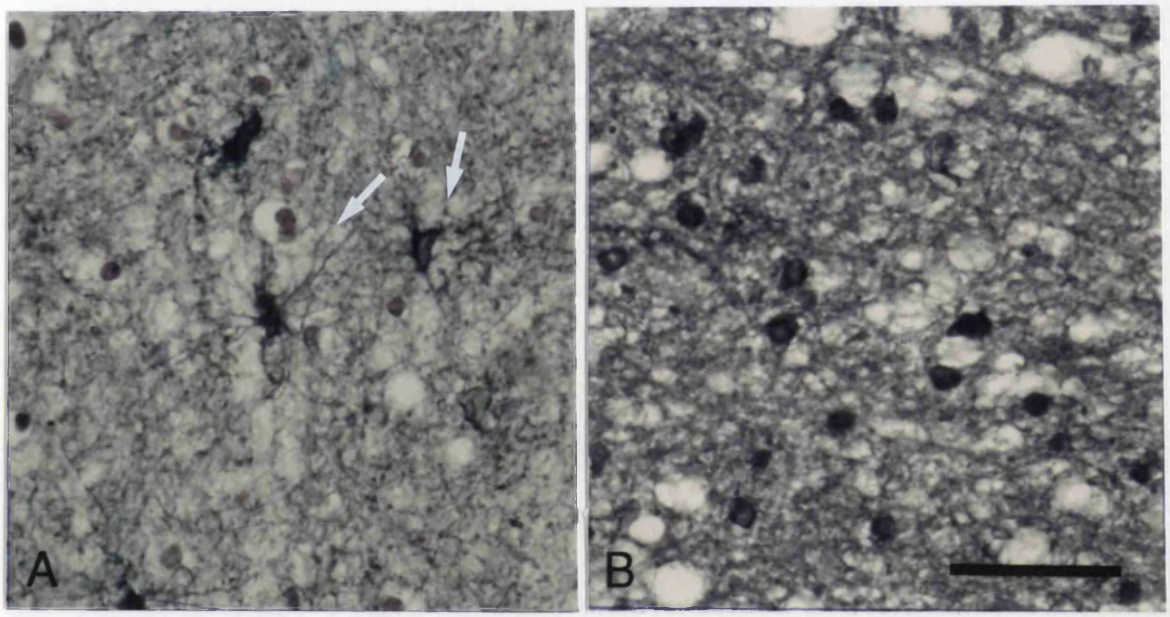



**Figure 34.** Identification of tau-positive glia in human post-mortem tissue. Sections of the corpus callosum of a patient dying 10 h following severe head injury were immunostained with A) GFAP as a marker for astrocytes shown in grey and Tau 1 shown in purple. Tau-positive cells were GFAP negative and morphologically distinct from astrocytes which displayed an elaborate array of processes (arrows) or B) transferrin as a marker for oligodendrocytes shown in grey and Tau 1 shown in purple. Tau-positive glial were positive for transferrin and Tau 1, the dark purple colour showing areas a overlapping immunostaining. Tau-positive cells detected in human post-mortem tissue after acute brain injury were therefore immunologically and morphologically similar to oligodendrocytes. Scale bar = 50μm.

## 3.3 Tau-positive oligodendrocytes following permanent focal cerebral ischaemia in the rat.

The presence of tau-positive oligodendrocytes in human brain following acute brain injury confirmed that the appearance of tau in oligodendrocytes was not a phenomenon unique to rat brain, but a response of these cells to acute brain injury. In order to further characterise the presence of tau in oligodendrocytes, the next study was designed to determine the earliest time following focal cerebral ischaemia in the rat that tau-positive oligodendrocytes could be detected. In addition, in order to determine whether the number of tau-positive oligodendrocytes increased over time, quantitative analysis of cell density was performed. Permanent MCAO in the rat was performed by Dr. K. Yatsushiro as originally described by Tamura et. al. (1981). Twenty min (n=3), 40 min (n=4) and 80 min (n=3) following the induction of cerebral ischaemia, the animals were killed by transcardiac perfusion and the brains processed for tau, MAP2 and MAP5 immunohistochemistry. The distribution of tau, MAP2 and MAP5 immunoreactivity in neurons was also investigated for comparison.

### 3.3.1 MAP2 as a marker of ischaemic damage

MAP2 immunostaining has been shown to be a sensitive marker of ischaemic damage in animal models of cerebral ischaemia (Dawson and Hallenbeck, 1996; Kitagawa, et. al. 1989; Yamamoto, et. al. 1986; Yanagihara, et. al. 1990). Although traditional histological stains such as H&E have been employed within 4 h of permanent focal ischaemia at shorter survival times, these stains are difficult to interpret. Therefore, in this study involving very short survival times following MCAO, the anatomical extent of ischaemic damage was detected using MAP2 immunostaining (Fig. 35). Twenty min post-MCAO, an area where MAP2 immunostaining was decreased was detected within the caudate nucleus in the ipsilateral hemisphere. Forty and 80 min post-occlusion, MAP2 immunostaining was decreased within the caudate nucleus within the territory ipsilateral to the occluded MCA. In addition, areas of decreased immunoreactivity were also detected within the ipsilateral cortex, however this was variable and patchy in its distribution. Decreased MAP2 immunostaining was detected in all animals used in this study. For a more detailed description of altered MAP2 distribution in neurons following ischaemic brain injury see section 3.3.3.

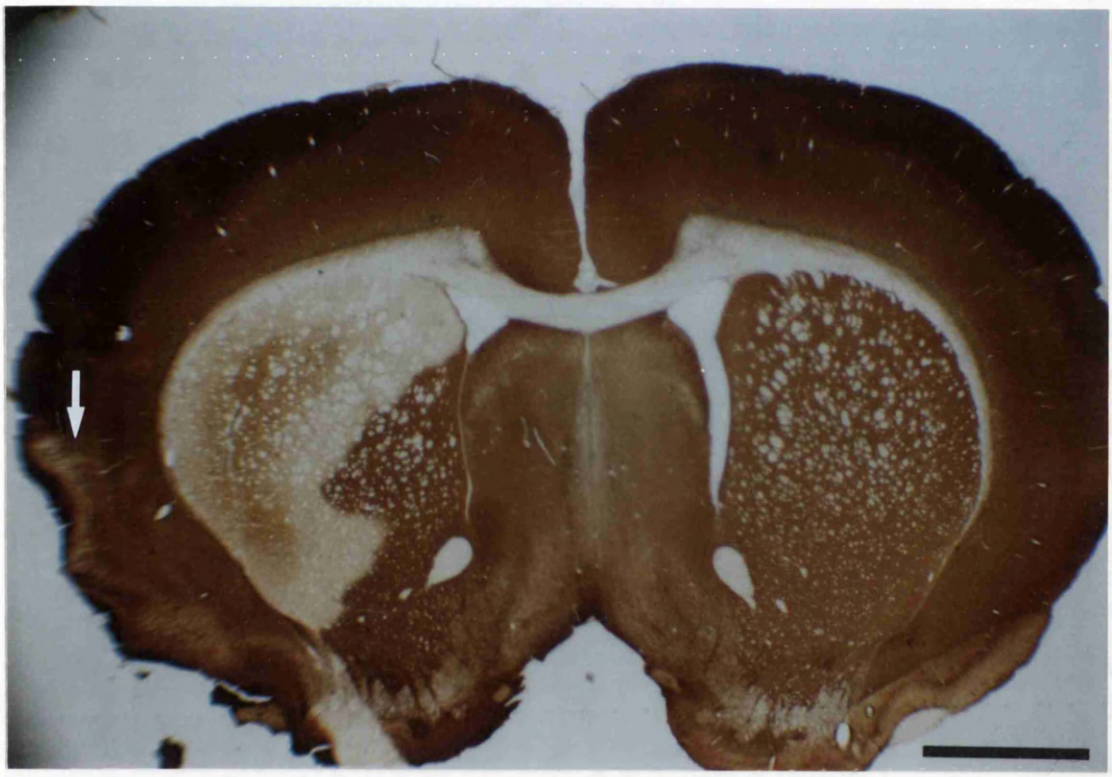


**Figure 35**. Photomicrograph of a rat brain section stained with MAP2 showing the area of ischaemic damage following 40 min MCAO. Immunoreactivity was markedly reduced in perikarya and neuropil of the caudate nucleus ipsilateral to the occluded MCA. The boundary of ischaemic tissue could also be detected in the cortex (arrow). All animals used in this study showed areas of decreased MAP2 immunoreactivity within the caudate nucleus, two animals also showed patches of decreased immunoreactivity in the cortex. Scale bar = 50μm.

### 3.3.2 Time course of increased tau immunoreactivity in oligodendrocytes following permanent MCAO in the rat.

In the hemisphere contralateral to the occluded MCA of all animals in this study the distribution of Tau 1, TP70 and TP007 immunoreactivity within the cortex and caudate nucleus was predominantly located within neurons. In previous studies no tau-positive oligodendrocytes were detected in rat tissue with any of the tau antibodies before or after dephosphorylation of the tissue (see section 3.1). In this study however, occasional tau-positive oligodendrocytes were detected in the subcortical white matter of the contralateral hemisphere 20 min following MCAO, using Tau 1, TP70 and TP007. This number increased slightly with time, however the number of tau-positive oligodendrocytes / $mm^2$ of subcortical white matter remained low compared to the contralateral hemisphere (Fig. 37). This suggests that the mechanisms underlying increased tau immunoreactivity in oligodendrocytes are induced throughout the entire corpus callosum following MCAO. Twenty min following MCAO, only a few tau-positive oligodendrocytes were detected within the ipsilateral subcortical white matter however this was greater than that in the contralateral hemisphere. In rats subjected to 40 min (Fig. 36 & 37) or 80 min of focal ischaemia, the number of tau-positive oligodendrocytes in the ipsilateral subcortical white matter, detected with all three tau antibodies, was increased compared to the contralateral white matter (Fig. 37). While increased tau immunoreactivity was most prominent within intrafasicular oligodendrocytes located in the subcortical white matter, oligodendrocytes were also present to a lesser extent in the grey matter of the caudate nucleus and the neocortex ipsilateral to the occluded MCA. These oligodendrocytes, detected using all three tau antibodies, were present in grey matter only within the boundary of ischaemic damage as determined by MAP2 immunostaining. Double label immunohistochemistry confirmed that similarly to the previous studies, these tau-positive glial cells with the morphological appearance of oligodendrocytes present in the subcortical white matter ipsilateral to the occluded MCA were morphologically and immunologically similar to oligodendrocytes (Fig. 38).

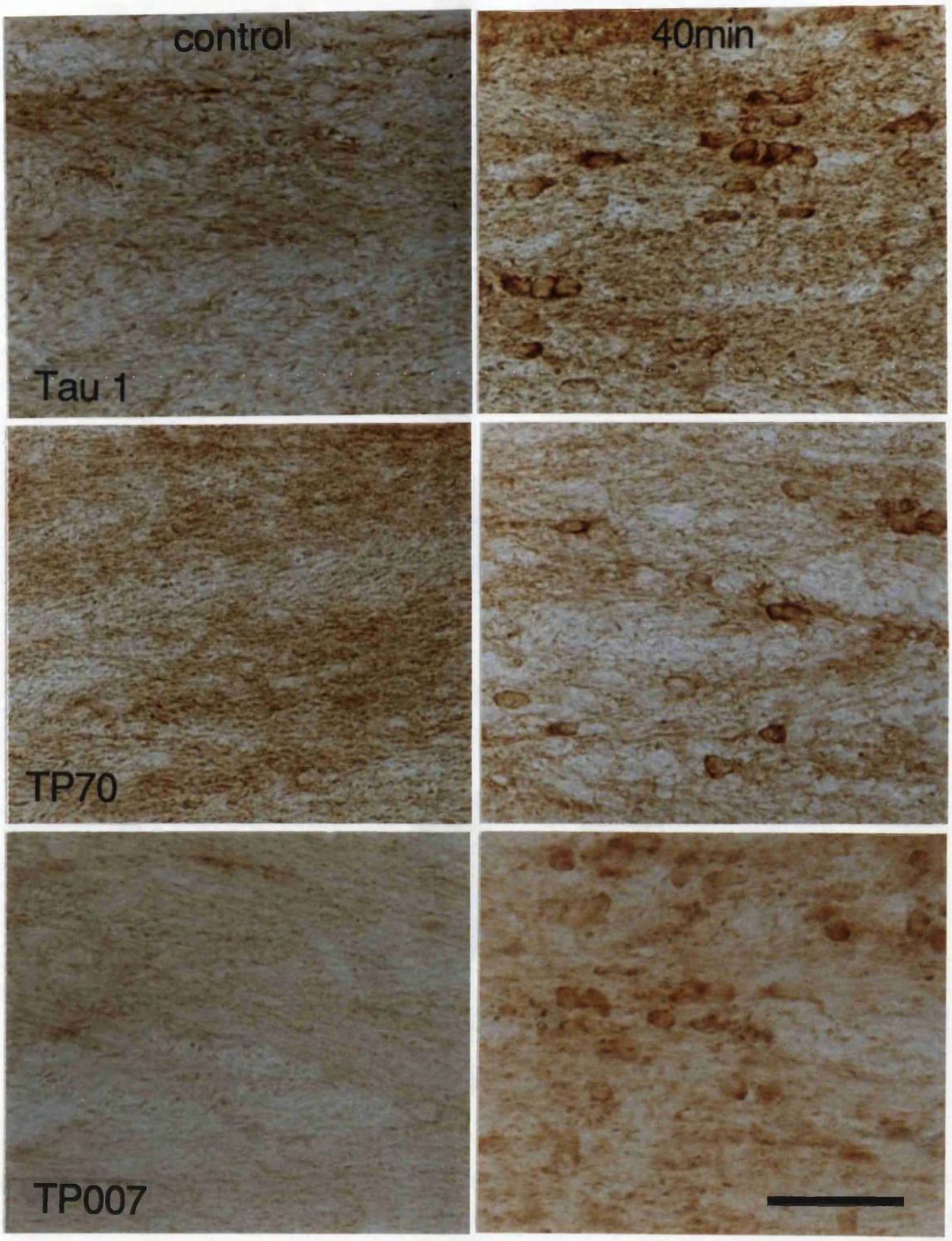



**Figure 36.** Tau immunostaining of subcortical white matter 40 min following MCAO in the rat. Tau immunostaining was localised mainly within the axons of the contralateral subcortical white matter 40 min following MCAO as detected with Tau 1, TP70 and TP007. In contrast, ipsilateral to the occluded MCA, numerous tau-positive oligodendrocytes were present in the subcortical white matter 40 min following the induction of cerebral ischaemia. Tau-positive oligodendrocytes in the ipsilateral hemisphere were detected with Tau 1, TP70 and TP007. Scale bar = 50μm.

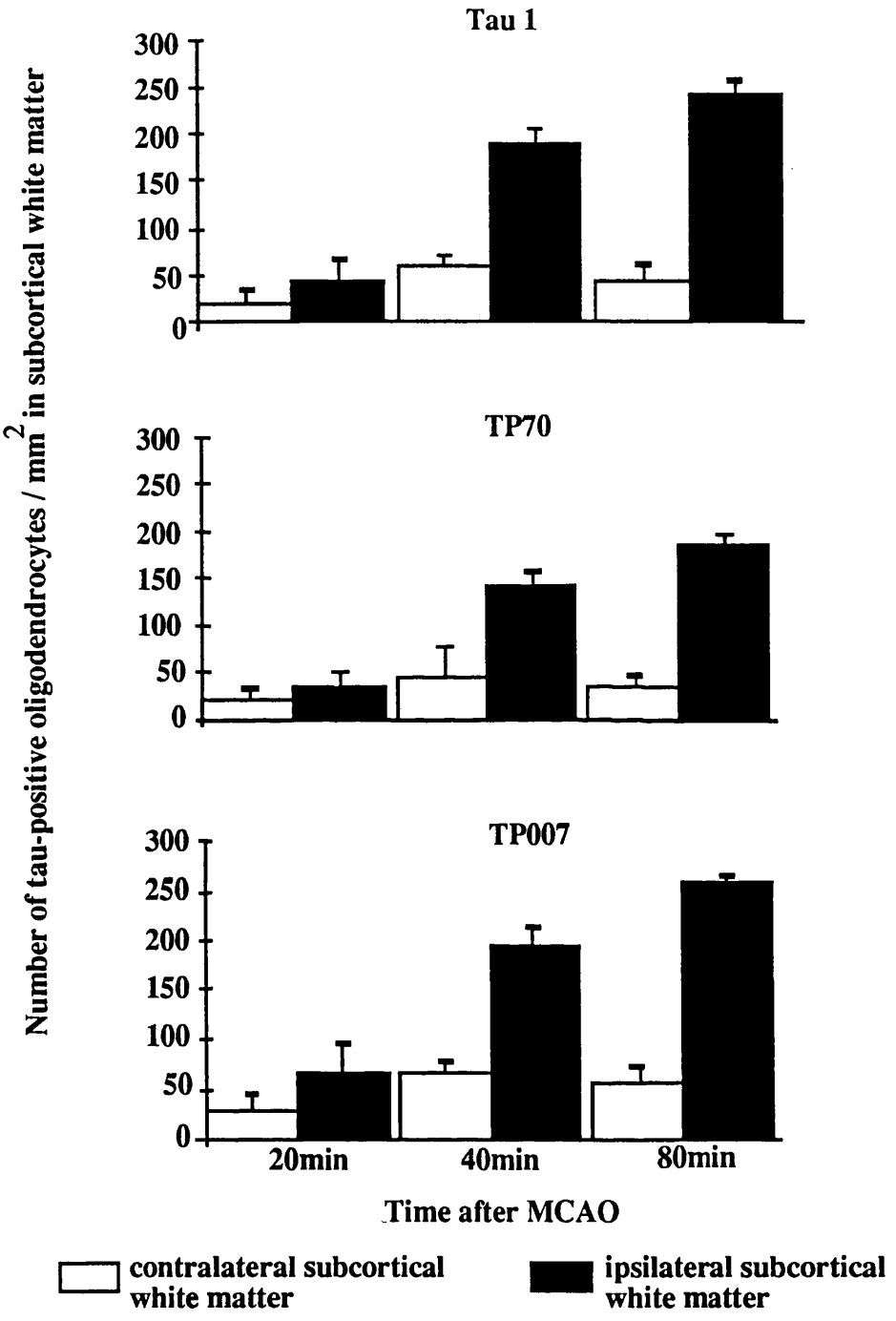



**Figure 37.** Quantification (described in section 2.6.4) showed the number of tau-positive oligodendrocytes to increase with time following MCAO. Twenty min following MCAO the number of tau-positive oligodendrocytes / $mm^2$ in the ipsilateral subcortical white matter was increased slightly as compared to contralateral white matter. The number of tau-positive oligodendrocytes / $mm^2$ increased significantly 40 and 80 min following MCAO in the white matter ipsilateral to the occluded MCA. Increased cell density was detected with all three antibodies : Tau 1, TP70 and TP007. Data are presented as Mean ± SEM, 20 min (n=3), 40 min (n=4) and 80 min (n=3).

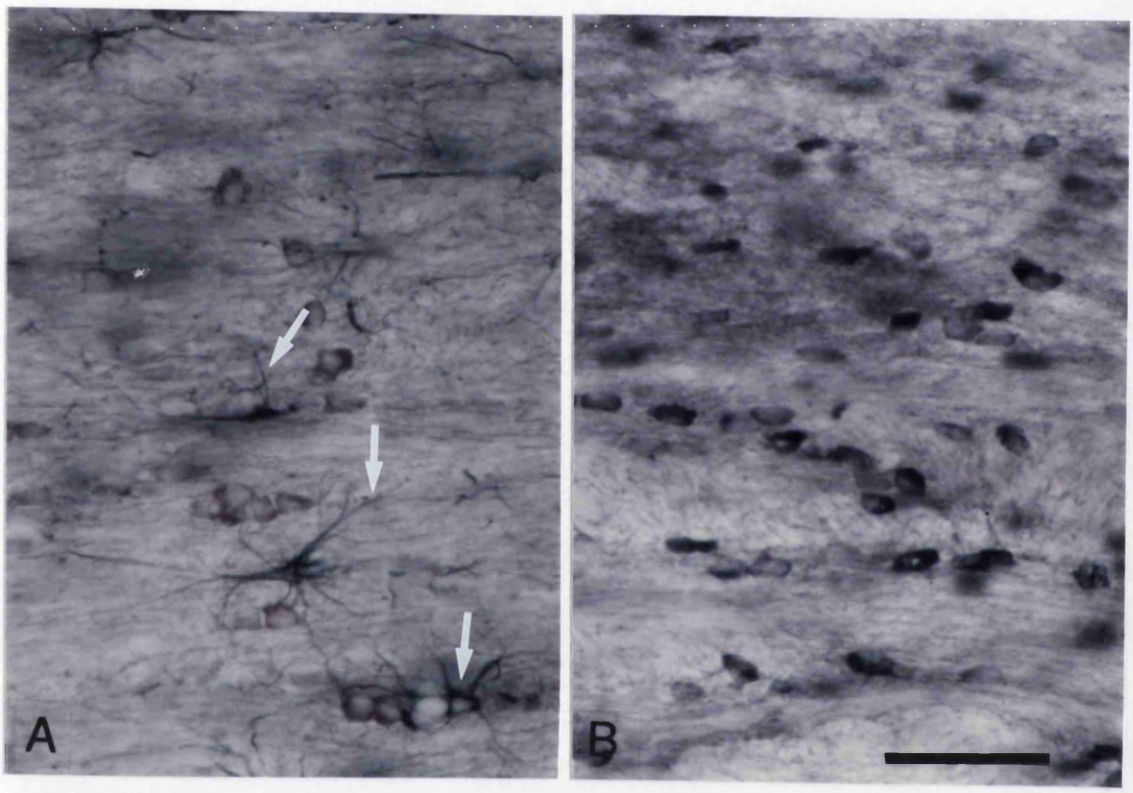



**Figure 38.** Identification of tau-positive glia present after MCAO as oligodendrocytes. A: Section labelled with Tau 1 shown in purple and GFAP shown in grey. Tau-positive cells were clearly GFAP-negative and therefore immunologically distinct from astrocytes. In addition tau-positive cells were morphologically distinct from astrocytes, which displayed an elaborate array of processes (arrows), having a compact, rounded appearance consistent with that of oligodendrocytes. B: Section double labelled with Tau 1 shown in purple and transferrin shown in grey. Tau-positive cells stained positive for transferrin, the coincident staining appears black. These cells were therefore immunologically and morphologically similar to oligodendrocytes. Scale bar = 50μm.

In contrast to those directed towards tau, oligodendrocytes were not detected with antibodies directed towards MAP2, MAP5 or c-fos in the subcortical white matter contralateral or ipsilateral to the occluded MCA at any time following the induction of cerebral ischaemia (Fig. 39).

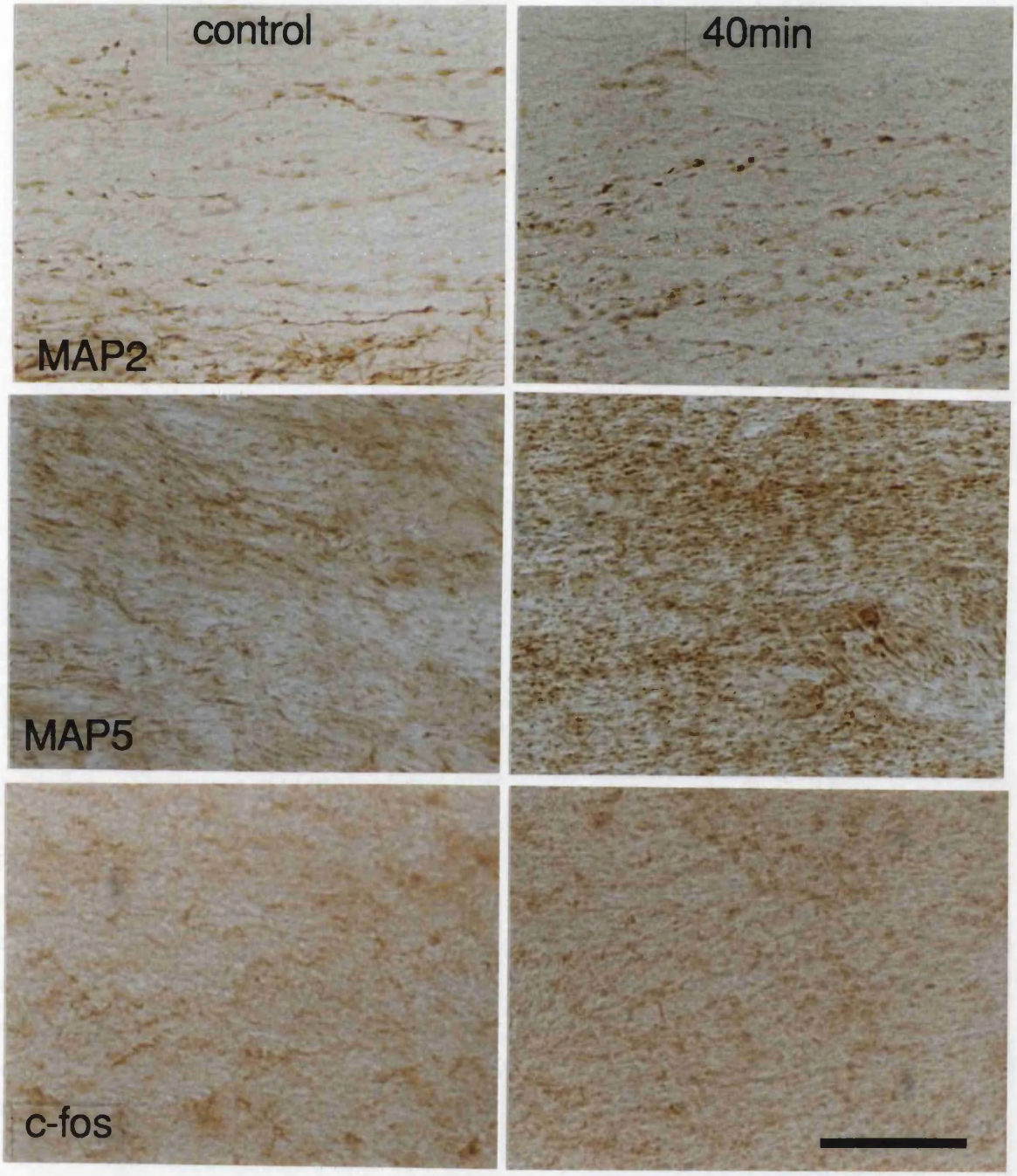



**Figure 39.** MAP2, MAP5 and c-fos immunostaining of subcortical white matter 40 min following the induction of focal cerebral ischaemia in the rat. In contrast to tau, MAP2, MAP5 or c-fos immunoreactivity was not detected in oligodendrocytes of the ipsilateral subcortical white matter 40 min following MCAO. Together this data suggests that the induction of tau immunoreactivity in oligodendrocytes following MCAO in the rat is a specific response of these cells to injury. Scale bar = 50μm.

### 3.3.3 Neuronal distribution of MAPs following permanent focal cerebral ischaemia in the rat

The purpose of the study described above was to examine tau immunostaining in oligodendrocytes in response to MCAO. However, in addition to changes in tau described within oligodendrocytes in the previous section, changes in the neuronal distribution of tau were also observed. Alteration to the neuronal distribution of tau were distinct from those observed in oligodendrocytes and therefore for comparison the following section describes these changes.

*Tau*

The distribution of Tau 1 immunoreactivity was predominantly located within axons of the hemisphere contralateral to the occluded MCA (Fig. 40). Twenty min following MCAO, Tau 1 immunoreactivity was increased within neuronal perikarya of both the cortex and caudate nucleus ipsilateral to the occluded MCA (Fig. 40). In contrast, there was no significant alteration in Tau 1 staining of the neuropil within these areas. Forty min after MCAO, Tau 1 immunoreactivity was increased within neuronal perikarya of both the cortex and caudate nucleus. In addition, increased immunoreactivity was detected within the neuropil of the caudate nucleus (Fig. 40). Eighty min following MCAO, the number of Tau 1 positive neuronal perikarya detected within the cortex, ipsilateral to the occluded MCA, decreased as compared to that present 40 min after MCAO. Within this area of the cortex, decreased neuropil staining was also detected as compared to the contralateral hemisphere. Tau 1 staining remained increased within the neuropil of the caudate nucleus ipsilateral to the occluded MCA.

TP70 and TP007 detected tau both within neuronal perikarya and axons in the hemisphere contralateral to the occluded MCA (Fig 41 & 42). TP70 and TP007 immunoreactivity remained relatively unchanged within neuronal perikarya and neuropil of the cortex and caudate nucleus ipsilateral to the occluded MCA, 20 min following the induction of cerebral ischaemia. In contrast to Tau 1, TP70 (Fig. 41) and TP007 (Fig. 42) staining of neuronal perikarya was decreased in the cortex and caudate nucleus, ipsilateral to the occluded MCA, 40 and 80 min following the induction of cerebral ischaemia, as compared to the contralateral hemisphere. TP70 immunostaining was decreased slightly within the neuropil of the cortex and caudate nucleus, ipsilateral to the occluded MCA (Fig. 41), in contrast TP007 immunoreactivity remained relatively unaltered (Fig. 42).

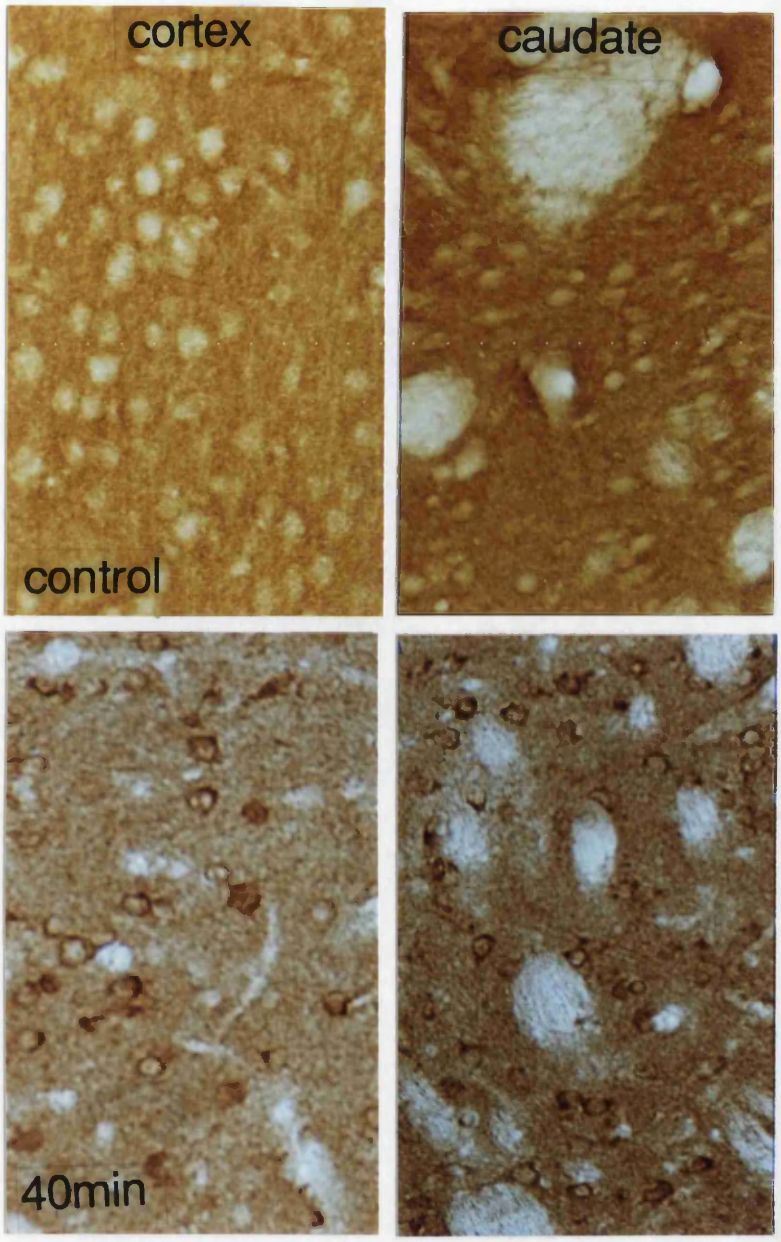



**Figure 40**. Tau 1 immunoreactivity within neurons 40 min following MCAO in the rat. Tau 1 immunoreactivity was increased in neuronal perikarya of the cortex and caudate nucleus in the hemisphere ipsilateral to the occluded MCA, 40 min after the induction of cerebral ischaemia as compared to controls. Axonal staining was not significantly altered within the cortex as compared to controls, however increased axonal staining was detected in the caudate nucleus 40 min after MCAO as compared to controls. Scale bar = 100μm.

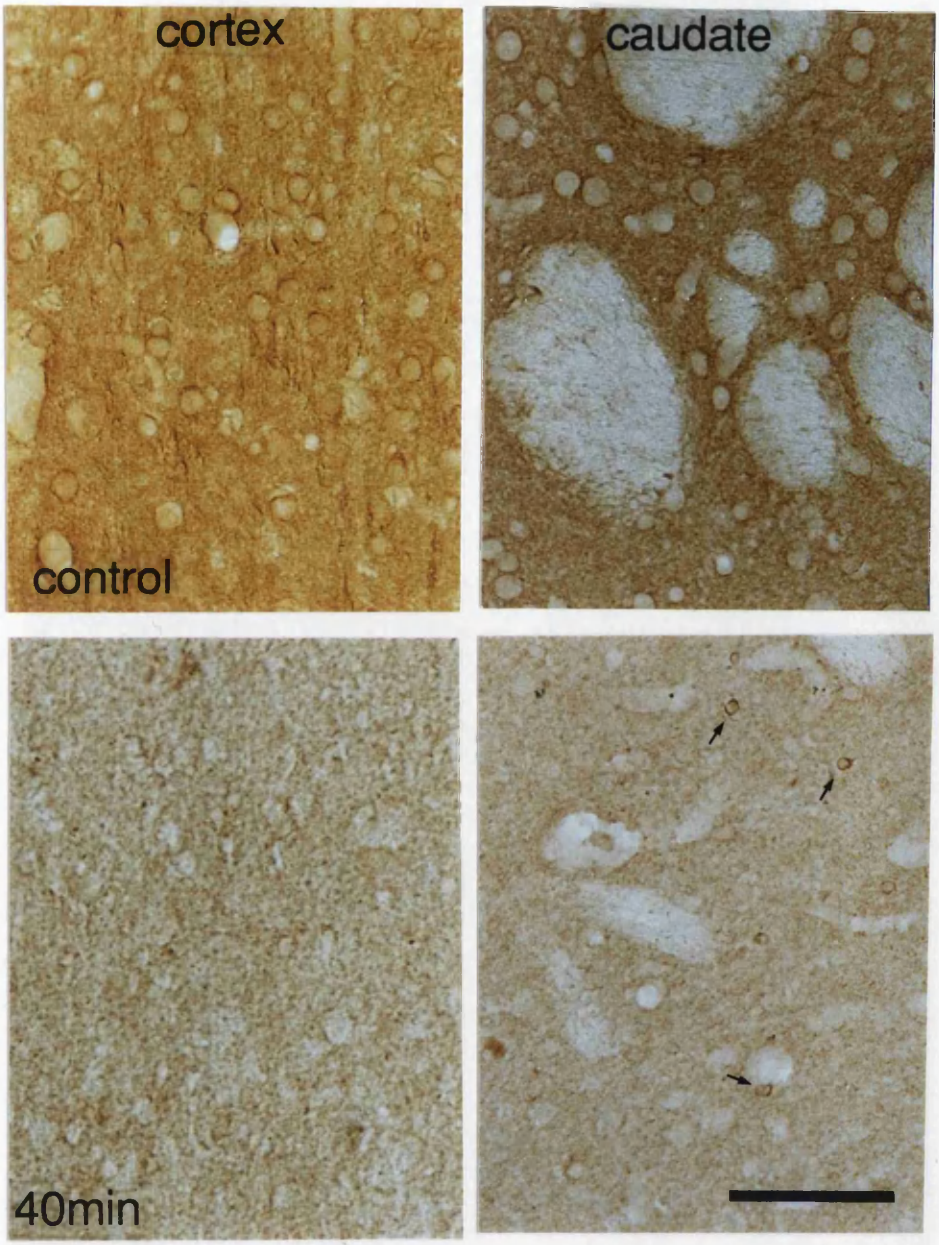



**Figure 41.** TP70 immunoreactivity within neurons 40 min following MCAO in the rat. In contrast to Tau 1, TP70 immunoreactivity was decreased within neuronal perikarya of the cortex and caudate nucleus ipsilateral to the occluded MCA, 40 min following the induction of cerebral ischaemia, as compared to control. Axonal staining was decreased within the cortex and caudate nucleus ipsilateral to the occluded MCA 40 min following MCAO. Note the tau positive glia (arrows) within the caudate nucleus. Scale bar = 100μm.

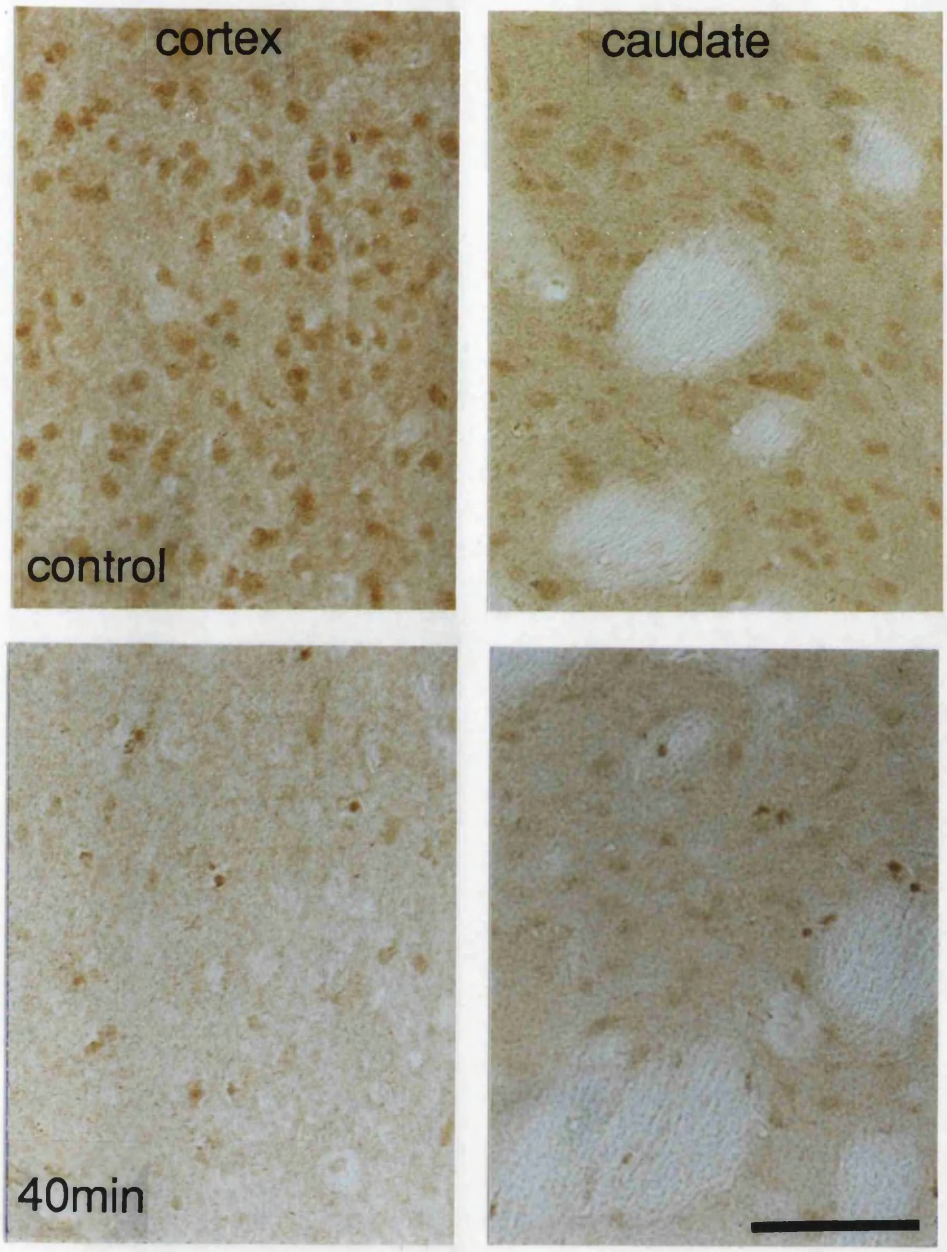



**Figure 42.** TP007 immunoreactivity within neurons 40 min following MCAO in the rat. Similarly to TP70, TP007 immunoreactivity was decreased within neuronal perikarya of the cortex and caudate nucleus ipsilateral to the occluded MCA, 40 min following the induction of cerebral ischaemia as compared to control. Axonal staining was not significantly altered in the cortex or caudate nucleus ipsilateral to the occluded MCA. Scale bar = 100μm.

*MAP2*

Twenty min following MCAO, MAP2 immunoreactivity was increased in neuronal perikarya of both the cortex and caudate nucleus ipsilateral to the occluded MCA as compared to the contralateral hemisphere (Fig. 43). An area where MAP2 staining was decreased within neuropil outlined the boundary of tissue affected by MCAO within the caudate nucleus and cerebral cortex. Forty or 80 min following MCAO, decreased MAP2 immunoreactivity was detected within neuronal perikarya and neuropil of the caudate nucleus within the hemisphere ipsilateral to the occluded MCA (Fig. 43). MAP2 immunoreactivity was increased in neuronal perikarya and decreased in the neuropil of the cortex, however the number of positive cells was decreased compared to that present following 20 min MCAO (Fig. 43).

*MAP5*

MAP5 immunoreactivity was detected both within neuronal perikarya and neuropil of the cortex and caudate nucleus contralateral to the occluded MCA (Fig. 44). Twenty min following MCAO, increased MAP5 immunoreactivity was detected in neuronal perikarya of both the cortex and caudate nucleus ipsilateral to the occluded MCA as compared to the contralateral hemisphere. No significant difference in MAP5 immunostaining was detected within the neuropil. Forty and 80 min after MCAO MAP5 immunoreactivity remained increased in perikarya of the cortex and caudate nucleus ipsilateral to the occluded MCA, however the number of cells stained with MAP5 was decreased in the cortex as compared to that following 20 min MCAO (Fig. 44). Decreased staining of the neuropil was detected within the cortex ipsilateral to the occluded MCA, as compared to that of the contralateral hemisphere.

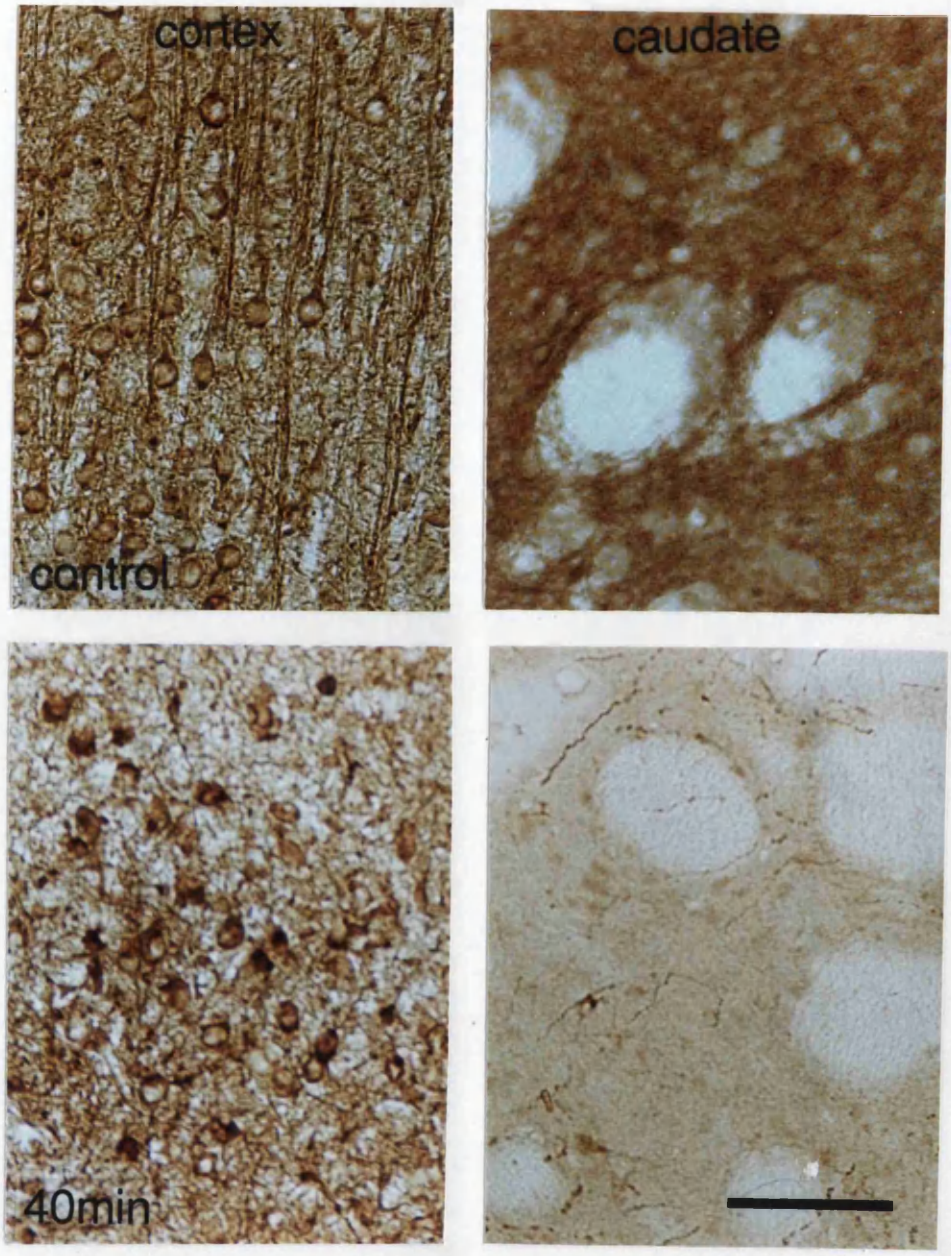



**Figure 43**. MAP2 immunoreactivity within neurons 40 min following MCAO in the rat. Increased MAP2 immunoreactivity was detected in neuronal perikarya, while neuropil staining decreased within the cortex ipsilateral to the occluded MCA 40 min following the induction of cerebral ischaemia. In the hemisphere ipsilateral to the occluded MCA, MAP2 staining was decreased within both the neuronal perikarya and neuropil of the caudate nucleus, as compared to the contralateral hemisphere. Scale bar = 100μm.

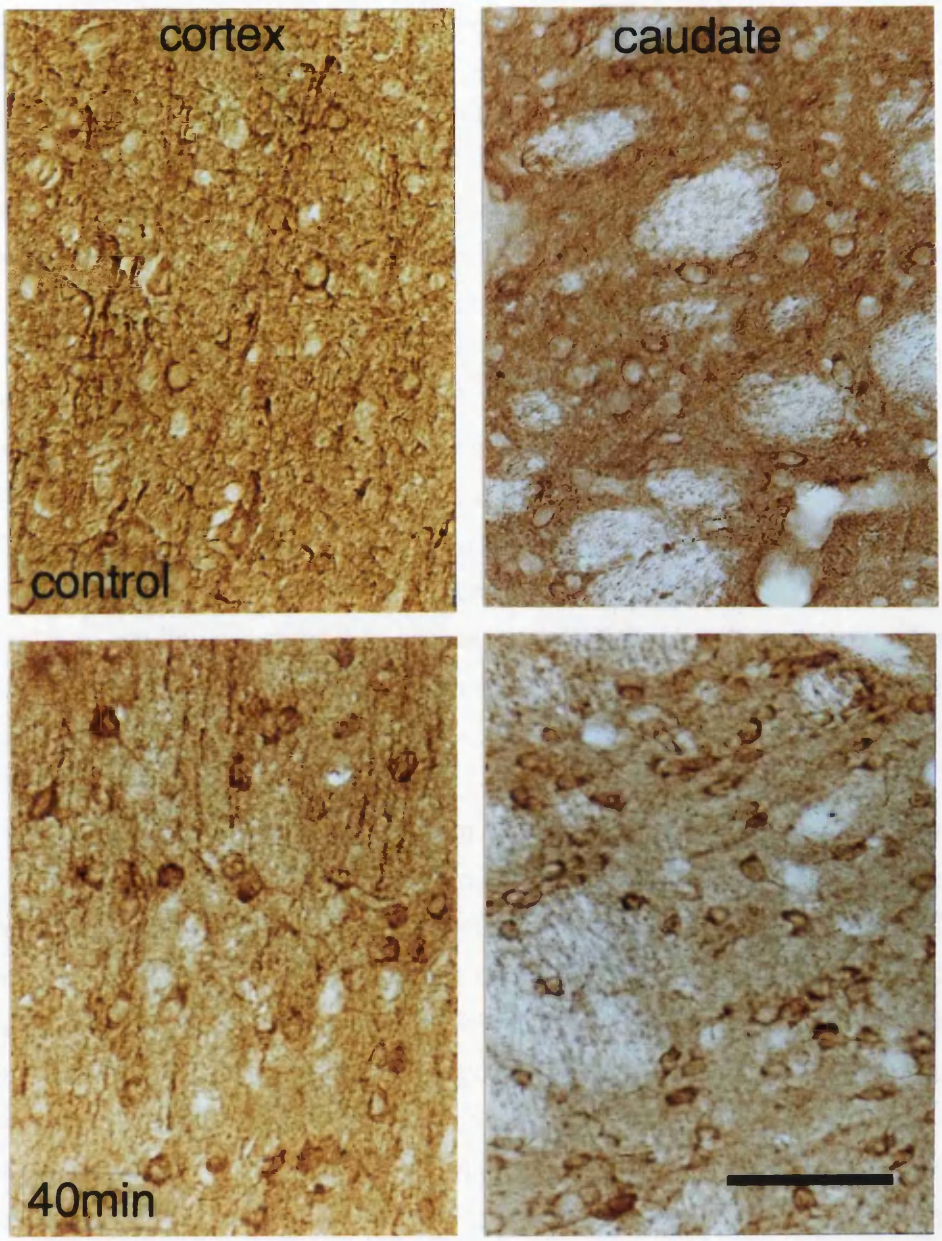



**Figure 44**. MAP5 immunoreactivity in neurons 40 min following MCAO in the rat. Increased MAP5 immunoreactivity was detected in neuronal perikarya within the cortex and caudate nucleus, in the hemisphere ipsilateral to the occluded MCA 40 min following the induction of cerebral ischaemia as compared to control. In the hemisphere ipsilateral to the occluded MCA, MAP5 immunoreactivity was decreased in neuropil within the cortex and caudate nucleus as compared to the contralateral hemisphere. Scale bar = 100μm.

### 3.3.4 Mechanisms underlying increased tau immunoreactivity in oligodendrocytes following focal cerebral ischaemia.

Glutamate excitotoxicity is involved in the mechanisms underlying the neurodegeneration which occurs following ischaemic brain injury (for review see McCulloch, et. al. 1991) and intracortical perfusion in the rat induced tau-positive oligodendrocytes. Therefore it might be speculated that the presence of tau immunoreactivity within oligodendrocytes in the focal ischaemia model may be induced by the actions of elevated extracellular concentrations of glutamate (Butcher, et. al. 1990). However the ability of intracortical NaCl perfusion to induce a similar response suggests that additional mechanisms may be involved in the induction of tau immunoreactivity within these cells. In addition to glutamate receptor activation, free radical mediated mechanisms have also been implicated in the pathology of cerebral ischaemia (see section 1.2.3). *In vitro* studies employing pure oligodendrocyte cultures have shown that oligodendrocytes are susceptible to both glutamate toxicity and free radical mediated damage (see section 1.5.7). The next study was therefore designed to determine whether the presence of tau immunoreactivity within oligodendrocytes following focal cerebral ischaemia in the rat, was initiated through glutamate receptor activation or free radical mediated mechanisms. In order to do this, rats were pre-treated with the NMDA receptor antagonist MK-801, the AMPA receptor antagonist NBQX or the spin trap agent PBN. Previous studies using the MCAO rat model of focal ischaemia have shown these agents at the doses used in this study reduced the volume of infarcted tissue following permanent MCAO in the rat (Cao and Phillis, 1994; Gill, et. al. 1992; Park, et. al. 1988).

The time course study described in section 3.3.2 demonstrated that the number of tau-positive oligodendrocytes / $mm^2$ detected in the subcortical white matter ipsilateral to the occluded MCA was markedly increased 40 min after the induction of focal cerebral ischaemia in the rat. This was the earliest time point following MCAO that significant numbers of tau-positive cells were detected within the subcortical white matter ipsilateral to the occluded MCA and was therefore selected for the drug intervention study. Similarly to the previous quantitative study, for each animal the total number of tau-positive oligodendrocytes in the entire subcortical white matter, extending from that underlying the perirhinal cortex to that underlying cingulate cortex was counted at two distinct brain levels, as described in section 2.6.4. The area of subcortical white matter within which tau-positive cells were counted was measured for each animal and the number of tau-positive cells / $mm^2$ subcortical white matter was calculated for both the ipsilateral and contralateral hemispheres. Thirty min prior to MCAO rats received PBN (100mg/kg; n=5), MK801 (0.5mg/kg, n=5) or NBQX (30mg/kg; n=4), NBQX was administered again 30 min following MCAO. Forty min following MCAO animals were killed by transcardiac perfusion of 4%

paraformaldehyde, and the brains processed for tau immunohistochemistry. Animals subjected to 40 min MCAO with no therapeutic intervention served as controls in this study.

*Ipsilateral hemisphere*

Pre-treatment with the spin trap agent PBN reduced the number of tau-positive oligodendrocytes / $mm^2$ by approximately 50% within the subcortical white matter ipsilateral to the occluded MCA of animals subjected to 40 min cerebral ischaemia as compared to untreated animals (Fig. 45). This marked reduction in the number of oligodendrocytes / $mm^2$ subcortical white matter ipsilateral to the occluded MCA was consistently detected using all 3 tau antibodies : Tau 1 (recognises a dephosphorylated epitope between residues 199-202); TP70 (directed towards the C-terminal of tau) and TP007 (directed towards the N-terminal of tau) (Fig 45).

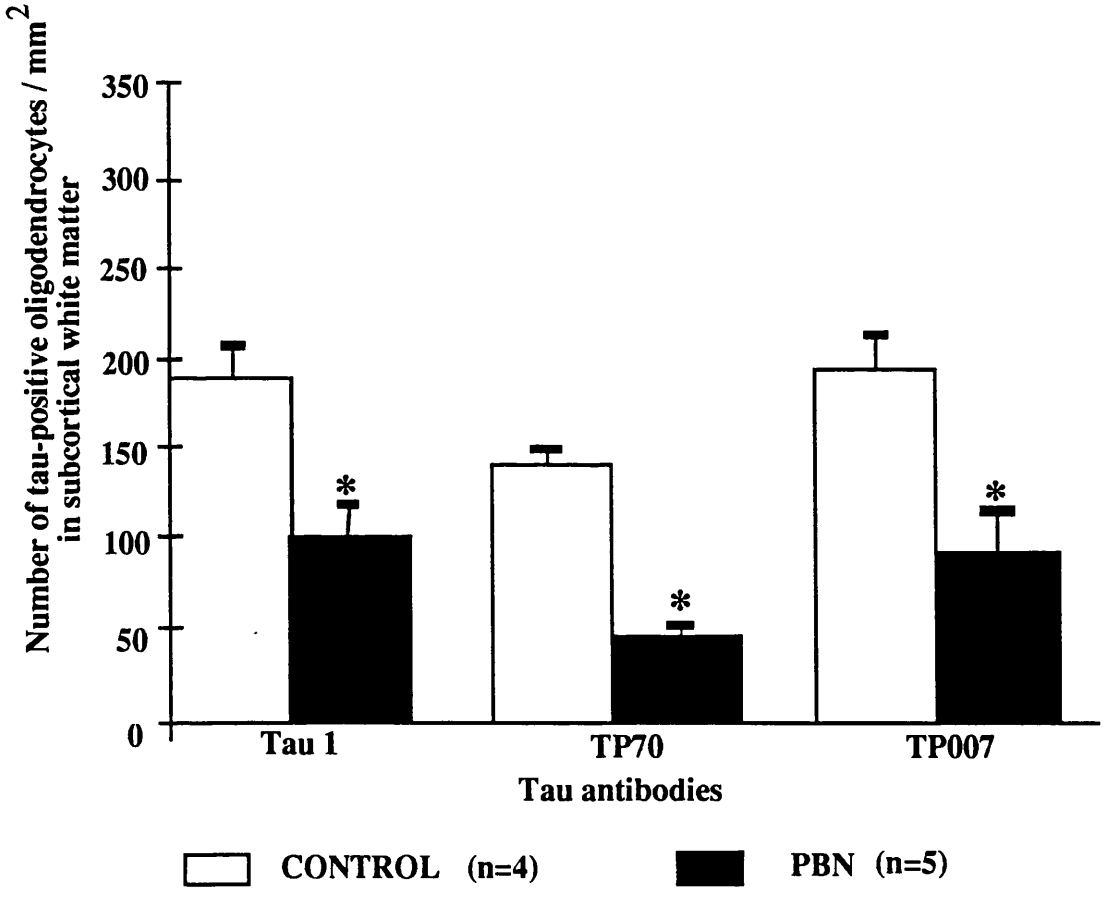



**Figure 45.** Effect of PBN pre-treatment on the number of tau-positive oligodendrocytes present in the ipsilateral sucortical white matter 40 min following MCAO. Pre-treatment with the spin trap agent PBN (100mg/kg) significantly reduced the number of tau-positive oligodendrocytes / $mm^2$, as detected with Tau 1, TP70 and TP007, in the ipsilateral subcortical white matter as compared to untreated animals (* $p<0.05$). Data is presented as Mean ± SEM. Statistical analysis consisted of ANOVA followed by Student's *t*-test with Bonferroni correction.

By contrast, pre-treatment with the glutamate receptor antagonists MK801 or NBQX failed to decrease the number of tau-positive oligodendrocytes / $mm^2$, as detected with Tau 1, TP70 and TP007, in the subcortical white matter ipsilateral to the occluded MCA compared to untreated animals (Fig. 46). Moreover, pre-treatment with MK801 and NBQX increased the number of tau-positive oligodendrocytes in the ipsilateral subcortical white matter by up to 50% and 25% respectively. The statistical significance of this increase was not tested due to the *a priori* hypothesis being tested i.e. that drug intervention would decrease the response in the ischaemic hemisphere. Together these data suggest that the mechanisms which lead to increased tau immunoreactivity in oligodendrocytes following focal cerebral ischaemia at least in part involve free radicals but not glutamate receptor activation.

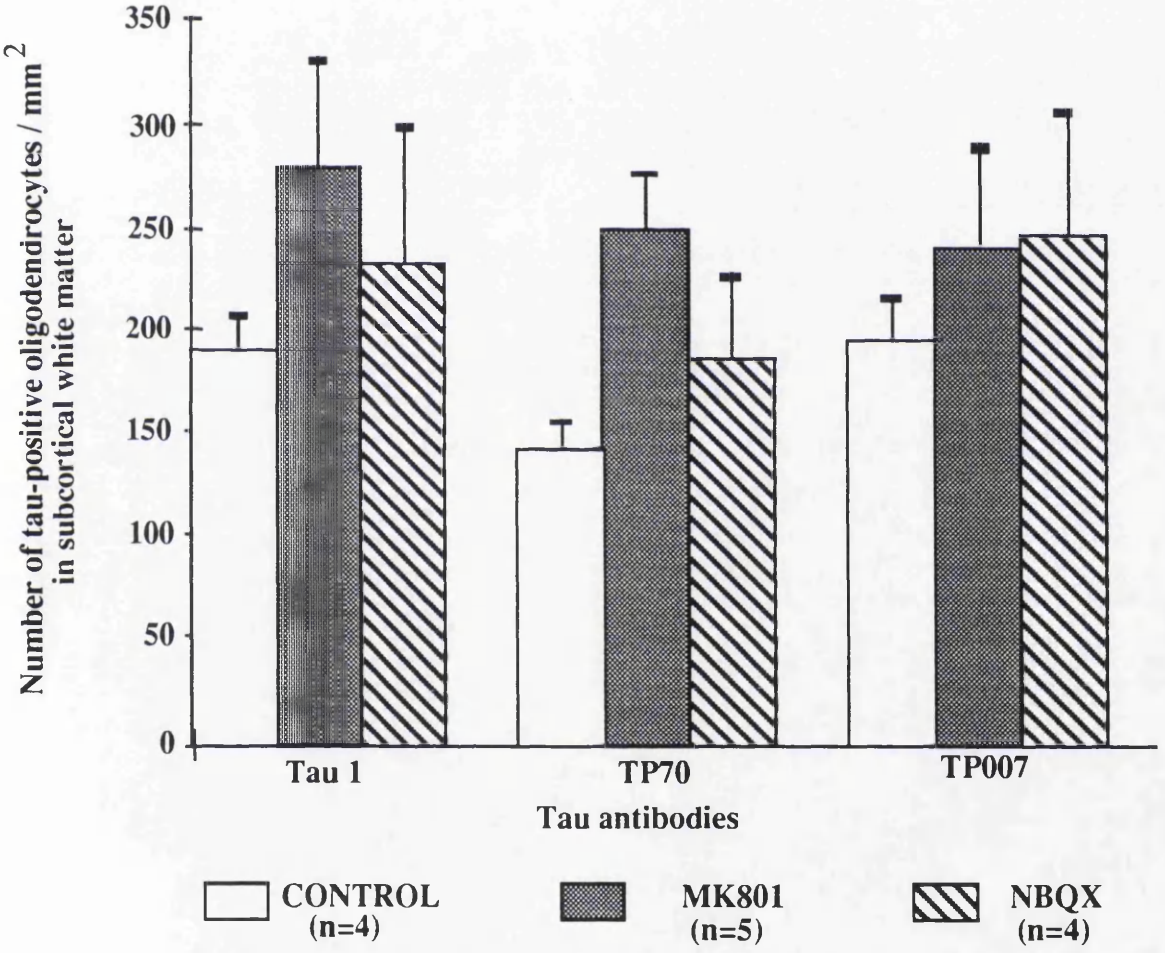



**Figure 46.** Effect of MK801 and NBQX pre-treatment on the number of tau-positive oligodendrocytes 40 min following MCAO. Pre-treatment with the NMDA receptor antagonist MK801 (0.5mg/kg) or the AMPA receptor antagonist NBQX (30mg/kg, x2) failed to significantly reduced the number of tau-positive oligodendrocytes / $mm^2$, as detected with Tau 1, TP70 and TP007, in the ipsilateral subcortical white matter compared to untreated animals. Data is presented as Mean ± SEM. Statistical analysis consisted of ANOVA followed by Student's *t*-test with Bonferroni correction.

*Contralateral hemisphere*

Due to the unilateral nature of the ischaemic brain damage resulting from MCAO, the contralateral hemisphere served as a control for the effect of drug treatment alone on tau immunoreactivity in oligodendrocytes. In animals pre-treated with PBN, the number of tau-positive oligodendrocytes / $mm^2$ in the contralateral hemisphere 40 min following MCAO was not significantly different from that present in untreated animals (Fig. 47). This shows that PBN alone did not have an effect on tau immunoreactivity within oligodendrocytes distant from the area of ischaemic brain injury.

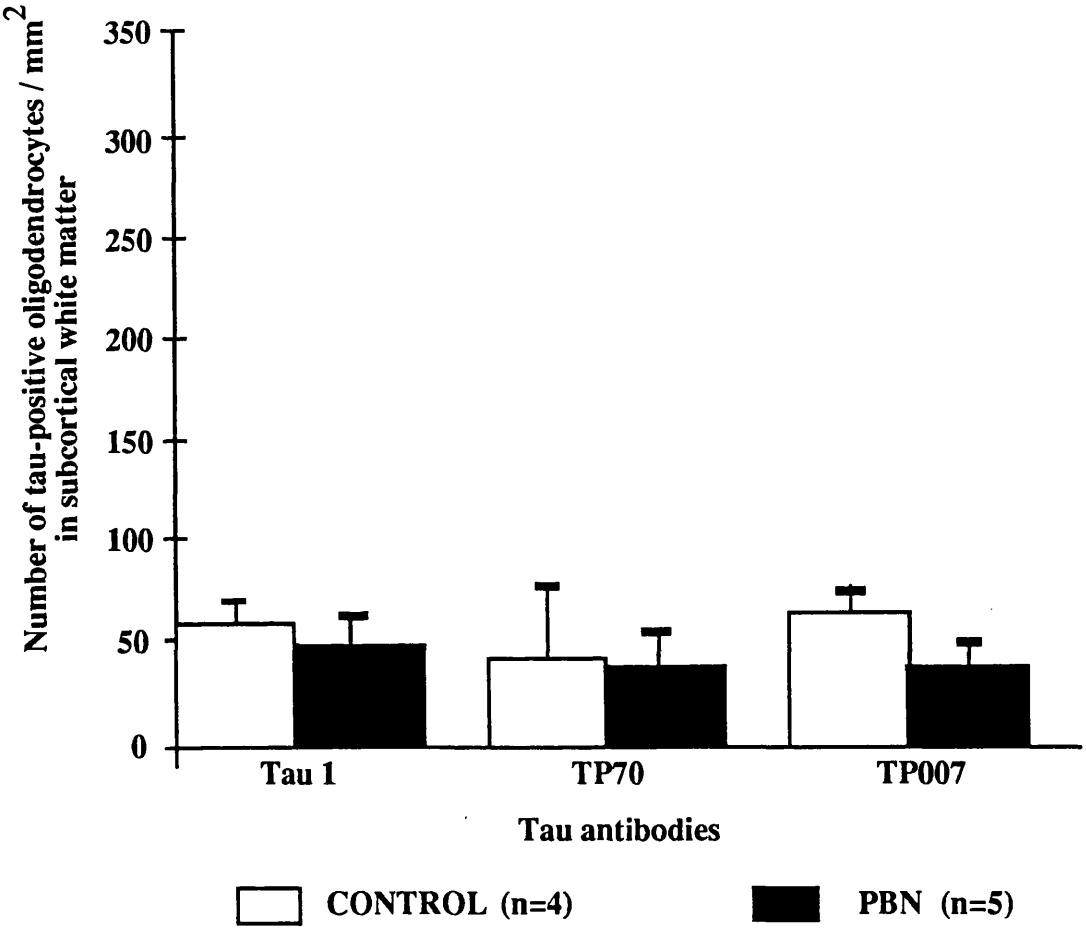



**Figure 47.** The effect of PBN pre-treatment on tau-positive oligodendrocytes within the contralateral subcortical white matter 40 min following MCAO. Pre-treatment with PBN (100mg/kg) did not significantly alter the number of tau-positive oligodendrocytes / $mm^2$, as detected with Tau 1, TP70 or TP007 as compared to untreated animals. This shows that PBN alone at this concentration does not have an effect on tau immunoreactivity in oligodendrocytes. Data is presented as Mean ± SEM.

Pre-treatment with MK801 or NBQX however increased the number of tau-positive oligodendrocytes in the contralateral subcortical white matter compared to untreated animals (Fig. 48). Thus treatment with these glutamate receptor antagonists alone at the concentrations employed in the present study have an effect on tau in oligodendrocytes distant from the area of ischaemia. Again the statistical significance of this increase was not tested due to the *a priori* hypothesis being tested i.e. that drug intervention would decrease the response in the ischaemic hemisphere.

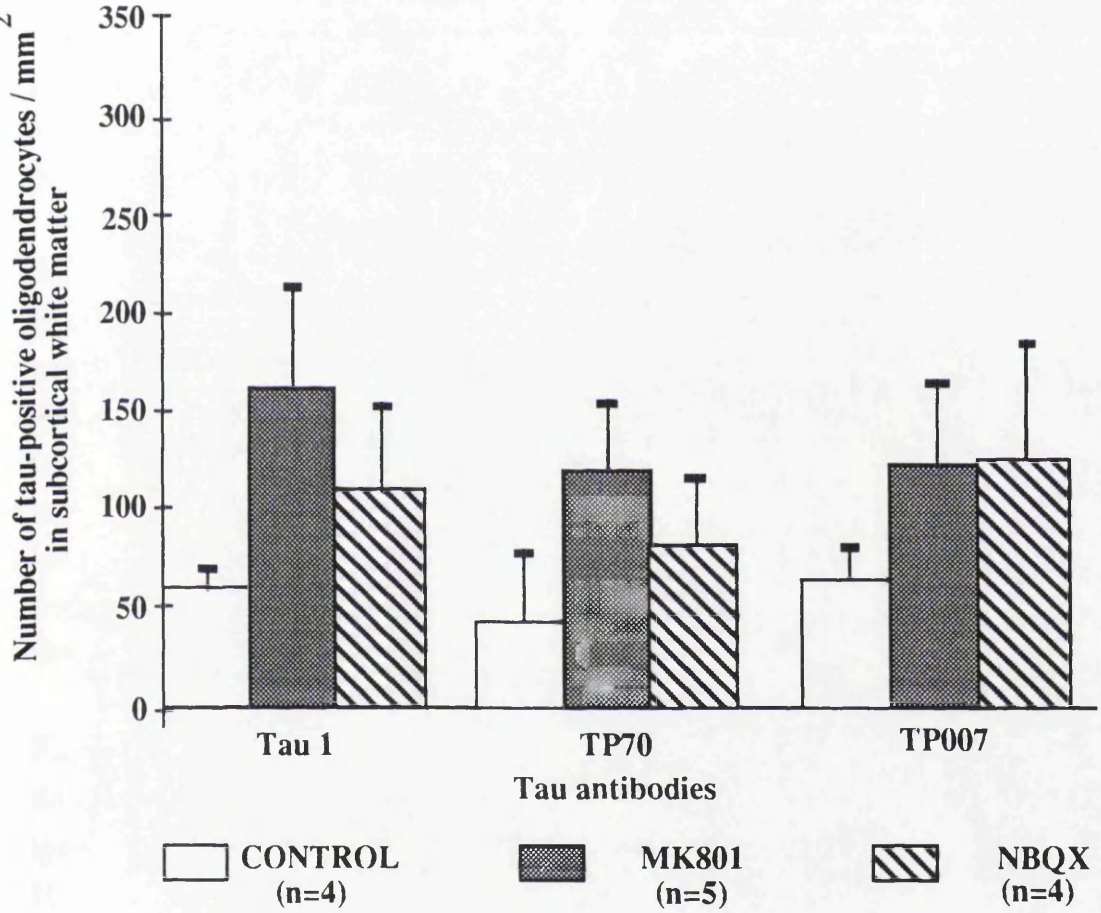



**Figure 48.** The effect of MK801 and NBQX pre-treatment on tau-positive oligodendrocytes within the contralateral subcortical white matter 40 min following MCAO. Following pre-treatment with MK801 (100mg/kg) or NBQX (2 x 30mg/kg), the number of tau-positive oligodendrocytes / $mm^2$ subcortical white matter increased by up to 300% and 100% respectively as compared to untreated animals. This increase was detected with each of the 3 tau antibodies used : Tau 1; TP70 and TP007, showing that MK801 and NBQX alone have an effect on tau immunoreactivity in oligodendrocytes. Data is presented as Mean ± SEM.

## 3.4 The effect of permanent focal cerebral ischaemia on the synthesis of tau in oligodendrocytes

In all the experimental models used so far and in the human post-mortem brain tissue, increased tau immunoreactivity was detected by antibodies directed towards the N- and C-terminals, as well as the middle portion of the protein. This suggests that full-length tau may be present in oligodendrocytes and that increased staining represents increased protein levels. The next question to be addressed therefore, was whether tau synthesis was increased under the same pathological circumstances as those in which increased immunoreactivity was present. In situ hybridisation studies were performed in order to determine whether tau immunoreactivity detected within oligodendrocytes following permanent MCAO in the rat reflects increased de novo synthesis of the protein. In this study rats were killed 40 min (n=2), 120 min (n=2) and 240 min (n=2) after the induction of permanent cerebral ischaemia and the brains processed for in situ hybridisation.

An anti-sense $^{35}S$ labelled riboprobe was synthesized from a 1031bp fragment generated from the coding region of rat tau cDNA (see section 2.3). Dark field microscopy, revealed neurons of the cerebral cortex as the most intensely labelled cells in rat brain sections (Fig. 49). High signal was also detected in neurons of the caudate nucleus and other areas of grey matter, however these were labelled to a lesser extent than cortical neurons. Low levels of signal were detected in the subcortical white matter, however the cellular localisation of this signal was difficult to distinguish. The level of signal detected in the white matter was only slightly higher than that detected in sections labelled with a sense riboprobe, suggesting that this signal may represent non-specific hybridisation (Fig. 49).

Forty, 120 or 240 min following the induction of ischaemia, no significant difference in the distribution of tau mRNA in oligodendrocytes was detected within the white matter ipsilateral to the occluded MCA, as compared to the contralateral hemisphere (Fig. 50). However, 120 or 240 min after MCAO tau mRNA was decreased within the neurons of the cortex within the region of ischaemic tissue (Fig. 51)

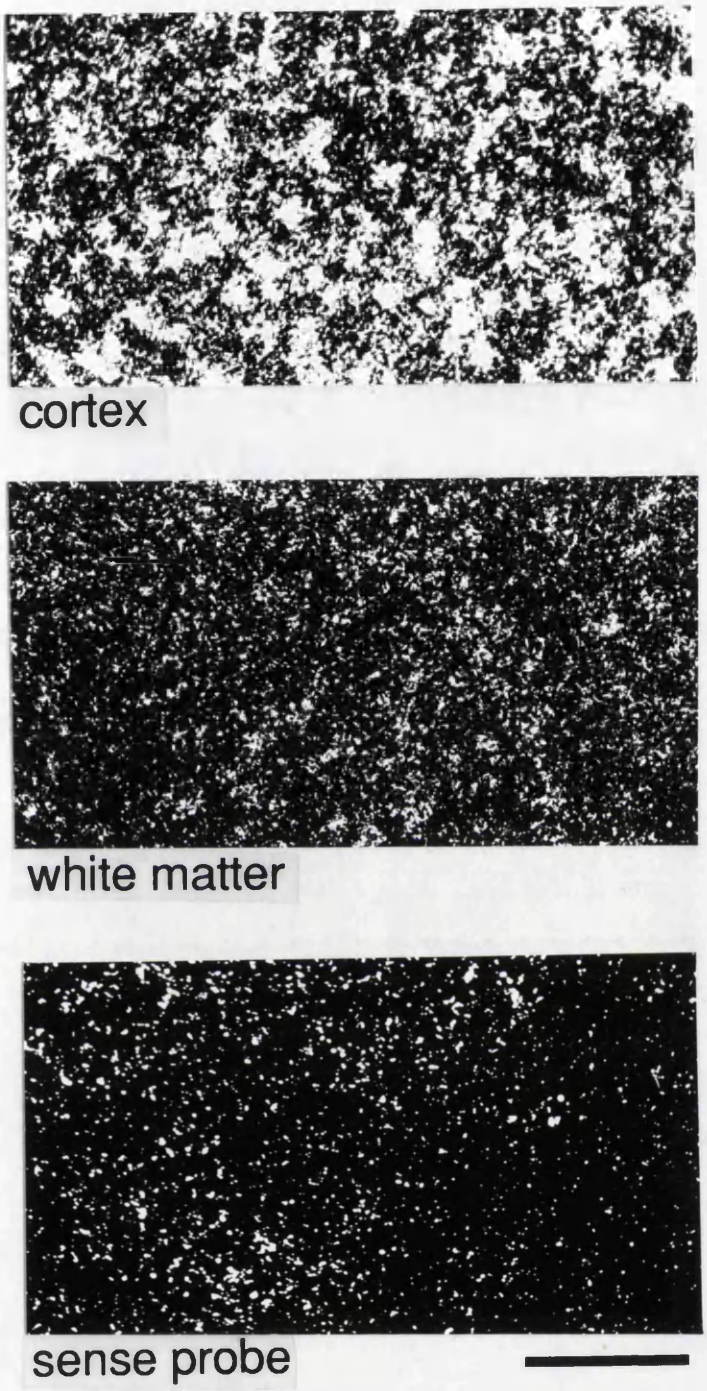



**Figure 49.** Distribution of tau mRNA in normal rat brain tissue. Tau mRNA was detected using a $^{35}S$ labelled riboprobe directed towards the coding region of rat tau cDNA. Dark field microscopy revealed that tau mRNA was detected predominantly within neurons of the cerebral cortex in histologically normal tissue. Signal present in the subcortical white matter was, by contrast, much lower than that present in grey matter. In situ hybridisation using a sense riboprobe resulted in similar levels of signal within the subcortical white matter, suggesting that signal detected in the subcortical white matter using the antisense ripboprobe represent non-specific hybridisation of the probe. Scale bar = 200μm.

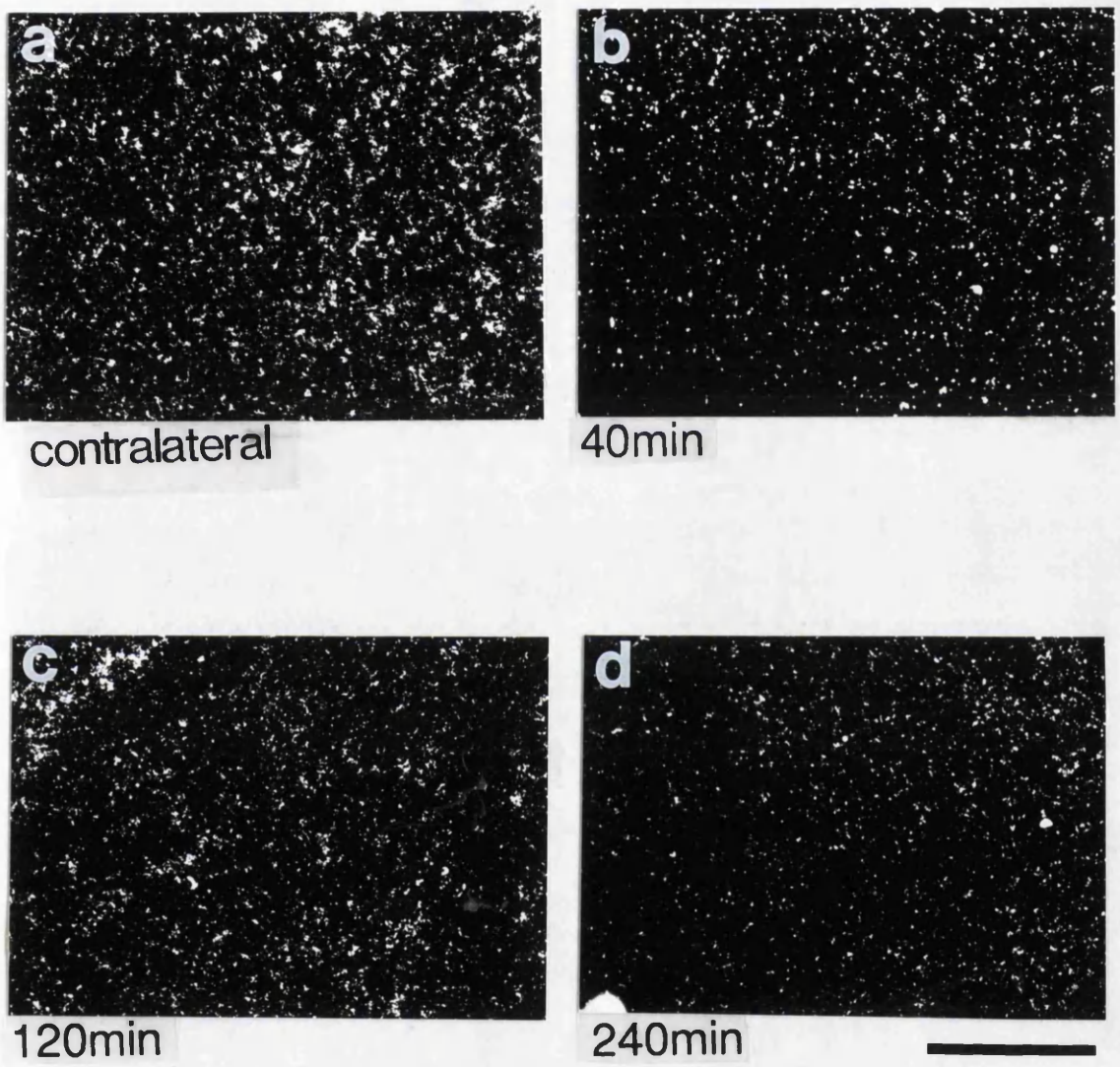



**Figure 50**. Distribution of tau mRNA in the subcortical white matter following MCAO in the rat. No significant increase in tau mRNA was detected in the subcortical white matter ipsilateral to the occluded MCA, 40 min (b), 120 min (c) or 240 min (d) following MCAO as compared to the contralateral white matter (a). Scale bar = 200μm.

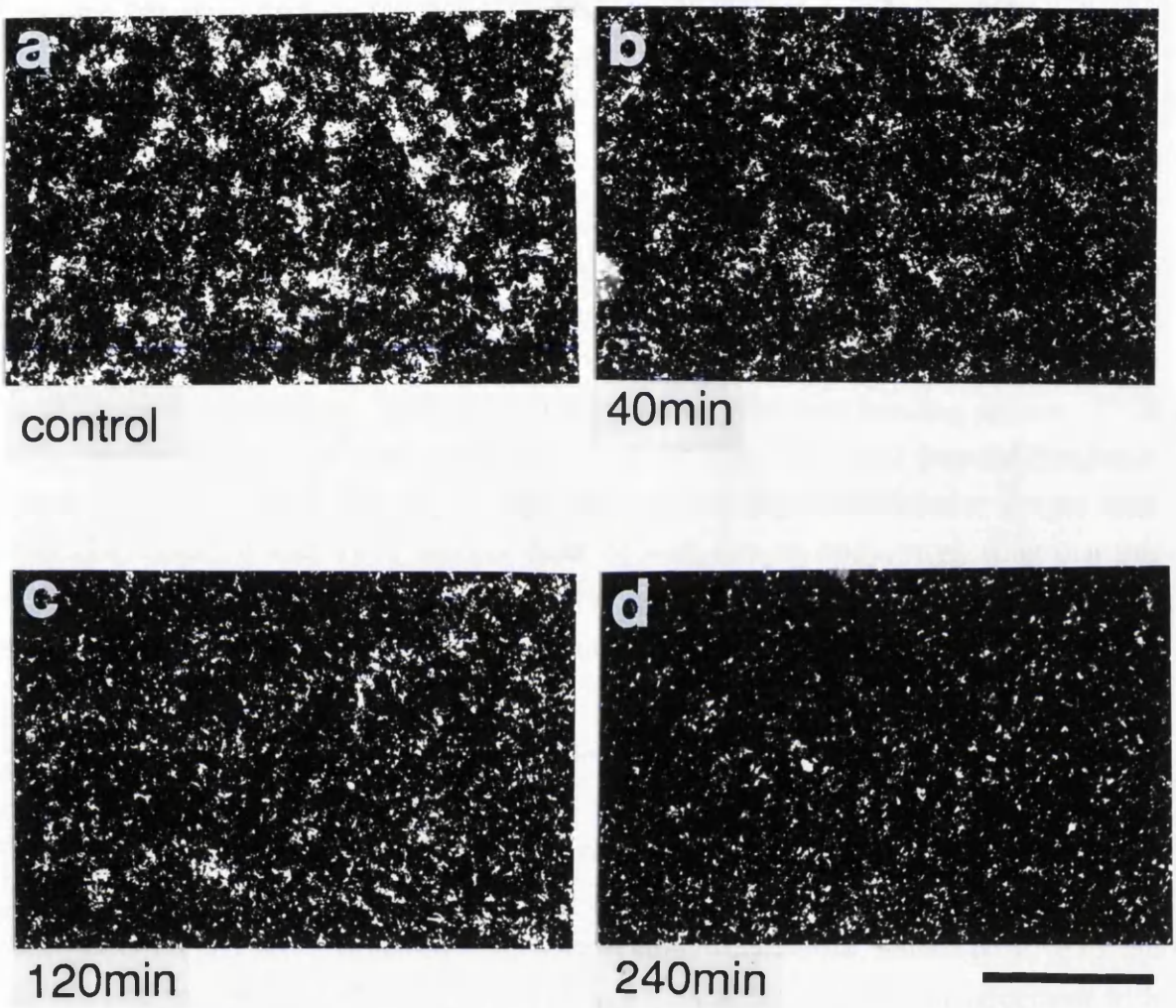



**Figure 51.** The distribution of tau mRNA in neurons following MCAO in the rat. Tau mRNA as detected with a $^{35}S$ riboprobe directed against the coding region of tau, was decreased in the ipsilateral hemisphere 120 min (c) and 240 min (d) following MCAO as compared to control (a).No significant alteration in the pattern of tau mRNA was seen 40 min following MCAO as compared to controls. Scale bar = 200μm.

## 3.5 Tau protein in an O-2A oligodendrocyte cell line

The previous experiments show that the induction of tau immunoreactivity in oligodendrocytes is a rapid response of these cells to acute brain injury and is initiated through free radical mediated mechanisms. However, whether the presence of tau in oligodendrocytes results through direct injury to these cells or is mediated through neuronal degeneration is not known. Pure oligodendrocyte cultures derived from an O-2A cell line, kindly prepared by Dr S.C. Barnett were therefore utilised to investigate the direct effects of pathological stimuli on tau immunoreactivity.

### 3.5.1 Western blot analysis of tau in oligodendrocyte cultures

In normal rat brain tau exists as six isoforms. To determine whether tau was present in oligodendrocytes in culture, extracts from these cultures were resolved on SDS-PAGE and blotted with Tau 1 and TP70 antibodies. Both antibodies detected multiple isoforms of tau in oligodendrocyte cultures, however each resulted in a different banding pattern. TP70 detected four major bands between 40kDa and 60kDa (Fig. 52). Tau 1 detected five bands within a similar molecular weight range, however the highest molecular weight band (~60kDa) detected with TP70 was not detected with this antibody, suggesting that this isoform may be phosphorylated at the Tau 1 epitope. These results show that oligodendrocyte cultures used in this study contain tau protein.

### 3.5.2 Tau immunoreactivity in oligodendrocyte cultures

***Tau immunoreactivity in control cultures***

In contrast to oligodendrocytes in normal rat brain tissue, oligodendrocyte cultures derived from an O-2A cell line, maintained under normal culture conditions, stained positive for tau as detected with Tau 1, TP70 and TP007 (Fig. 53, 54 & 55). This is in agreement with western blot analysis of these cultures which showed the presence of multiple tau isoforms in control cultures and suggests that full length tau protein is present in these cells. Each antibody showed a distinct pattern of immunoreactivity. TP70 and Tau 1 stained the cellular cytoplasm within the cell body and processes, while TP007 immunoreactivity appeared to be within the nucleus. Nuclear staining often represents non-specific binding, it is possible therefore that TP007 may recognise an unrelated epitope in the nucleus of these cells. TP007 immunoreactivity remained nuclear following exposure to glutamate and NaCl (Fig. 55) and the results were therefore disregarded.

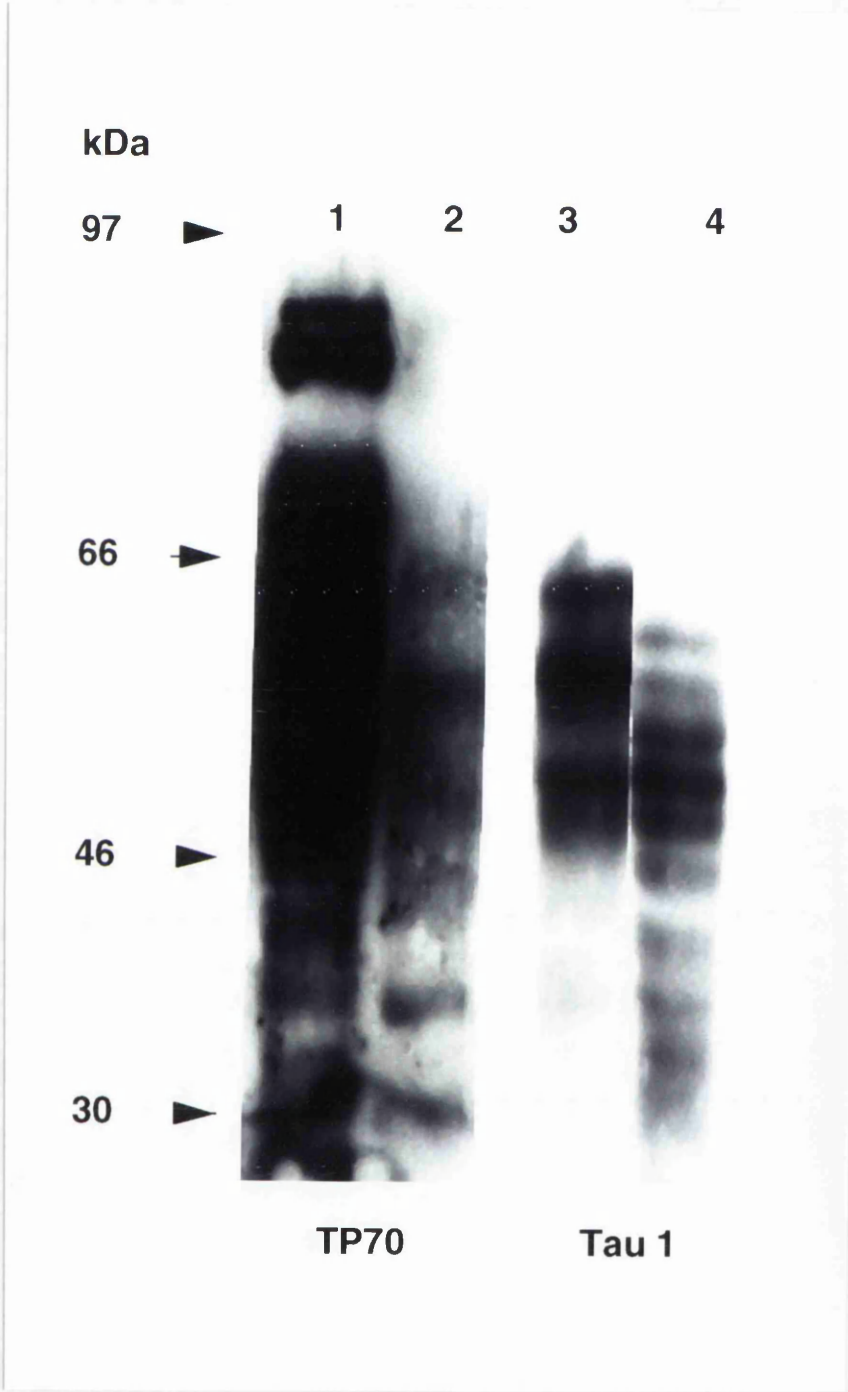



**Figure 52.** Western blot analysis of tau in cultured oligodendrocytes, using TP70 and Tau 1. Similarly to tau present in whole rat brain homogenates (lanes 1 & 3), tau extracted from oligodendrocyte cultures consisted of multiple isoforms (lanes 2&4). Rat brain homogenates displayed tau isoforms between 52 & 68kDa as described previously (Drubin, et. al. (1984). In oligodendrocyte extracts, TP70 detected 3 major bands ranging from 44-66kDa (lane 2). Tau 1 however detected 5 bands ranging from 44-60kDa (lane 4).

*Monosodium glutamate dose response curve*

All experiments described below were carried out in duplicate on three occasions using different batches of oligodendrocyte cultures. Pure oligodendrocyte cultures were exposed to 10μM, 100μM or 1mM monosodium glutamate for 4 h at 37°C in a $CO_2$ incubator. Immediately following incubation, the cells were rinsed and immunostained with Tau 1 (Fig. 53). Tau 1 immunoreactivity remained relatively unchanged following exposure of oligodendrocyte cultures to 10μM monosodium glutamate as compared to control cultures. Following exposure to 100μM monosodium glutamate Tau 1 immunoreactivity was significantly increased both within the cytoplasm of the cell body and processes. Tau 1 immunoreactivity was increased following exposure to 1mM monosodium glutamate as compared to those treated with 100μM monosodium glutamate. However, these tau-positive oligodendrocytes exhibited signs of degeneration, the cells were swollen in appearance and disintegration of processes was detected. From these results 100μM monosodium glutamate was selected to investigate the effect of sublethal glutamate induced injury on tau immunoreactivity in pure oligodendrocyte cultures.

***Effect of 100μM monosodium glutamate and NaCl on tau immunoreactivity in oligodendrocyte cultures***

As a control for these studies, oligodendrocyte cultures were exposed to 100μM NaCl for 4 h prior to tau immunostaining. Increased Tau 1 immunoreactivity was detected in oligodendrocyte cultures treated with either 100μM monosodium glutamate or 100μM NaCl (Fig. 54). However, the intensity of Tau 1 staining was less in NaCl treated cultures as compared to those treated with glutamate (Fig. 54). Dephosphorylation of control cultures prior to Tau 1 immunostaining resulted in a similar increase in the intensity of Tau 1 staining following treatment with 100μM monosodium glutamate and NaCl (Fig. 54). In contrast to the increased Tau 1 immunoreactivity detected following exposure of cultures to 100μM monosodium glutamate or NaCl, TP70 staining remained largely unchanged as compared to controls (Fig. 55). Together these results suggest that, in contrast to the accumulation of tau in oligodendrocytes detected in vivo following glutamate or NaCl perfusion, tau undergoes dephosphorylation following cytotoxic injury in vitro. All results were obtained in duplicate on three separate occassions.

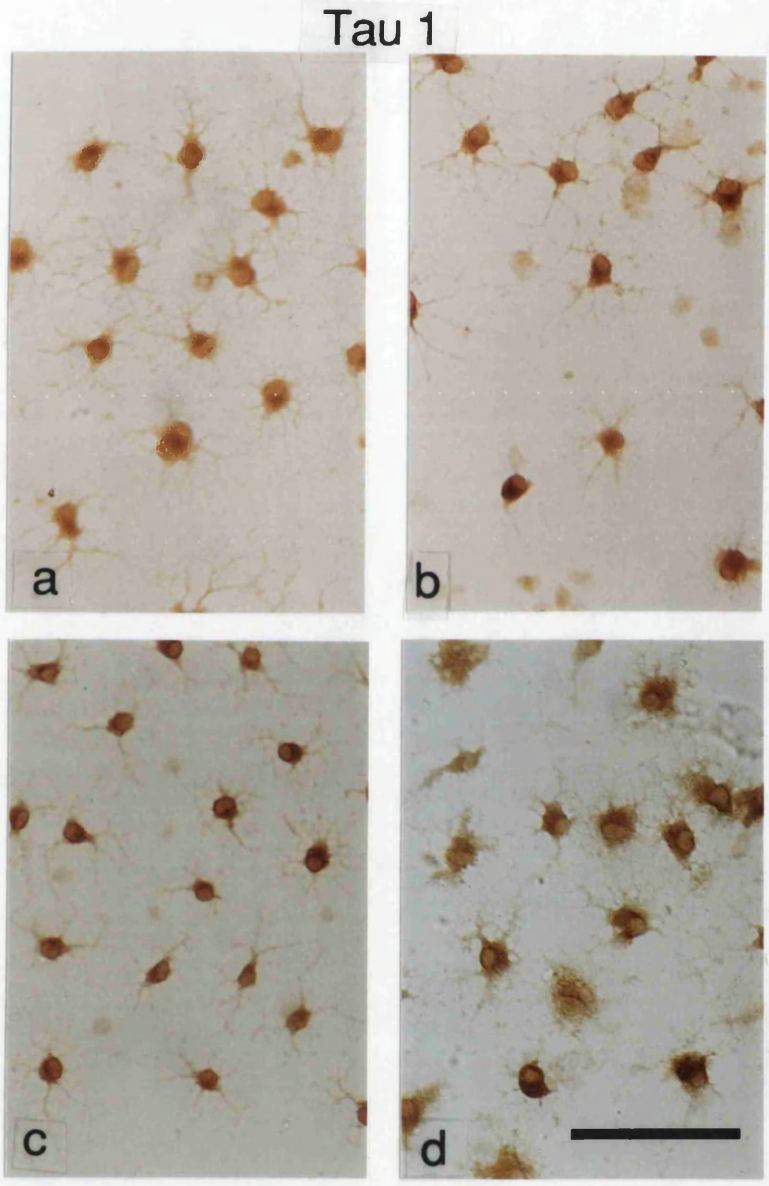



**Figure 53.** Tau 1 immunoreactivity in oligodendrocyte cultures treated with 10μM, 100μM or 1mM monosodium glutamate for 4 h. Similarly to that present in control cultures (a), Tau 1 immunoreactivity was detected within the cytoplasm of the cell body and processes of oligodendrocytes following exposure of these cultures to 10μM monosodium glutamate (b). However, following exposure to 100μM monosodium glutamate (c), Tau 1 immunoreactivity was increased both within the cell body and processes. Treatment of cultures with 1mM monosodium glutamate resulted in increased Tau 1 imunoreactivity, however cells showed signs of degeneration, such as the disintegration of cellular processes (arrows) and swelling of the cell body (d). Scale bar = 100μm.

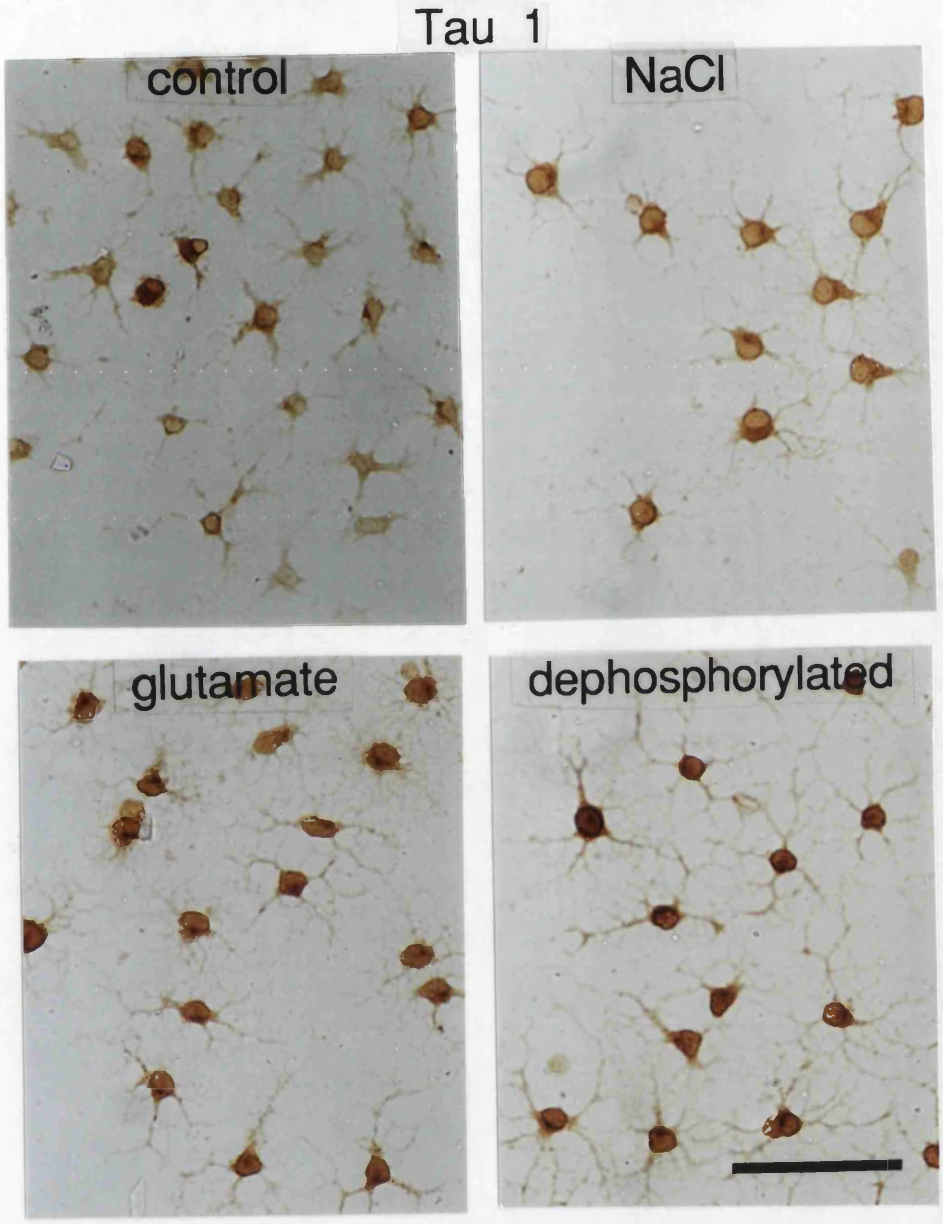



**Figure 54.** Tau 1 immunoreactivity in oligodendrocyte cultures derived from an O-2A cell line. Tau 1 immunoreactivity was detected within the cytoplasm of the cell body and processes of oligodendrocytes maintained in Sato's growth medium. Following exposure of these cultures to 100µM monosodium glutamate or 100µM NaCl, Tau 1 immunoreactivity was increased both within the cell body and processes. Dephosphorylation of control cultures prior to immunostaining resulted in a similar pattern of Tau 1 immunoreactivity within these cells as detected following treatment with 100µM monosodium glutamate or NaCl. Scale bar = 100µm.

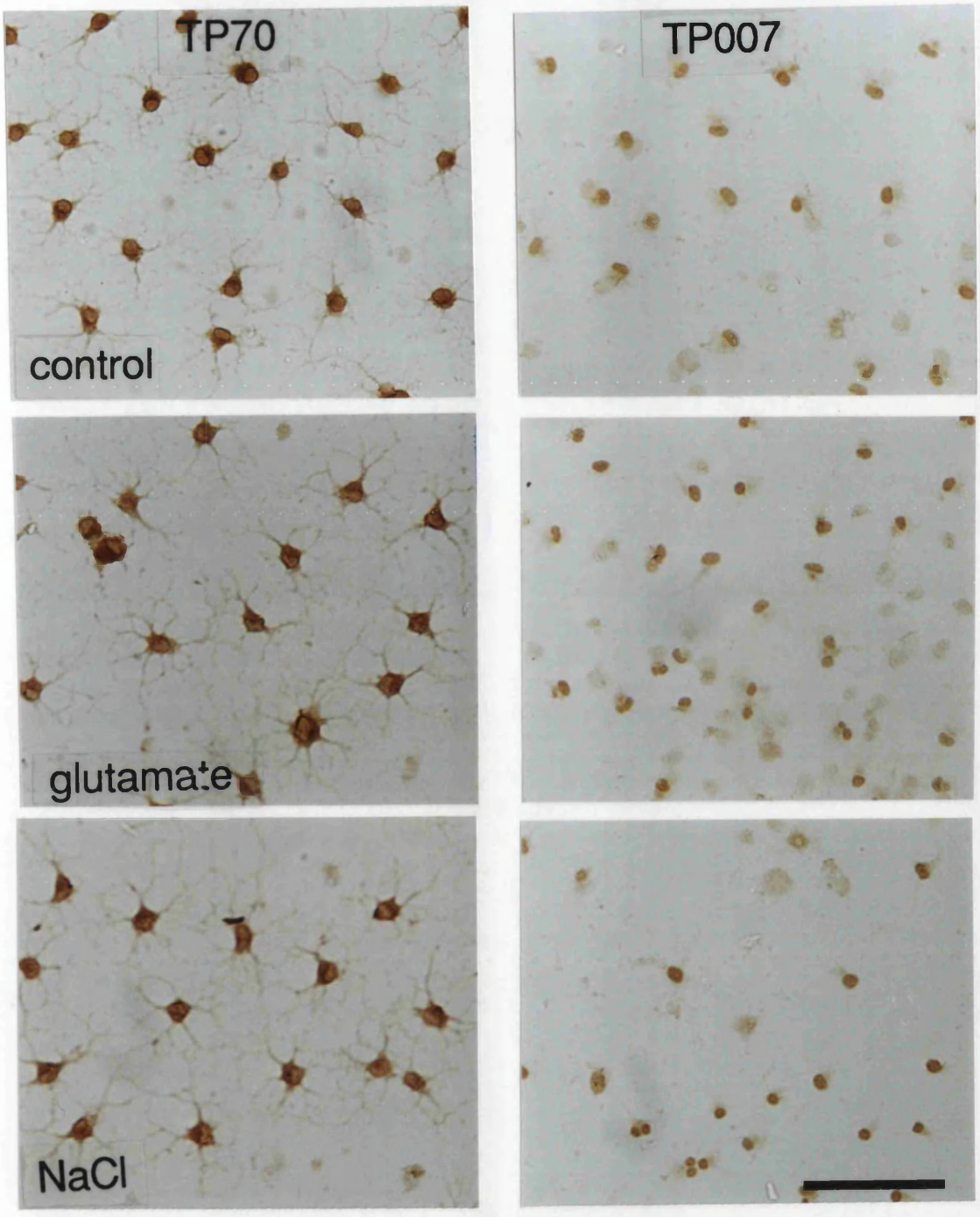



**Figure 55.** TP70 and TP007 immunoreactivity in oligodendrocyte cultures derived from an O-2A cell line. TP70 immunoreactivity was detected in oligodendrocyte cultures maintained in Sato's growth medium. Immunoreactivity was detected within the cytoplasm of the cell body and processes, while TP007 was localised mainly to the cell nucleus. Following treatment of cultures with 100μM monosodium glutamate or 100μM NaCl, the distribution of TP70 and TP007 immunoreactivity was relatively unchanged. The localisation of TP007 immunoreactivity to the nucleus, suggests that this antibody may bind non-specifically to an unrelated protein within these cells. Scale bar = 100μm

# CHAPTER 4 DISCUSSION

The original aim of my thesis was to investigate the possible mechanisms underlying cytoskeletal breakdown in neurons following acute brain injury. However, in the first study investigating the possible role of glutamate excitotoxicity in this process, tau immunoreactivity was detected within oligodendrocytes. This intriguing finding indicated that cells other than neurons within the CNS may respond to acute brain injury with changes in tau protein. My studies were therefore directed at further characterising the presence of tau in oligodendrocytes following acute brain injury and determining the mechanisms underlying this response. Until recently oligodendrocytes have been considered to be relatively inactive during cerebral ischaemia, however the work in this thesis shows that oligodendrocytes have the ability to respond rapidly to acute brain injury and may therefore play an important role in the progression of ischaemic brain damage. I will firstly describe changes in the oligodendrocyte cytoskeleton that occur following acute brain injury and then go on and compare these to the alterations of the cytoskeleton which occur in neurons.

## 4.1 Detection of the "neuronal" protein tau in oligodendrocytes following acute brain injury

### 4.1.1 Distribution of tau in histologically normal brain tissue

The normal distribution of tau in the CNS has been well documented (see section 1.4.2) and it is generally accepted that tau is predominantly located within neurons where it is most abundant in the axonal compartment (Binder, et. al. 1985; Brion, et. al. 1988; Papasozomenos and Binder, 1987; Trojanowski, et.al. 1989). Moreover, tau mRNA was shown to be located specifically within neurons *in situ* (Goedert, et. al. 1989; Kosik, et. al. 1989a; 1989b). In the studies carried out throughout this thesis, tau immunoreactivity was found located mainly within neurons in histologically normal rat and human brain tissue, supporting a predominantly neuronal localisation of tau. Tau immunoreactivity was not detected within glia in histologically normal tissue using antibodies directed towards various regions of tau : Tau 1 (recognises a dephosphorylated epitope in the middle portion of the protein); TP70 (raised against C-terminal portion of tau); TP007 (raised against the N-terminal portion of tau) and B19 (raised against full-length tau). In addition, dephosphorylation of histologically normal rat or human brain tissue failed to induce Tau 1 immunoreactivity in glial cells, suggesting that the lack of Tau 1 immunoreactivity in these cells is not due to masking of the protein by phosphorylation. In support of this TP70, TP007 and B19 antibodies, which detect tau independent of its phosphorylation state, also failed to detect tau in glial cells in normal rat or human brain tissue. These results suggest that tau may be absent from glial cells in histologically normal human and rat brain tissue, or

may reflect very low levels of tau within mature glia *in vivo* which are undetectable using standard immunohistochemical techniques. This is in contrast to a previous study which reported the presence of tau immunoreactivity in glial cells and neurons (Migheli, et. al. 1988). In addition, Tau 1 immunoreactivity was detected in perineuronal oligodendrocytes and astrocytes but not intrafasicular oligodendrocytes in rat brain following enzymatic dephosphorylation of the tissue (Papazosomenos and Binder, 1987). However, in agreement with the present study, Lopresti, et. al. (1995) failed to detect oligodendrocytes in normal rat brain using Tau 1 however did detect tau in intrafasicular oligodendrocytes using Tau 5, an antibody raised specifically towards glial tau. The possibility that tau present in intrafasicular oligodendrocytes in normal brain may be folded in a particular conformation within the oligodendrocyte thus preventing antibody binding cannot be ruled out. However, it is difficult to envisage that epitopes located in the middle of the protein and at the N- and C- terminals of the protein would all be masked under normal conditions and all uncovered following injury. Furthermore the immunostaining detected using the polyclonal antibodies TP70 and TP007 raised against synthetic fragments of tau is unlikely to be altered by conformational change in the protein.

### 4.1.2 Tau immunoreactivity in oligodendrocytes following acute brain injury

Due to the vast amount of evidence suggesting that tau is a neuron-specific protein and the inability of the tau antibodies used throughout this thesis to detect tau in glial cells in histologically normal brain tissue, the most intriguing and novel finding of this thesis was the induction of tau immunoreactivity in small cells with the morphological appearance of glia following acute brain injury. Double label immunohistochemistry, utilising GFAP as a cellular marker for astrocytes and transferrin as a cellular marker for oligodendrocytes, identified these tau-positive glial cells as oligodendrocytes. Tau-positive oligodendrocytes were detected in all animal models of acute brain injury and in post-mortem brain tissue from patients who died following a stroke or head injury. These tau-positive cells were found located in both white and grey matter depending on the location of the brain injury. In white matter these cells were arranged in rows with the appearance of intrafasicualr oligodendrocytes, while those in grey matter were found located around neurons and were therefore assumed to be perineuronal oligodendrocytes. Tau immunostaining was observed with antibodies directed towards full length tau, and the N- and C-terminals of the protein suggesting the accumulation of full-length tau protein within these cells. Tau-positive oligodendrocytes were also detected using the Tau 1 antibody, showing that tau present in these cells following injury was dephosphorylated at the Tau 1 epitope. It is possible that injury to oligodendrocytes may result in a conformational change in tau which results in increased antigenicity, however as discussed above it is unlikely that all the epitopes

examined in this study would be masked and unmasked equally before and after injury. Together these findings do not support the complete absence of tau in intrafasicular or perineuronal oligodendrocytes within the adult rat and human brain. In addition, to the evidence described above showing tau within oligodendrocytes in normal rat brain tissue, glial inclusions characteristic of chronic neurodegenerative conditions such as MSA, PSP and PD (Abe, et. al. 1992; Iwatsubo, et. al. 1994; Nishimura, et. al. 1992; Yamada, et. al. 1992) have been shown to stain positive for tau. Tau present in these cells can be detected with antibodies directed towards PHF tau, suggesting that in addition to neurons, tangle formation may also occur in glial cells during chronic progressive degeneration of the brain.

Although oligodendrocytes contain abundant microtubules and would therefore be expected to contain many MAPs, in contrast to tau, neither MAP2 nor MAP5 immunoreactivity was detected in oligodendrocytes before or after acute brain injury in human or rat brain tissue. This is in contrast to previous studies which report the presence of both MAP2 and MAP5 immunoreactivity in oligodendrocytes (Fischer, et. al. 1990; Ulloa, et. al. 1994b; Vouyiouklis and Brophy, 1995). The possibility that oligodendrocytes *in situ* express levels of these proteins below the detection limits of standard immunohistochemical techniques cannot be ruled out. However, mRNA for MAP2 and MAP5 has been located only within neurons *in vivo* (Garner, et. al. 1988; Landry, et. al. 1994; Papadrikopoalou, et. al. 1986; Tucker, et. al. 1989) suggesting that glial cells *in situ* do not express these proteins. These studies highlight the caution that should be exercised when extrapolating events occurring *in vitro* to those occurring *in vivo* where the cellular milieu is more complex.

### 4.1.3 Tau in oligodendrocyte cell cultures

Further supporting the presence of tau in non-neuronal cells, the results presented in this thesis show the presence of tau in pure oligodendrocyte cultures derived from an O-2A cell line. In the adult rat brain, tau protein comprises six isoforms produced by alternative splicing of a single mRNA transcript. Similarly, tau present in the oligodendrocyte cultures also comprised several isoforms. This finding is in agreement with those of Lopresti, et. al. (1995) who also showed the presence of multiple tau isoforms in primary oligodendrocyte cultures. Immunoblotting with TP70 detected 3 major bands ranging from 40-66kDa. In contrast Tau 1 detected 5 major bands within a similar molecular weight range, however failed to detect the highest molecular weight isoform detected with TP70. In addition to the multiple isoforms reported here, Lopresti, et. al. (1995) also showed the presence of a higher molecular weight band of approximately 80kDa. The distinct pattern of isoform detection with Tau 1 and TP70 suggests that, as with neuronal tau, isoforms present in oligodendrocytes are modified by phosphorylation. Together these results confirm the

presence of tau in oligodendrocytes maintained in normal culture conditions. To ascertain whether these bands represent an oligodendrocyte specific isoform pattern or whether each band represents differentially phosphorylated forms of a single isoform would require isolation and characterisation of the tau protein and characterisation of the tau transcripts present within these cells.

In contrast to oligodendrocytes present in the normal rat CNS, tau as detected with Tau 1 and TP70 was present within oligodendrocytes *in vitro* maintained in normal growth conditions. In addition, the distribution of tau was different in oligodendrocyte cultures as compared to those *in situ* following acute brain injury. In oligodendrocytes resident within adult rat brain, tau immunoreactivity was located only within the cell body, no staining was present in the cellular processes. This is probably not surprising however due to the intricate architecture of the mature CNS; oligodendrocyte processes are not readily detected *in situ* at the light microscope level. *In vitro* however TP70 and Tau 1 immunoreactivity was detected both within the cytoplasm of the oligodendrocyte cell body and the cellular processes. The possibility that discrepancies between *in vivo* and *in vitro* studies is due to the accesability of the tau epitopes to the antibodies cannot be ruled out.

Detection of tau with TP70 and to a lesser extent Tau 1, suggests the presence of full-length tau protein in these cells under normal culture conditions. It should be noted that although oligodendrocytes were detected with Tau 1, the intensity of this staining was far less than that of TP70, suggesting that a proportion of tau present in these cells is phosphorylated at the Tau 1 epitope. Immunostaining with Tau 1 and TP70 was continuous along the length of the oligodendrocyte processes. This contrasts with the findings of Lopresti, et. al. (1995) who reported discontinuous staining within the cellular processes. In their studies, tau staining was segmental, present at the tips of the processes and in the membrane expansions present at the end of each process. It is possible to speculate that discrepancies between these 2 studies may reflect differential stages of maturity of the cultures used in the 2 studies, since cells used in the investigations carried out in this thesis did not show any membrane expansions present in their cultures. Alternatively this may reflect species differences since cultures used throughout this thesis were derived from rat tissue while those employed by Lopresti, et. al. 1995 were derived from ovine brain. In contrast to these 2 studies Vouyiouklis and Brophy (1995) failed to show the presence of tau in rat primary oligodendrocyte cultures using the monoclonal antibody Tau 2 directed towards full-length tau. This may be partly explained by the poor cross-reactivity of this antibody with rat tissue (Binder, et. al. 1985).

The apparent lack of tau immunoreactivity in oligodendrocytes *in situ* may reflect levels of protein in these cells which are below the limits of detection using standard immunohistochemical techniques, however tau protein may be more abundant in oligodendrocyte cultures than those present *in vivo*. Cell cultures are derived from immature tissue and, although these cells were grown for 5 days and showed immunological characteristics of mature oligodendrocytes, it is possible that they may still be undergoing development. Oligodendrocytes have to extend multiple processes required for the effective myelination of axons and in addition, proteins required for myelin formation have to be transported to the site of myelinogenesis. There is a general consensus that the oligodendrocyte cytoskeleton is involved in oligodendrocyte process extension and myelin assembly (Brophy, et. al. 1993). Tau has been shown to be involved in process extension in neurons (Cáceres and Kosik, 1990; Cáceres, et. al. 1991) and presuming that tau has similar functional properties in both neurons and oligodendrocytes, tau may also play a key role in process extension in oligodendrocytes. Supporting this theory, the tips of growing oligodendrocyte processes, like neuronal growth cones are known to be actin rich (Kachar, et. al. 1986) and previous studies have shown that tau binds to actin *in vitro* (Seldon and Pollard, 1983). Taken together this data supports a role for tau in oligodendrocytes similar to that proposed for neuronal tau in the growth cones of cerebellar neurons. That is, mediating interactions between elements of the growth cone cytoskeleton, therefore maintaining its structural organization (DiTella, et. al. 1994). It is possible therefore that until the extension of processes and myelination is established, oligodendrocytes may express high levels of tau protein. Therefore, oligodendrocytes present in culture, although expressing markers of mature oligodendrocytes, may still be extending processes and thus contain high levels of tau protein. In contrast, fully developed oligodendrocytes present within the adult CNS may lack the necessity for high levels of tau protein.

The differential distribution of tau present in oligodendrocytes *in vitro* and *in vivo* highlight the care that should be exercised when extrapolating findings *in vitro* to events occurring within the adult CNS where the cellular milieu is more complex. To ascertain whether tau present in these cells is similar to that present in neurons requires further characterisation, however together the results presented in this thesis strongly indicate that tau is present in oligodendrocytes and therefore can no longer be viewed as a neuron-specific protein.

## 4.2 The induction of tau immunoreactivity in oligodendrocytes is a rapid response to acute brain injury

The results presented in this thesis provide compelling evidence that the induction of tau immunoreactivity in oligodendrocytes is a rapid response of these cells to acute brain injury. The first study in this thesis, showed the presence of tau-positive oligodendrocytes 4 h following intracortical perfusion of 1M monosodium glutamate both within the resulting cortical lesion and in the subcortical white matter immediately underlying this area. The presence of tau-positive oligodendrocytes in human post-mortem tissue from patients who died within 2 h following head injury confirmed that the induction of tau immunoreactivity in oligodendrocytes was not a phenomenum characteristic of rat brain, but also occurred rapidly in human brain. Tau-positive cells were detected within and immediately surrounding areas of tissue shown by decreased H&E staining to be damaged by the injury. Following severe head injury in the human, the proportion of tau-positive cells detected increased with survival time and it is possible to speculate that this pattern may reflect the evolution of brain damage over time following injury. The detection of tau-positive oligodendrocytes in post-mortem brain tissue of patients who died 23 h following head injury and several weeks following a stroke suggests that the induction of tau immunoreactivity in these glial cells is maintained over long periods of time. In order to determine just how rapidly the presence of tau-positive oligodendrocytes could be detected, animals were killed 20, 40 or 80 min following MCAO and the brains processed for tau immunohistochemistry. Twenty minutes following MCAO the number of tau-positive oligodendrocytes / $mm^2$ subcortical white matter was not significantly different in the ipsilateral hemisphere compared to the contralateral hemisphere. However, 40 and 80 min following MCAO the number of tau-positive oligodendrocytes / $mm^2$ subcortical white matter in the ischaemic hemisphere, as detected with all three antibodies : Tau 1, TP70 and TP007, was increased compared to the contralateral hemisphere. Tau-positive oligodendrocytes were located predominantly within the subcortical white matter ipsilateral to the occluded MCA where they were found aligned in rows with the appearance of intrafasicular oligodendrocytes. In addition tau-positive perineuronal oligodendrocytes were also found scattered throughout the caudate nucleus and cortex within the area of ischaemic tissue as defined by decreased MAP2 immunostaining. Thus, tau immunostaining in oligodendrocytes increased within 40 min of the ischaemic challenge, indicating that these glial cells, like neurons, have the ability to respond rapidly to injury.

Proteins coded by proto-oncogenes such as *c-fos,* act to carry cytoplasmic signals to the nucleus where they interact with the DNA enhancing the expression of several genes (Morgan and Curran, 1989; 1991). *C-fos* mRNA expression has been shown to increase

rapidly within neurons following ischaemic brain injury in the rat (Abe and Kogure, 1993; Hsu, et. al. 1993). Increased expression of *c-fos* and other immediate early genes following brain injury is thought to be involved in the ability of neurons to survive following injury (Magnusson and Wieloch, 1989), however, the real significance of this reponse is unknown. Since tau immunoreactivity in oligodendrocytes is detected rapidly following injury, it is possible to speculate that this may be part of a "stress" response of these cells to brain injury. *C-fos* has recently been shown to be induced in oligodendrocytes progenitor cells *in vitro* following exposure to glutamate (Liu and Almazan, 1995), suggesting that increased *c-fos* may occur in response to injury in these cells. The distribution of *c-fos* immunoreactivity in oligodendrocytes was therefore examined in the rat brain following MCAO this gene product was also increased in these oligodendrocytes following injury *in vivo*. In contrast to increased *c-fos* levels seen in neurons 20 min after MCAO, and in oligodendrocyte progenitor cells in vitro, *c-fos* was undetectable within oligodendrocytes *in situ* before or after MCAO. Together these results suggest that acute brain injury in the rat specifically alters tau protein in oligodendrocytes and the alteration is not due to a generalised "stress" response within these cells.

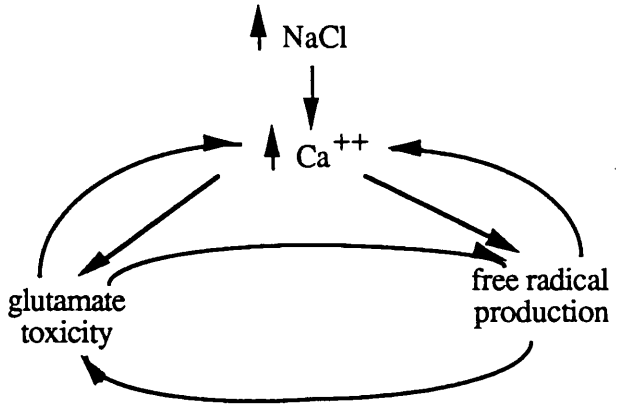
## 4.3 Mechanisms underlying the induction of tau immunoreactivity in oligodendrocytes

### 4.3.1 Glutamate toxicity *in vivo* induces tau immunoreactivity in oligodendrocytes

The *in vivo* model of glutamate toxicity used in this study has been well characterised previously (Landolt, et. al. 1993; Fujisawa, et. al. 1993; 1996) and provides a means of studying the effects of glutamate toxicity without the interference of factors such as reduced blood flow, present in models of cerebral ischaemia. In the *in vivo* study described in section 3.1, 1M monosodium glutamate was perfused through the dialysis probe, at a concentration far in excess of those used in previous *in vitro* studies (μM range). However, due to the dynamics of the dialysis membrane, only approximately 10% of the solution being perfused reaches the tissue where glutamate uptake into astrocytes (Hertz, et. al. 1992; Schousboe, et. al. 1988) further reduces the concentration of extracellular glutamate remaining in the tissue. The extracellular concentration of glutamate decreases further in relation to the distance from the probe as the glutamate diffuses through the tissue. The extracellular concentration of glutamate reaching the corpus callosum is therefore estimated to be within the μM-mM range.

Tau immunoreactivity was detected in intrafasicular and peri-neuronal oligodendrocytes within the subcortical white matter and the cortical lesion respectively, following

intracortical perfusion of 1M monosodium glutamate, suggesting that glutamate may be involved in the accumulation of tau in oligodendrocytes following acute brain injury. Monosodium glutamate was chosen to avoid the use of glutamic acid which proves problematic when maintaining a neutral pH and 1M NaCl was therefore the control solution for this study. The ability of NaCl perfusion to induce tau immunoreactivity within oligodendrocytes however, suggests that mechanisms other than glutamate toxicity may be involved in this response. It is possible to speculate that the mechanisms underlying tau accumulation in oligodendrocytes involves the presence of excessive extracellular $Na^+$. Glutamate excitotoxicity to neurons has been shown to consist of both a NaCl component and a $Ca^{++}$ component (Dessi, et. al. 1994). Influx of NaCl results in neuronal swelling and can induce neuronal degeneration without concurrent influx of $Ca^{++}$. In addition, increased extracellular sodium exacerbates the damage produced by glutamate, possibly through the disturbance of the $Na^+/K^+$ glutamate transporter. Cells from the oligodendrocyte lineage have been shown to express many ion channels *in vitro* including two types of voltage dependant $Ca^{++}$ channels (Blankenfield, et. al. 1992; Verkhratsky, et al. 1990), and $Na^+$ channels (for review see Barres, et. al. 1990). In light of this it is possible to envisage that high extracellular concentrations of $Na^+$ would result in $Na^+$ influx through $Na^+/K^+$ channels, resulting in membrane depolarisation. Such depolarisation of the membrane may lead to the initiation of $Ca^{++}$ influx through voltage gated $Ca^{++}$ channels and through the mobilisation of intracellular $Ca^{++}$ stores. Moreover, it is possible that glutamate toxicity in oligodendrocytes may also consist of both a NaCl and a $Ca^{++}$ component, since glutamate and the non-NMDA receptor agonists, kainate and AMPA have been shown to cause an influx of $Na^+$ in oligodendrocyte cultures (Borges and Kettenman, 1995). Furthermore glutamate toxicity in oligodendrocytes *in vitro* has also been shown to be $Ca^{++}$ dependent (Yoshioka, et. al. 1995). Disruption of $Ca^{++}$ homeostasis is thought to be involved in the formation of free radicals (Siesjö, et. al. 1989). Once free radicals are formed these cause further disruption of calcium homeostasis and therefore a vicious circle continues. It is possible to speculate therefore that free radical mediated mechanisms initiated through increased intracellular $Ca^{++}$ levels, whether through glutamate receptor activation or excess $Na^+$ ions, may be involved in the induction of tau immunoreactivity in oligodendrocytes as outlined below.




In support of this theory oligodendrocytes have been shown to be particularly susceptible to free radical damage (Kim and Kim, 1991; Oka, et. al. 1993; Thorburne and Juurlink, 1996). Moreover, in the present studies pre-treatment of animals with the spin trap agent PBN significantly reduced the number of tau-positive oligodendrocytes / $mm^2$ present in the subcortical white matter following focal cerebral ischaemia. This will be discussed more extensively in section 4.3.3.

### 4.3.2 Increased Tau 1 immunoreactivity in oligodendrocytes is a direct response of these cells to cytotoxic injury

Experiments employing pure oligodendrocyte cultures provided evidence that changes in tau occurring in oligodendrocytes following cytotoxic injury are induced through a direct effect on oligodendrocytes and is not mediated through neuronal degeneration. Electron microscopy showed oligodendrocytes did not show significant signs of degeneration following *in vivo* perfusion of 1M monosodium glutamate or NaCl. The *in vitro* study was therefore designed to treat the cells with a sublethal dose of monosodium glutamate and NaCl. Exposure to 100μM glutamate over 24 h has previously been reported to be sublethal to oligodendrocyte cultures (Oka, et. al. 1993). In the present study oligodendrocyte cultures were exposed to 10μM, 100μM and 1mM monosodium glutamate for 4 h. Exposure of oligodendrocytes to 10μM monosodium glutamate failed to induce changes in tau immunoreactivity within these cells, in contrast tau immunoreactivity was increased following exposure to 100 or 1mM monosodium glutamate. However morphological signs of degeneration were detected oligodendrocyte cultures exposed to 1mM monosodium glutamate, therefore 100μM monosodium glutamate was selected for this study.

Tau 1 immunoreactivity was increased in the cytoplasm of both the oligodendrocyte perikarya and processes following treatment of the cells with 100μM monosodium glutamate or 100μM NaCl for 4 h. In contrast to the *in vivo* study however, TP70 immunoreactivity remained largely unchanged following exposure to 100μM monosodium

glutamate or NaCl, although staining within the cellular processes became slightly more prominent with TP70. Dephosphorylation of cultures maintained in Sato's medium resulted in a similar intensity of Tau 1 immunoreactivity present following treatment with 100μM monosodium glutamate or NaCl. Together these results suggest that rather than accumulation of tau protein within these cells as seen *in situ*, increased Tau 1 immunoreactivity reflects dephosphorylation of existing protein in response to glutamate or NaCl exposure. However the possibility that increased TP70 levels were not detected due to the high level of immunoreactivity present in control cells cannot be ruled out.

These results show that alterations to tau in oligodendrocytes following exposure to monosodium glutamate or NaCl is a direct response of these cells to injury. This suggests that the accumulation of tau in oligodendrocytes *in vivo* following acute brain injury is is not a consequence of neuronal degeneration. However they do highlight again the caution that must be exercised when relating events occurring *in vitro* to those occurring *in vivo* where the cellular milieu is more complex.

### 4.3.3 The spin trap agent PBN reduces the number of tau-positive oligodendrocytes following focal cerebral ischaemia in the rat

The presence of tau-positive oligodendrocytes following exposure to both glutamate and NaCl suggests that additional mechanisms other than glutamate receptor activation are involved in the initiation of this response. Both glutamate toxicity and free radical mediated mechanisms have been implicated in the evolution of ischaemic brain damage. Oligodendrocytes have been shown *in vitro* to be susceptible to both glutamate and free radical mediated toxicity therefore permanent MCAO in the rat was used to determine if glutamate receptor activation or free radical mediated events are involved in the mechanisms underlying the accumulation of tau in oligodendrocytes following ischaemic brain injury.

Pre-treatment of animals with the spin trap agent PBN prior to MCAO significantly reduced the number of tau-positive oligodendrocytes / $mm^2$ in the subcortical white matter ipsilateral to the occluded MCA as compared to untreated animals 40 min after the induction of focal cerebral ischaemia. This indicates that the initiating stimulus for the response involves events mediated by free radicals. Oligodendrocytes are thought to be particularly susceptible to free radical damage due to low levels of the free radical scavenger glutathione and high levels of iron which is a catalyst for free radical production (Thorburne and Juurlink, 1996). Primary cultures of bovine oligodendrocytes were shown to be susceptible to oxygen radicals derived from glucose/glucose oxidase and hypoxanthine/xanthine oxidase reactions (Kim and Kim, 1991). In that study, cytotoxicity was measured by tryptan blue exclusion and lactate dehydrogenase leakage, and it showed that oxygen radicals induce significant

membrane damage to oligodendrocytes within 2 h of exposure. In addition, Oka, et. al. (1993) showed in rat primary oligodendrocyte cultures that glutamate induced cell death was mediated, not through glutamate receptor activation, but through free radical mediated mechanisms. Excessive extracellular glutamate concentrations led to the efflux of cystine from these cells through the actions of the glutamate/cystine transporter which in turn resulted in glutathione depletion. Glutathione is an important protective agent against oxidative stress (for review see Bast et. al. 1991) and its depletion therefore renders the cell vulnerable to oxidative stress. PBN is a nitrone compound which reacts with many types of free radicals resulting in the formation of the stable nitroxide radical adducts (Oliver, et. al. 1990). Glutamate toxicity and free radical generation have been shown to be interdependent, many studies demonstrating that glutamate toxicity can be markedly reduced by treatment with free radical scavenger systems both *in vitro* (Bondy and Lee, 1993; Dykens, et. al. 1987; Murphy, et. al. 1990; Miyamoto, et. al. 1989; Nakao, et. al. 1996) and *in vivo* (Nakao, et. al. 1996; Schulz et. al. 1995). Extracellular glutamate concentrations peak at 40 min following MCAO in the rat (Butcher, et. al. 1990) and it is possible to speculate therefore that treatment with PBN protects oligodendrocytes indirectly from glutamate toxicity and directly from free radical mediated damage resulting from ischaemic injury and thus prevents accumulation of tau within these cells.

In contrast to PBN neither the NMDA receptor antagonist MK801 nor the AMPA receptor antagonist NBQX prevented increased tau immunoreactivity in oligodendrocytes following 40 min cerebral ischaemia further indicating that glutamate receptor activation is not involved in the response. However, both primary oligodendrocyte cultures and immortalised cultures derived from rat brain express non-NMDA receptor subtypes (Patneau, et. al. 1994; Puchalski, et. al. 1994; Yoshioka, et. al. 1995). Moreover, damage to these cells was mediated by non-NMDA receptor activation. Thus in our *in vivo* experimental paradigm, while it is possible that activation of AMPA receptors by excessive extracellular glutamate concentrations may damage oligodendrocytes, this signalling pathway does not appear to be involved in the tau protein response. Alternatively, the rapid desensitisation of AMPA receptors in oligodendrocytes in response to glutamate (Yoshioka, et. al. 1995) may have masked any effect of receptor blockade by NBQX. Finally although the selection of the drug doses were based on those previously shown to reduce infarct volume following permanent focal cerebral ischaemia in the rat (Cao and Phillis, 1994; Gill, et. al., 1992; Park, et. al. 1988), the possibility that the concentration of NBQX used in this study may be too low to prevent ischaemia induced accumulation of tau in oligodendrocytes cannot be ruled out. Mature oligodendrocytes have been reported to contain non-NMDA receptors, although mRNA for NMDA receptors was not detected (Patneau, 1994; Puchalski, et. al. 1994; Yoshioka, et. al 1995) and therefore it is perhaps not surprising that MK801 failed to

prevent increased tau immunoreactivity in these cells following ischaemic injury. However, the mean value of tau-positive oligodendrocytes present in both the ipsilateral and contralateral white matter was greater following MK-801 treatment compared to controls. This suggests that MK-801 alone may be able to induce increased tau in oligodendrocytes. In the absence of a direct effect on oligodendrocytes themselves, increased tau immunoreactivity within these cells may arise as an indirect consequence of neuronal changes both in proximity and distant to ischaemia. MK-801 and other NMDA ion channel blockers induce a range of marked neurochemical alterations within neurons which include glucose utilisation and heat shock protein gene expression (Kurumaji et. al., 1988; Sharp, et. al. 1992). In light of the growing literature on neuronal-glia communication it may be envisaged that MK-801-induced changes in neuronal activity in turn induce responses in associated oligodendrocytes. Alterations in the cytoskeletal protein tau may represent such a response. Together the results of these studies show that the induction of tau immunoreactivity in oligodendrocytes is initiated through free radical mediated mechanisms.

## 4.4 Functional significance of tau in oligodendrocytes following injury

### 4.4.1 Protective response ?

At this stage the functional significance of tau in oligodendrocytes following acute brain injury is unknown. If tau is a neuron-specific protein it is intriguing that oligodendrocytes express a neuronal protein following injury. It is possible to speculate the accumulation of tau in oligodendrocytes following injury may be part of a protective response in that dephosphorylated tau is able to bind to and stabilise microtubules (Brandt and Lee, 1994; Kosik, 1993; Trinczek, et. al. 1995). Increased tau immunoreactivity was detected with antibodies directed towards full length protein and both the N- and C-terminals of the protein suggesting the accumulation of full length protein within these cells following acute brain injury both in the rat and the human. *In situ* hybridisation studies were therefore carried out to determine if this increased immunoreactivity in oligodendrocytes following ischaemic brain injury reflects increased *de novo* synthesis of tau.

*In situ* hybridisation using a $^{35}S$-labelled riboprobe synthesized from a 1031bp fragment of the coding region of adult rat tau cDNA revealed that tau mRNA was predominantly located within the neurons of the cerebral cortex in rat brain contralateral to the occluded MCA. A low level of message was detected in white matter tracts, however the cellular localisation of this signal was difficult to interpret. Control experiments using a sense riboprobe generated from the same coding region of tau cDNA revealed a similar level of signal within the subcortical white matter, suggesting that white matter signal reflects non-specific

hybridisation of the riboprobe. This is in agreement with previous studies which detected tau mRNA in neurons, however failed to detect tau mRNA in glial cells (Goedert, et. al. 1989; Kosik, et. al. 1989). Following 40, 120 or 240 min MCAO in the rat, no significant changes in the pattern of tau mRNA distribution was detected ipsilateral to the occluded MCA. This suggests that increased tau immunoreactivity within oligodendrocytes following ischaemic brain injury does not reflect increased *de novo* synthesis of the protein. This is maybe not surprising considering the rapidity of tau accumulation in oligodendrocytes following injury. However the possibility that our detection system was not sensitive enough to detect small increases in mRNA levels within these cells cannot be ruled out.

In addition, it is possible to speculate that mRNA present in oligodendrocytes may differ from that present in neurons. Recently a 2kb and 8kb tau mRNA have been detected in a neuroblastoma cell line and the peripheral nervous system respectively, encoding nuclear tau and big tau, as opposed to the 6kb mRNA originally described for neuronal tau (Couchie, et. al. 1992; Goedert, et. al. 1992; Loomis, et. al. 1990; Wang, et. al. 1993). The riboprobe used in the present study was derived from a 1031bp fragment of the coding region of a tau cDNA encoding the 6kb mRNA of tau which includes exons 2, 3 and 10 (Kosik, et. al. 1989). However, it is unknown what type of tau mRNA is present in oligodendrocytes. Tau cDNA encoding the 8kb mRNA, contains an extra 762bp region encoded by exon 4A situated down stream of exons 2 and 3 which encode the N-terminal inserts (Goedert, et. al. 1992). In addition exon 6 further downstream from exon 4A has also been reported to be expressed in this larger isoform of tau (Couchie, et. al. 1992). The riboprobe generated in the present study encompasses 1031bp of the coding region from the 3' end of the tau cDNA. It is possible to speculate therefore that the expression of exon 4A and // or 6 would reduce the strength of hybridisation of the probe with this area of the mRNA. Signal obtained from weaker hybridisation reactions between the probe and the mRNA therefore may be lost during the stringency washes designed to reduce non-specific hybridisation.

Recently increased ferritin synthesis was reported in oligodendrocytes following 6h hypoxia *in vitro* (Qi and Dawson, 1992, 1994, 1995). In these studies the authors showed that increased protein levels were due to a post-transcriptional modification of protein synthesis. It is possible to speculate therefore that increased tau immunoreactivity within oligodendrocytes following acute brain injury may also represent post-transcriptional modification of protein synthesis, which may account for the rapid appearance of tau in these cells. If increased tau immunoreactivity does reflect a protective response of these

cells to injury, it is possible to envisage that post-transcriptional modification of protein synthesis would result in the rapid increase in tau protein levels required for their protection.

There is growing evidence that the oligodendrocyte cytoskeleton is involved in process extension and the transport of myelin proteins to the site of myelin assembly (Brophy, et. al. 1993). The tips of growing oligodendrocyte processes, like neuronal growth cones are known to be actin rich (Kachar, et. al. 1986) and previous studies have shown that tau binds to actin *in vitro* (Seldon and Pollard, 1983). Taken together this data supports a role for tau in oligodendrocytes similar to that proposed for neuronal tau in the growth cones of cerebellar neurons (DiTella, et. al. 1994). Tau has been shown to be involved in process extension in neurons (Cáceres and Kosik, 1990; Cáceres, et. al. 1991) therefore it is possible to speculate that tau also plays a key role in the extension of oligodendrocyte processes. Oligodendrocytes contain abundant microtubules which have been postulated to be key elements in the transportation of myelin proteins to the site of myelin assembly (Barbarese, et. al. 1991; Brophy, et. al. 1993; Colman, et. al. 1982). Purified MBP can form complexes with tubulin and actin (Modesti and Barra, 1986), and using cell culture both MBP and CNP have been shown to associate with the cytoskeleton of oligodendrocytes (Colman, et. al. 1982; Gillespie, et. al. 1989). Both CNP and MBP are synthesized on free ribosomes and have to be transported into the processes for myelin formation. It is possible to speculate therefore that microtubular networks within these cells are essential for the transportation of the machinery required for myelin formation into the processes. Recently MBP and tau have been shown to co-localize within the cell soma, processes and myelin expansions of oligodendrocyte cultures (Lopresti, et. al. 1995) and tau has also been postulated to play a role in the transportation of MBP mRNA and protein to the site of myelinogenesis (Lopresti et. al. 1995). It is attractive to hypothesise therefore that increased levels of dephosphorylated tau in these cells following injury may act to stabilise existing processes and increase the transportation of myelin components to sites where myelin sheaths have been damaged.

Petito, et. al. (1985) showed that medium-light oligodendrocytes increased in size following permanent focal cerebral ischaemia in the rat (Petito, et. al. 1985). These cells showed increased microtubule profiles and accounted for approximately 50% of all glial cells within 2 hr of injury, however the number present decreased to 6.5% by 3 h. Petito, et. al. suggest that cell loss may represent a transition of medium-light oligodendrocytes to intermediate glia which have some characteristics of both oligodendrocytes and astrocytes. This may represent an "active" oligodendrocyte. Since tau is postulated to be involved in process expansion and therefore cell morphology, it is possible to speculate that increased tau levels within these cells following injury may be involved in the morphological changes

described following ischaemia and may therefore represent a marker for "active" oligodendrocytes. In addition oligodendrocytes have been shown to proliferate 24 hr following trauma in the mouse (Ludwin, 1984). It could be envisaged that the accumulation of tau following acute brain injury may be involved in the proliferation of oligodendrocytes, which would require the extension of new cellular processes. Tau-positive oligodendrocytes found following acute brain injury may thus represent cells about to undergo or are undergoing proliferation.

### 4.4.2 Degenerative response ?

In contrast to a protective role, the accumulation of tau in oligodendrocytes may represent an early stage of cellular degeneration. Tau has recently been reported to be present in glial inclusions characteristic of chronic neurodegenerative conditions such as MSA, PSP and PD (Abe, et. al. 1992; Iwatsubo, et. al. 1994; Nishimura, et. al. 1992; Yamada, et. al. 1992). Tau present in these glial inclusions can be detected using antibodies directed towards PHF tau, suggesting that tau in these glial inclusions is abnormally phosphorylated. The detection of tau immunoreactivity in oligodendrocytes present in human post-mortem brain tissue from patients who died following a stroke or head injury indicates that the accumulation of tau occurs in both acute and chronic neurodegenerative conditions. In contrast to tau present in glial inclusions (Abe, et. al. 1992; Iwatsubo, et. al. 1994; Kato, et. al. 1991; Nishimura et. al., 1992; Yamada , 1992; Yamada and McGeer, 1990) however, tau present in oligodendrocytes early following acute brain injury is dephosphorylated at the Tau 1 epitope. In one patient who survived 23 h following severe head injury, and one patient surviving several weeks following stroke, TP70 and TP007 immunoreactivity was found accumulated to one side of oligodendrocyte cytoplasm with the morphological appearance similar to the tau-positive glial inclusions previously described in MSA (Abe, et. al. 1992; Iwatsubo, et. al. 1994; Kato, et. al. 1991). These cells were detected within areas of tissue severely affected by head injury and within infarcted tissue of the stroke patient, as determined by decreased H&E staining. Together these results suggest that accumulation of tau in oligodendrocytes is an acute response of these cells to injury, however the formation of cytoplasmic inclusions may occur over time. However such tau-positive inclusions were not detected in tissue obtained from patients dying up to 12 days following a stroke, suggesting that factors other than survival time following injury may be involved in the formation of these tau positive accumulations. Tau present in the cytoplasm of oligodendrocytes with a morphology similar to glial inclusions, were not detected with Tau 1 and in addition the number of tau-positive oligodendrocytes detected with Tau 1 was decreased 23 h following head injury, as compared to earlier survival times, within these areas of tissue where these inclusion like accumulations were detected, suggesting that tau within these cells may be phosphorylated at this epitope. Failure of AD2, an antibody

directed towards PHF tau, to detect tau in oligodendrocytes showed that tau present within these cells, in contrast to PHF tau, was not phosphorylated at the AD2 epitope. The possibility that tau is phosphorylated at other sites similar to PHF tau cannot be ruled out. It is attractive to speculate therefore that tau accumulation in oligodendrocytes may be a rapid response of these cells to acute injury, however changes in antigenicity similar to those of PHF tau may occur over longer periods of time. NFT formation is thought to lead to neuronal degeneration in a variety of chronic degenerative disorders such as AD, PD and PSP, it is possible to speculate that tangle formation in oligodendrocytes will also lead to the subsequent degeneration of these cells.

## 4.5 The neuronal distribution of MAPs following acute brain injury

### 4.5.1 Tau

Following intracortical perfusion of glutamate, axonal Tau 1 staining was reduced while perikaryal staining was increased within the core of the lesion. This *in vivo* result concords with previous *in vitro* studies which showed increased Tau 1 immunoreactivity within cultured neuronal perikarya following glutamate exposure (Bigot and Hunt, 1990; Davis, et. al. 1995; Pizzi, et. al. 1993; 1994; 1995). Studies using antisense oligonucleotides suggested that increased tau immunoreactivity *in vitro* reflected increased synthesis of the protein supported by the increased Tau 2 staining which recognises full length tau protein (Pizzi, et. al. 1993; 1994; 1995). This is in contrast to the results presented in this thesis, which suggests that rather than accumulation of protein, increased Tau 1 immunoreactivity reflects dephosphorylation of existing protein in response to glutamate toxicity *in vivo*. In support of this theory AT8 immunoreactivity was lost from neuronal perikarya within the lesioned area which is consistent with dephosphorylation of tau at this epitope. In addition, accumulation of the protein was not indicated with B19, which labels full length tau, or with tau antibodies directed at the N- and C-terminals of the protein.

In contrast to the findings of the present study, other *in vitro* studies have shown the converse; increased AT8 immunoreactivity following glutamate exposure (Sindou, et. al. 1994). Although increased Tau 2 immunoreactivity was detected by Sindou and colleagues they suggested that the accumulation of phosphorylated tau was not due to increased synthesis of the protein (Sindou, et. al. 1992). Discrepancies between different *in vitro* studies may be related to the maturation stage of the neuronal cultures since immature tau has antigenic properties similar to that present in neurofibrillary tangles. It is interesting to note that in experiments employing neuronal cultures grown for 8 days or less (Mattson, et. al. 1990; Sautiere, et. al. 1992; Sindou, et. al. 1994) glutamate was proposed to induce

changes in tau similar to those found in neurofibrillary tangles. However in cultures grown for more than 8 days tau was dephosphorylated at the Tau 1 epitope in response to glutamate (Bigot and Hunt, 1990; Pizzi, et. al. 1993; 1994; 1995). It is also interesting to note that these investigators showed using antisense oligonucleotides, that increased tau immunoreactivity within these older neuronal cultures did represent increased synthesis of the protein (Pizzi, et. al. 1993; 1994; 1995). Such discrepancies between *in vitro* and *in vivo* again highlight the care that should be taken when relating observations made in cell cultures to pathophysiological events proposed to occur in the intact CNS, where the cellular milieu is more complex.

Monosodium glutamate was used in the present study and it is possible therefore that some of the changes in tau immunoreactivity observed may be due to the presence of excessive sodium ions. NaCl however failed to induce increased Tau 1 immunoreactivity in perikarya seen following glutamate perfusion, indicating that glutamate was responsible for the altered pattern of Tau 1 immunoreactivity. However, NaCl perfusion resulted in decreased tau immunoreactivity within the neuropil, suggesting that this pattern of immunoreactivity also seen following glutamate perfusion may result from excessive levels of sodium ions. In addition, decreased B19, TP007 and TP70 was also detected in perikarya following NaCl perfusion *in vivo* further implicating a role for $Na^+$ ions in the disruption of cytoskeletal proteins.

Increased Tau 1 immunostaining within neuronal perikarya of the cortex and caudate nucleus was detected within 20 min following MCAO in the rat. 40 min following the onset of cerebral ischaemia, TP70 and TP007 staining was decreased within neuronal perikarya of the cortex and caudate nucleus, ipsilateral to the occluded MCA. Together these results suggest that neuronal tau undergoes rapid dephosphorylation, which is closely followed by loss of C- and N- terminal immunoreactivity suggesting degradation of the protein, both of which may be mediated through glutamate excitotoxicity. In agreement with this, the *in situ* hybridisation studies carried out showed that tau mRNA was decreased early following ischaemic brain injury, which implies that tau mRNA is degraded following focal cerebral ischaemia. This is in agreement with a previous study showing increased Tau 1 and decreased TP70 immunoreactivity within neuronal perikarya of the cortex and caudate nucleus 2 h and 6 h following permanent MCAO in the rat (Dewar and Dawson, 1995). These results suggest that tau undergoes rapid alteration following MCAO in the rat and that these alterations are maintained up to 6 h or more following the induction of cerebral ischaemia. However in axons, while Tau 1 immunoreactivity was reduced by both glutamate and sodium chloride perfusion *in vivo*, Tau 1 immunoreactivity was increased in the axons of the cortex and caudate nucleus ipsilateral to the occluded MCA 40 min

following the induction of cerebral ischaemia. A similar increase in Tau 1 staining of axons was reported in permanent MCAO in the rat by Dewar and Dawson, (1995). Together this suggests that mechanisms other than those involving high extracellular glutamate and / or $Na^+$ levels may be involved in the changes in tau occurring in the axonal compartment following ischaemic brain damage.

### 4.5.2 MAP2 and MAP5

Both MAP2 and MAP5 immunoreactivity was increased in neuronal perikarya and decreased in neuropil within the lesion resulting from the intracortical perfusion of 1M monosodium glutamate. In contrast to tau which undergoes dephosphorylation and / or degradation, MAP2 and MAP5 appear to undergo redistribution from the neuropil to the perikarya. This is in agreement with previous studies showing a similar redistribution of MAP2 within neurons in culture following exposure to glutamate (Bigot and Hunt, 1990). Similarly, MAP2 and MAP5 immunoreactivity was increased in neuronal perikarya ipsilateral to the occluded MCA 20 min following the induction of cerebral ischaemia in the rat. 40 min following the onset of ischaemia both MAP2 and MAP5 immunoreactivity was decreased in the neuropil of the cortex of the ipsilateral hemisphere, implicating glutamate excitotoxicity in the mechanisms underlying the redistribution of these proteins following focal cerebral ischaemia. In addition, MAP2 immunoreactivity was decreased within both neuronal perikarya and neuropil of the caudate nucleus and clearly delineated the region of ischaemic tissue. This is similar to the findings of Dawson and Hallenbeck, (1996) who reported decreased MAP2 immunoreactivity within neuropil and neuronal perikarya of the caudate nucleus and cortex 1 h following focal cerebral ischaemia in the rat. This study further demonstrates the sensitivity of MAP2 to cerebral ischaemia, highlighting its use as a sensitive marker for ischaemic damage as early as 40 min of MCAO. NaCl perfusion also resulted in reduced MAP2 staining in perikarya and dendrites suggesting that excess $Na^+$ ions may be involved in the loss of MAP2 immunoreactivity following 40 min cerebral ischaemia.

In conclusion the present study demonstrates that neuronal tau, MAP2 and MAP5 undergo marked alteration in their cellular distribution as early as 20 min following MCAO in the rat indicating that the neuronal cytoskeleton is extremely vulnerable to ischaemic brain injury. Glutamate perfusion *in vivo* resulted in many of the alterations of tau, MAP2 and MAP5 described following cerebral ischaemia in the rat; it is possible to speculate therefore that in conditions such as cerebral ischaemia and head injury, where excessive extracellular concentrations of glutamate are present, alterations in cytoskeletal proteins may be involved in the evolution of irreversible brain damage. These results have important implications for the development of neuroprotective strategies, since cytoskeletal abnormalities are detected

within neurons as early as 20 min following the induction of cerebral ischaemia which may represent the initial stages of irreversible brain damage.

## 4.6 Oligodendrocyte versus neuronal tau following acute brain injury

Following acute brain injury in the rat and the human, increased tau imunoreactivity was detected in oligodendrocytes using a variety of tau antibodies, suggesting the accumulation of full-length protein within these cells. In contrast, neuronal tau appears to undego dephosphorylation and/or degradation in response to acute brain injury. The accumulation of tau in oligodendrocytes was detected as early as 40 min following MCAO in the rat suggesting that the cytoskeleton within these cells undergoes rapid alteration following acute brain injury. As early as 20 min following MCAO in the rat, increased tau immunoreactivity was detected in neuronal perikarya with Tau 1, however this was not detected with antibodies raised against the N- and C-terminals of the protein. Together these results suggest that in contrast to the accumulation of full-length tau in oligodendrocytes, neuronal tau becomes dephosphorylated and/or degraded following acute brain injury in the rat. Dephosphorylation of tau in neurons was detected as early as 20 min following MCAO in the rat, however tau-positive oligodendrocytes were not observed until 40 min after the induction of cerebral ischaemia. In addition degradation of tau in neurons but not in oligodendrocytes suggests that tau present in neuronal perikarya may be more susceptible to ischaemic brain damage than that in oligodendrocytes.

Dephosphorylated tau has the ability to bind to and stabilise microtubules. It is therefore possible to speculate that increased Tau 1 immunoreactivity detected in both these cell types following acute brain injury is an attempt to try and stabilise the cytoskeletal architecture. The observation that tau may have undergone some degree of proteolysis within neurons may reflect a loss or modification of the C-terminal of the protein which encompasses the microtubule binding domain, dephosphorylated tau present in neurons following acute brain injury may therefore lack the ability to bind to and stabilise microtubules. In contrast, the presence of full length tau within oligodendrocytes following injury suggests that successful microtubule stabilisation may occur within these cells therefore helping to maintain their cytoskeletal integrity. In contrast to the accumulation of MAP2 and MAP5 in neurons following acute brain injury, these proteins were not detected in oligodendrocytes. It is possible to speculate that MAP2 and MAP5 remain unaltered in oligodendrocytes following injury, however the possibility that oligodendrocytes do not contain these proteins cannot be ruled out. Breakdown of the neuronal cytoskeleton is thought to represent a common final pathway of irreversible cell damage following ischaemic brain injury. Neurons are generally considered to be the most vulnerable cells of the CNS to cerebral ischaemia, while

oligodendrocytes are thought to be fairly resistant. The differential sensitivities of the cytoskeleton to cellular injury in these two cell types may help explain their differential sensitivity to acute brain injury.

In addition, the mechanisms underlying the altered distribution of tau in neuronal perikarya and oligodendrocytes appear to be different. Glutamate excitotoxicity appears to mediate the changes in tau occurring within neurons, while free radical mediated mechanisms appear to be responsible for those occurring in oligodendrocytes. Activation of the NMDA receptor and subsequent influx of $Ca^{++}$ is thought to be one of the most important mechanism underlying glutamate mediated neurotoxicity following cerebral ischaemia (see McCulloch, et. al. (1991) for review). In contrast to neurons which contain abundant NMDA receptors, oligodendrocytes only contain mRNA encoding non-NMDA glutamate receptors (for review see Gallo and Russell, 1995). Glutamate release occurs immediately following MCAO (Butcher, et. al. 1990) and is thought to lead to the release of free radicals (Bondy and Lee, 1993; Dykens et. al. 1987; Miyamoto, et. al. 1989; Nakao, et. al. 1996; Schulz, et. al. 1995). It is tempting to speculate therefore that neurons are susceptible to the initial release of glutamate following the onset of ischaemia, however oligodendrocytes which lack the presence of NMDA receptors are more susceptible to the secondary release of free radicals. Indeed oligodendrocytes are thought to be particularly susceptible to free radical damage due to their high levels of iron and low levels of the endogenous antioxidant glutathione (Thorburne and Juurlink, 1996). Further supporting this theory glutamate toxicity has been shown to be mediated not through receptor activation, but through free radical mediated mechanisms in oligodendrocyte cultures (Oka, et. al. 1993; Yonezawa, et. al. 1996).

In conclusion, the results of this thesis demonstrate that tau is present in oligodendrocytes, which undergoes rapid alteration in response to acute brain injury. Alterations in the distribution of microtubule-associated proteins in neurons following injury are thought to represent altered cytoskeletal integrity which may result in irreversible neuronal damage. It is possible to speculate therefore that alterations to tau in oligodendrocytes, following acute brain injury may also represent early stages of cytoskeletal breakdown within these cells. Oligodendrocytes have been until recently considered to be relatively unresponsive to ischaemic brain injury, and neuroprotective agents have been evaluated solely on their ability to reduce neuronal damage. One of the most successful strategies involves the blockade of glutamate receptor activation which was without effect in the reduction of the induction of tau immunoreactivity in oligodendrocytes following focal cerebral ischaemia. The protection of neurons without concurrent protection of glial cells following acute brain injury would lead to delayed neuronal death due to the degeneration of glia rather than complete protection of the brain following injury.

# Appendix

## Growth media

### *SOC medium*

1litre - 20g tryptone, 5g yeast extract, 0.5g NaCl in distilled water. Add 10ml of 250mM KCl and adjust to pH7 using NaOH and sterilise by autoclaving for 20 min at 15lb/in$^2$. Once cooled to <60°C 20ml of filter sterilised, 1M glucose solution was added.

### *Luria-Bertrani (LB) agar indicator plates*

1litre- 20g Tryptone, 5g Yeast extract, 10g NaCl; dissolved in distilled water and adjusted to pH7; 12g agar/L added and the solution sterilised by autoclaving for 20 min at 15lb/in$^2$. Agar was allowed to cool to 50°C and the following added prior to pouring the plates: 0.004% X-gal (5-Bromo-4-chloro-3-indoyl-ß-L-galactoside), 0.012% IPTG (isopropylthio-ß-D-galactoside) and 100µg/ml ampicillin.

### *Terrific Broth*

500ml - 6g Tryptone, 12g Yeast extract, 2ml glycerol, make up to 450ml on distilled water (soln A). 0.17M$KH_2PO_4$ and 0.72M $K_2HPO_4$ in 100ml distilled water (soln. B). Autoclave both solutions separately for 20 min at 15lb/in$^2$, allow to cool and add 50ml of soln B and 100µg/ml ampicillin to soln A.

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## Abstracts

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