THE EFFECT OF TRAUMATIC BRAIN INJURY ON THE NEURONAL CYTOSKELETON AND THE AXOLEMMA.

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CONTENTS

CHAPTER 1 - INTRODUCTION

CHAPTER 2 - MATERIALS AND METHODS

2.1 ACUTE SUBDURAL HAEMATOMA IN THE RAT.

2.2 ACUTE SUBDURAL HAEMATOMA IN HUMAN POST MORTEM TISSUE.

2.3 AXOLEMMAL PERMEABILITY FOLLOWING TRAUMATIC BRAIN INJURY.

CHAPTER 3 - RESULTS

3.1 ALTERATIONS IN THE NEURONAL CYTOSKELETON FOLLOWING ACUTE SUBDURAL HAEMATOMA IN THE RAT.

3.4 ALTERATIONS IN AXOLEMMAL PERMEABILITY FOLLOWING TRAUMATIC BRAIN INJURY.

CHAPTER 4 - DISCUSSION.

4.1 ALTERATIONS IN THE NEURONAL CYTOSKELETON FOLLOWING ACUTE SUBDURAL HAEMATOMA IN THE RAT

LIST OF TABLES

LIST OF FIGURES

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SUMMARY

Head injuries are a major cause of morbidity and mortality in western society. Despite advances in the detection and management of head injury there has been limited improvements in outcome. It is clear that a better understanding of the pathophysiology of traumatic brain injury is essential. Without this scientific basis, the future for patients sustaining severe head injuries remains bleak.

The neuronal cytoskeleton consists of proteins which serve structural and functional roles within the neurone. Microtubules are a major component of the cytoskeleton and are essential for normal neuronal functioning. Specifically, they are intimately involved in the process of fast axoplasmic transport in which proteins and organelles are transported from the neuronal cell body to synapses. Disruption of this process may be important in the pathogenesis of traumatically induced neuronal injury. It is hypothesised that alterations in the cytoskeletal framework may represent a common final pathway in the mediation of traumatic insults in the brain. Therefore, elucidation of the mechanisms involved in this process would provide valuable information which could be used in the design of future therapeutic strategies.

Alterations in the microtubule component of the neuronal cytoskeleton following production of an acute subdural haematoma in the rat.

Immunohistochemistry and Western blot analysis were used to examine specific components of the neuronal cytoskeleton at 30 minutes, 2 hours and 4 hours following production of an acute subural haematoma in the rat. The microtubule associated proteins, MAP 2 and Tau, are preferentially localised to dendrites and axons respectively. β–tubulin is one of the major constituent polypeptides of microtubules and provided a useful marker of microtubules. A series of investigations demonstrated that the microtubule component of the neuronal cytoskeleton was disrupted following induction of an acute subdural haematoma. There was a dramatic loss in MAP 2 immunostaining in the cortex underlying the subdural haematoma within 2 hours of production of the haematoma. However, there was not uniform loss of MAP 2 immunostaining throughout the cortical layers suggesting that certain neurones are less susceptible to the mechanisms leading to neuronal cell death. Specific alterations in Tau-1 and β–tubulin immunoreactivity were demonstrated in both the cerebral cortex and the myelinated fibre tracts of the ipsilateral corpus callosum. This important finding demonstrates that traumatically induced axonal injury may contribute to the pathogenesis of acute subdural haematoma. Quantitative Western blot analysis supported the hypothesis that degradation of the neuronal cytoskeleton occurs following traumatic brain injury. In addition there were alterations in the electrophoretic profile of Tau detected which were consistent with changes in the phosphorylation state of the protein.

To examine the morphological correlate of these alterations, transmission electron microscopy was employed in a group of 4 hour animals. There was clear evidence of disruption of the dendritic and axonal cytoskeletons. Specifically, following induction of an acute subdural haematoma, the dendritic microtubules demonstrated an abnormal helical configuration compared to the normal linear arrangement. Transverse sections from myelinated fibres in the corpus callosum demonstrated loss of microtubules and compaction of neurofilaments.

Overall, this series of investigations supports the central hypothesis that disruption of the neuronal cytoskeleton may represent a common final pathway of neuronal injury.

The role of calpain in traumatically induced cytoskeletal injury.

Calpain is a family of calcium dependent proteolytic enzymes. Calpain activation is believed to occur when intracellular free calcium levels surpass a certain threshold. Substrates for activated calpain include cytoskeletal proteins. Using a polyclonal antibody that recognises calpain mediated breakdown products (BDPs) of spectrin, the distribution and time course of calpain activation following acute subdural haematoma in the rat was characterised. A series of experiments demonstrated that prolonged activation of calpain occurred following induction of a subdural haematoma. Spectrin BDPs were identified predominantly in the hemisphere underlying the haematoma, located primarily in the cortex

which exhibited features of the ischaemic cell process. Calpain mediated spectrin breakdown was detected in dendrites and neuronal perikarya as early as 2 hours after induction of the haematoma. In the ipsilateral white matter, spectrin breakdown was detected in a small proportion of axons subjacent to the cortical region of maximal tissue injury. The results suggest that calpain may play an important role in the pathobiology of traumatic brain injury.

Alterations in the neuronal cytoskeleton following acute subdural haematoma in humans.

Immunohistochemistry was employed to investigate alterations in the neuronal cytoskeleton in fatally injured patients sustaining an acute subdural haematoma. Post-mortem sections from the superior parietal cortex were examined and compared to neurological controls. There was loss of MAP 2 immunolabelling in the cortex underlying the haematoma. Tau-1 immunoreactivity was increased in some neuronal perikarya following acute subdural haematoma. However, the intensity of immunoreactivity in both controls and haematoma cases was poor for reasons which remain unclear. There was an increase in somato-dendritic β−tubulin immunoreactivity in the cortex after acute subdural haematoma. The results from the human post mortem study are similar to those obtained in the series of experiments using the rat model of acute subdural haematoma. This suggests that the animal model is suitable for the analysis of aspects of the pathogenesis of traumatic brain injury.

Alterations in axolemmal permeability following traumatic brain injury.

Traumatic brain injury can be associated with alterations in the axolemma. It is unclear if there exists a spectrum of axolemmal failure following the traumatic event or if the passage of macromolecules occurs in an "all or none" fashion. The present study used two extracellular tracers of different molecular weights to investigate this specific feature. In normal conditions these macromolecules are confined to the extracellular space by the intact axolemma. Following traumatic injury, the axolemma is altered and becomes permeable to macromolecules which can then be detected intra-axonally. Anaesthetised, mechanically ventilated rats received intrathecal horseradish peroxidase (HRP) or microperoxidase (MP), and were then subjected to an impact acceleration closed head injury. Animals were perfusion killed at various intervals after injury and the brains processed for the visualisation of peroxidase activity at both light and electron microscopic level. The results demonstrated that both HRP and MP were detectable within injured axons at all time points after injury. However, quantitative analysis confirmed that HRP demonstrated more reactive axons in the sections examined compared to MP. The reason for this is unclear but may be related to the biochemical binding properties of MP.

PREFACE AND DECLARATION

This thesis primarily represents results from a series of investigations which were designed to examine the role of the microtubule component of the neuronal cytoskeleton in the pathobiology of acute subdural haematoma. The main techniques employed were immunohistochemistry, Western blot analysis and transmission electron microscopy. An additional study was carried out to investigate the nature of the altered axolemmal permeability which occurs in traumatic brain injury.

Hypothesis to be presented

The central hypothesis is that, in traumatic head injury, the changes that take place in the microtubule component of the cytoskeleton are the end-product of a final common pathway that is triggered by the influx of calcium, through areas of membrane disruption, activating a variety of enzymes including calpains.

Investigations were carried out in four broadly defined areas:

1. To examine the microtubule component of the axonal and dendritic cytoskeleton in the rat brain following induction of an acute subdural haematoma. In addition, to determine the temporal profile of the development of cytoskeletal pathology.

2. To determine the role of calpain mediated proteolysis in the pathogenesis of acute subdural haematoma.

3. To detect alterations in the microtubule component of the neuronal cytoskeleton in post mortem tissue from fatally injured patients who sustained an acute subdural haematoma.

4. To examine the nature of disruptions in the axolemma following traumatic brain injury in the rat impact acceleration model of closed head injury.

Results from these studies are presented and discussed separately. In the discussion I have attempted to highlight the implications of this work and indicate future directions which subsequent investigations may address.

This thesis comprises my own original work and has not been presented previously as a thesis in any form. Certain parts of this work involved my collaboration with other workers. Western blot analysis and electron microscopy were performed in collaboration with Dr Karen Horsburgh and Dr William Maxwell respectively. I acknowledge the assistance of Professor John T. Povlishock in the interpretation of electron micrographs.

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CHAPTER 1 - INTRODUCTION

1.1 Head Injury

Head injuries are currently the most important cause of death in young adults in the United States of America. Throughout western society a high proportion of young chronically disabled individuals become dependent on care because of its effects. In contrast to stroke and coronary artery disease the incidence of head injury is increasing[1].

In the United Kingdom, one million patients per annum attend hospital after sustaining a head injury. Almost half of these are children less than 15 years old and males outnumber females by more than two to one. The majority of cases are the result of falls (41%) or assaults (20%). Road traffic accidents account for 13% of all patients attending hospital, but approximately one third of all acute cases transferred to neurosurgical units and 58% of all deaths in these units. Moreover, head injury accounts for 15%-20% of all deaths between the ages of 5 and 35 [2].

Over the last 20 years, major advances have been made in the management of head injury. Unfortunately these have not been translated into widespread improvement in outcome. In spite of improved access to better quality intensive care facilities and wider use of computerised axial tomography (CAT) scanning, neither the mortality rate or the incidence of preventable brain damage identified at postmortem, have decreased more than 5-10% over the last 15 years [3-5]. Intense research in recent years has increased our understanding of the pathophysiology of head injury and there are promising indications from experimental studies of benefit

from a range of neuroprotective drugs[6,7]. However, if this progress is to continue, further elucidation of the mechanistic basis of traumatic brain injury is essential.

1.2 Traumatic Brain Injury

Brain damage after a head injury can be classified by time course and by pattern. From a neuropathological point of view, there are two main stages in the development of brain damage after a head injury[8]. Primary damage occurs at the moment of injury and takes the form of scalp lacerations, fracture of the skull, surface contusions, lacerations of the brain, diffuse axonal injury and intracranial haemorrhage. Secondary damage ,which is produced by complicating processes that are initiated at the moment of injury but do not present clinically for a period of time, include ischaemia, raised intracranial pressure, brain swelling and hypoxia amongst others [2,9].

In recent years the specialised techniques of modern neuroradiology have contributed to the classification of traumatic brain injury by providing functional correlates of the structural damage identified by CAT scanning or at autopsy. Advances made in computerised tomography, magnetic resonance imaging and, more recently positron emission tomography, led to the introduction and wide acceptance of an alternative classification of traumatic brain damage based on the pattern of damage which could be either focal of diffuse[10-12]. It must be noted that in many patients the most accurate description may be of a multifocal distribution [2].

The principal types of focal brain damage are contusions on the surface of the brain, intracranial haematoma and the various types of brain damage secondary to intracranial expanding lesions and a high intracranial pressure (Figure 1). With the availability of sophisticated scanning, the clinician usually knows that lesions of this type are present during life. Diffuse brain damage is more subtle and consists of axonal injury, hypoxic brain damage, vascular injury and brain swelling. The clinician usually suspects this type of brain damage when faced with an unconscious patient who does not have an intracranial haematoma. As a result of a comprehensive clinical study on head injuries it became apparent that two lesions - diffuse axonal injury and acute subdural haematoma - were responsible for two thirds of the deaths, that is, more than all other lesions combined [13].

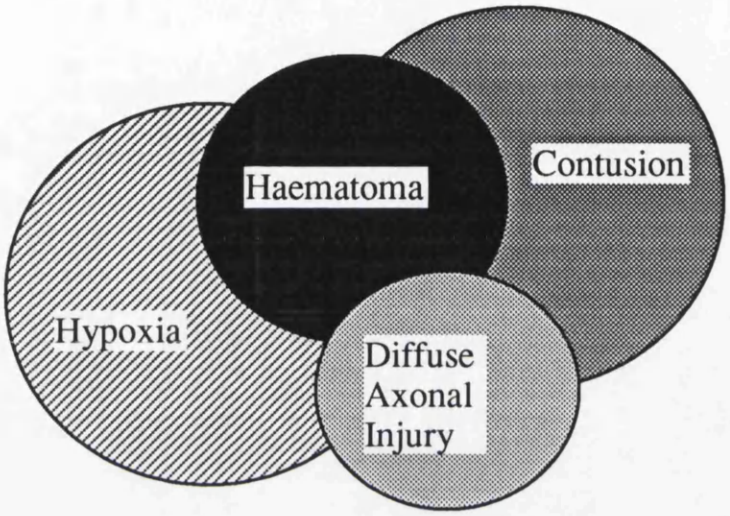


Figure 1

The inter-relationship of different pathophysiological processes responsible for brain damage in patients who die after head injury.

1.3 Diffuse Axonal Injury

Damage to axonal pathways in the brain is now recognised as the single most important lesion underlying traumatic brain injury with consequences ranging from brief concussion to profound vegetative state[2]. There is also increasing recognition of its importance as an initiating or accelerating factor in the development of cognitive decline and even Alzheimer's disease in later life[14].

The distinctive clinicopathological entity which is now internationally termed, diffuse axonal injury, can be defined as the occurrence of diffuse damage to axons in the cerebral hemispheres, in the corpus callosum, in the brain stem and, less commonly, the cerebellum resulting from a head injury[8,9,15-18]. Three grades of severity can be identified [8]. In grade 1, abnormalities are limited to histological evidence of axonal damage throughout the white matter without there being focal lesions in either corpus callosum or in the brain stem. Cases are designated as grade 2 if, in addition to widely distributed axonal injury, there is also a focal lesion in the corpus callosum. Grade 3 represents the most severe end of the spectrum and takes the form of diffuse damage to axons and focal lesions in both the corpus callosum and in the brain stem.

The severe end of the spectrum of diffuse axonal injury is characterised by a statistically lower incidence of lucid interval, fracture of the skull, cerebral contusions, intracranial haematomas, and evidence of a raised intracranial pressure, as compared with patients without this type of brain

damage. There is an increased incidence of gliding contusions and small deeply seated intracerebral haematomas and also a higher incidence of head injury due to road traffic accidents in comparison to falls [15].

Typically the axonal damage associated with traumatic brain injury is scattered throughout the brain and brain stem, in clear distinction to the focal contusions and mass lesions long thought to be the major complications of the traumatic insult. The diffuse injury to axons can only be idenfified microscopically. In patients with severe axonal injury who survive for only a short time (days), there are vast numbers of reactive axonal swellings sometimes called axonal bulbs or retraction balls [15]. It is important to appreciate that these terms are not synonyms. Indeed, the terminology used in the literature has resulted in confusion in the comparison of studies. A current review article has proposed clear definitions which should clarify this issue and hopefully avoid future confusion [19]. For all purposes the term "axonal swelling" refers to focal increases in axonal diameter which are still in continuity with the rest of the axon at both ends. On the other hand, the terminal increase in axonal diameter found after axonal disconnection should be referred to only as axonal bulbs. Thus the occurrence of axonal swellings precedes that of axonal bulbs in damaged nerve fibres. In this proposal, the term "retraction ball" corresponds to "axonal bulb" and its use is largely confined to historical perspective.

Axonal bulb formation is essential for a definitive diagnosis of irreversible axonal injury, and it is generally accepted that in the human

brain after head injury, using conventional silver staining methods, a survival time of 18-24 hours is required for incontravertible identification[8]. The axonal bulbs can be seen in sections stained by haemotoxylin and eosin as small round eosinophilic masses but are more easily seen in sections impregnated with silver. Recently developed immunohistochemical techniques are more sensitive in the detection of axonal bulb formation in human head injured brain sections[20-22]. Using an antibody to the β–amyloid precursor protein (β-APP), evidence of axonal damage can be seen after a survival of between 2 and 3 hours[22,23]. Histological studies demonstrated that axonal bulbs may be seen in only 30% of cases stained by silver impregnation, whereas if the same sections are immunostained using the antibody to β-APP, evidence of axonal bulb formation may be identified in nearly 92% of cases[22]. Despite these advances, a definitive diagnosis of diffuse axonal injury cannot be made in patients who survive for only a short time (1-2 hours) after their head injury [24].

Over the course of the past 25 years it has become increasingly clear, to both the neurosurgical and neuropathological communities, that in patients sustaining mild, moderate and severe forms of traumatic brain injury, axonal damage can occur, and based on the number of axons involved and their distribution, varying degrees of morbidity can ensue. Following upon the initial neuropathological observations [25-28] it became clear that damage to cerebral white matter was an important feature of head injured patients. During this period the term "shearing injury " was introduced since it was assumed that the forces of injury

immediately tear axons, causing them to retract and expel a ball of axoplasm, forming a retraction ball, the traditional histologic correlate of axonal injury. Controversy as to the nature of the brain damage and its pathogenesis continued for many years. At various stages it was referred to as diffuse damage to the white matter of immediate type [29], diffuse white matter shearing injury [30] and inner cerebral trauma [31].

Adams and colleagues more fully characterised the structural features and time course of the axonal damage occurring with traumatic brain injury and were responsible for clearly defining the concept of diffuse axonal injury [8,16,29,32,33]. Through the systematic study of a large population of patients who had sustained severe head injury, a direct link between the extent of diffuse axonal injury and the ensuing patient morbidity was established. This important observation was subsequently expanded upon in the laboratory [17]. The well controlled subhuman primate model of head injury demonstrated that the overall distribution and number of damaged axons directly correlated with the morbidity seen in the injured animals following angular acceleration injury. Studies conducted in humans who had sustained only mild or moderate traumatic brain injury also identified the presence of damaged axons[15,25,27,28,34]. Although reduced in both number and overall anatomical distribution in comparison to the severe traumatic state, their very occurrence suggested that axonal damage occurs across the spectrum of traumatic injury. This provides strong evidence that diffuse axonal injury is part of a clinical continuum of diffuse brain damage that clinically ranges from concussion up to and including persistent post traumatic coma. More recent findings support

this hypothesis. Using an antibody to the β–amyloid precursor protein (β-APP) axonal damage has been demonstrated in a series of 5 patients who died from unrelated causes after a mild head injury [35].

Numerous animal models of traumatic brain injury have been employed to investigate axonal injury. It is important to appreciate that, to date, only one animal experimental model precisely replicates the features of the human condition recognised as diffuse axonal injury. The angular acceleration model of head injury in the subhuman primate [17] results in axonal bulb formation in the white matter of the brain which is similar in distribution to that found in human diffuse axonal injury. Nevertheless, axonal swellings, as described above, occur in all animal "models of diffuse axonal injury". However, the distribution of these swellings within the white matter, differs discretely from that described in the head-injured patient. Therefore use of the term "diffuse axonal injury" in animal models in direct comparison with human diffuse axonal injury is not valid. It is suggested that the term "diffuse traumatic brain injury" should be used in preference and that a clear statement made that the axonal injury described in any experimental animal is a model of human diffuse axonal injury but does not exactly mirror the spatial distribution of damaged axons found in diffuse axonal injury in the head-injured patient [36].

Despite the fact that most researchers now concur that axonal damage is a consistent feature of mild, moderate and severe traumatic brain injury and, as such, contributes to morbidity, [16,17] no consensus has evolved

regarding the actual pathogenesis of the observed axonal damage. Contemporary studies, conducted in both experimental animals and humans, have demonstrated that the pathogenesis of traumatically induced axonal damage is more complex than originally posited. In many cases it involves evolving intra-axonal changes that lead to progressive axonal swelling and detachment over a 12-24 hour period [8,37,38].

1.4 Acute Subdural Haematoma

An acute subdural haematoma constitutes one of the "worse case scenarios" within the severe head injury spectrum. It is the most common mass lesion that complicates severe head trauma and is found in 20-30% of patients with severe head injury. The mortality rate for acute subdural haematoma exceeds that for any other sub-group of the severely head-injured population[5,13,39-41]. Although between 55% and 70% of patients who develop an acute subdural haematoma have a lucid interval at some stage in their clinical course, nearly two-thirds will die or be left severely disabled such that they are incapable of independent life. This occurs despite the fact that surgery is typically performed within a few hours of injury [5].

Acute subdural haematoma poses many problems for the neurosurgeon. One of these is the clinical paradox in which the outcome for a given clinical state appears to be much worse with subdural haematomas than with extradural haematoma. The combination of high morbidity and mortality despite the presence of a "conscious interval" suggests that

potentially treatable factors are responsible for much of the ischaemic brain damage that occurs in patients with an acute subdural haematoma. However, once a subdural haematoma has been surgically evacuated, the inadequacy of therapeutic options available to control intra-cranial pressure and limit ongoing neurological damage means that these patients often present the neurosurgeon with particular difficulties in management. For these reasons a major challenge is therefore to elucidate the pathophysiologic mechanisms causing ischaemic brain damage and to devise strategies to minimise its development [5,39-41].

The majority of subdural haematomas are due to disruption of the parasagittal bridging veins at the time of injury : however some are arterial in origin [9]. Most acute subdural haematomas are associated with considerable brain damage. Attempts to age haematomas on the basis of histological studies have not proved very satisfactory. A working classification describes a subdural haematoma as being acute when it is composed of clot and blood (usually within 48 hours of injury), subacute when there is a mixture of clotted and fluid blood (developing in between 2 and 14 days after injury) and chronic when the haematoma is fluid (developing more than 14 days after injury) [5].

The most common neuropathological finding in humans with an acute subdural haematoma is ischaemic brain damage[4].This is distributed chiefly in the ipsilateral hemisphere and also in the hippocampus bilaterally [32,42]. If the intracranial pressure is severely raised there may be evidence of ischaemic damage in the contralateral cortex[4]. Acute

hemispheric swelling, raised intracranial pressure and death are frequent sequelae of acute subdural haematoma; yet the majority of patients have been conscious at some time after injury. This suggests that secondary factors may contribute to the pathology of this condition. At present it is unclear what these factors are. Most investigators agree that the aetiology of acute subdural haematoma will prove to be multifactorial and related to, amongst others, local pressure effects, excessive activation of post synaptic receptors and release of toxic substances from the blood itself.

The development of a clinically relevant animal model of acute subdural haematoma has allowed some element of the pathophysiology of this injury to be clearly elucidated. The rat model of acute subdural haematoma demonstrated a consistent zone of focal ischaemic brain damage within the hemisphere beneath the subdural haematoma, as is found in some humans who die after an acute subdural haematoma[43]. This zone of histologic cortical infarction corresponded to an area of markedly reduced cerebral blood flow [10].

Subsequent studies have tended to focus on the role of excitatory amino acids (EAA's) in the mediation of the observed ischaemic damage. Microdialysis techniques demonstrated that the acute subdural haematoma is associated with release of extracellular glutamate in the underlying cortex, to levels seven times above basal[44]. In addition, a severe reduction in cerebral glucose metabolism was detected in the cortex beneath the haematoma and a band of glucose hypermetabolism in the peri-ischaemic tissue, surrounding the infarcted tissue, and throughout

the hippocampus[45]. These reports suggested that glutamate induced neuronal damage may be a feature of acute subdural haematoma and raised the possibility that this "excitotoxic" process may be amenable to therapeutic intervention. Drugs that block the excitotoxic effects of glutamate at the N-methyl-D-aspartate (NMDA) receptor have generally been effective in reducing the ischaemic brain damage observed in other animal models of focal cerebral ischaemia [6,46]. Several investigations have now demonstrated a consistent neuroprotective effect with NMDA antagonists, both in pretreatment and in post treatment paradigms with the experimental model of acute subdural haematoma[47-50]. There is now great enthusiasm that the potent neuroprotective effects that have been shown for NMDA antagonists drugs in the laboratory can be translated into clinical benefit for head injured patients. Clinical trials are either underway or planned[2,6].

It is also possible that the release of vasoactive blood components from the haematoma initiates pathophysiological events in the underlying cortex and leads to ischaemic damage [43,51]. This is in accordance with the demonstration that the extent of ischaemic damage produced by an intracerebral injection of blood was significantly greater than that produced by an equivalent volume of inert oil or a microballoon[52]. In a closed cranium model of subdural haematoma a zone of ischaemia and neuronal cell loss was observed contiguous to the clot whereas an inert silicone mass of similar volume had significantly less effect , suggesting a neurotoxic effect of blood or a blood component. [53] In humans, blood in the subarachnoid space causes delayed ischaemia and is toxic to cerebral

arteries [54]. Likewise, the concentration of glutamate in serum is 10 times greater than that of extracellular glutamate and in the red blood cells it is at least 10 times greater than in serum [55]. Therefore, exposure of neurones to blood could be associated with glutamate toxicity [56]. Furthermore, a recent investigation suggested that haemoglobin may contribute to neuronal loss after cerebral haemorrhage by exacerbating excitotoxicity[57]. Models of sub-arachnoid haemorrhage have investigated blood components such as oxyhaemoglobin and lipid peroxides and concluded that these may also result in ischaemic changes[58,59]. However a recent study demonstrated that blood in prolonged contact with the exposed cortical surface did not result in infarction of the underlying neurones [51]. This is consistent with experience from the clinical setting since it is well recognised that neurosurgical procedures are not associated with infarction or neuronal loss at adjacent sites.

It therefore appears likely that the brain's of traumatised patients responds in a compromised manner. Perhaps, following traumatic head injury the presence of subdural or subarachnoid blood acts synergistically with processes initiated by existing insults, such as the mechanical forces of injury, raised intracranial pressure, hypoxia or ischaemia, to overwhelm the brains's compensatory mechanisms and lead to neuronal cell death. There are precedents for this type of synergistic effect in experimental traumatic brain injury. The addition of a low level ischaemic insult to a sub-threshold mechanical insult potentiated neuronal loss beyond that seen with either insult alone [60]. Recent work lead to the development of a combined fluid percussion brain injury and entorhinal cortical lesion as

a model for assessing the interaction between neuroexcitation and axonal injury. Early results have demonstrated that axonal injury and it's attendant deafferentation, when coupled with traumatically induced neuroexcitation, produce an enhancement of the morbidity associated with traumatic brain injury [61].

At present the role of the neuronal cytoskeleton in the mediation of traumatic brain injury is a major focus of investigation. It is recognised that, by using multiple research stategies, an insight into the mechanistic basis of traumatic brain injury will be gained. This knowledge should then allow the development of appropriate therapeutic interventions aimed at inhibiting the progression to neuronal cell death. Without such agents the prognosis for traumatically brain injured patients will remain bleak.

1.5 The Neuronal Cytoskeleton

Early neuroanatomists soon concluded that the long nerve processes contained ' fibrils ' and that these provided the necessary structural support [62,63]. The era of the cytoskeleton had arrived. In a cross sectional view most of the axon is filled with 8-12nm structures, neurofilaments, separated from each other by side arm spacers. The axon also contains microtubules and microfilaments (Figure 2). Membrane bound organelles, including mitochondria, appear to be preferentially associated with microtubules and microfilaments. Longitudinal sections of the axon reveal arrays of neurofilaments and microtubules as well as sidearms that seem to connect cytoskeletal elements to each other and to membrane bound vesicles. Essentially, the cytoskeleton consists of protein components of the neurone which form the structural elements within the cell. It comprises three main elements viz:

a) Microtubules.

b) Neurofilaments.

c) Microfilaments.

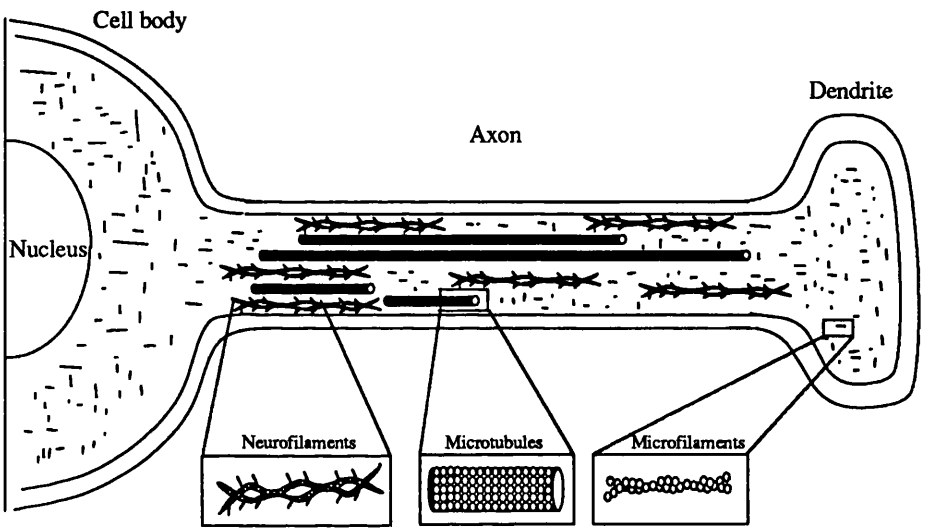


Figure 2

The components of the neuronal cytoskeleton.

Microtubules

Microtubules (MTs) are a major component of the cytoskeleton in nervous tissue and are especially prominent in neuronal processes. They play important functional and architectural roles in neurones. The primary polypeptides of MTs are alpha and beta tubulins and multiple genes exist for both tubulins including brain specific isoforms [64,65]. Both subunits have a molecular weight of approximately 50kDa and share considerable homology [66,67]. Within either family, individual subunits diverge from each other at less than 10% of amino acid positions [68]. For β– tubulin, five isotype clones have been identified and these contribute unique functional properties to the cell. Neuronal MTs are small tubules approximately 24nm in diameter with walls made up of 12-14 protofilaments which can be visualised in high resolution electron micrographs. In many cases, sidearms can be visualised projecting from the surface of the microtubule and these appear to interact with other MTs and surrounding structures. As such they constitute a relatively dynamic component of the cytoskeleton; a property known as "dynamic instability" [69]. MTs serve multiple roles in the neurone. In concert with actin microfilaments and neurofilaments, they establish and maintain the overall internal architecture of the cytoplasm and are a major determinant of neuronal size and shape[70,71]. MTs also have a critical role in intracellular transport. Video enhanced microscopy has demonstrated that the MT arrays in axons serve as tracks along which vesicles and cellular organelles are transported from the cell body to the synapse which may be up to one metre away[72]. The process of axoplasmic transport allows a

whole variety of proteins and organelles to be packaged in the cell soma and distributed throughout the neurone. This MT based motility is mediated by two families of proteins found in the nervous system; the kinesin and cytoplasmic dynein families[72]. Kinesin is a MT activated ATPase and it is involved in the anterograde movement of membrane bound organelles along the MTs in fast axonal transport[73]. Dyneins serve as the motor for retrograde axonal transport and may have a role in slow axoplasmic transport[74]. Disorganisation of the MT component would therefore interrupt these vital transport systems. Finally, MTs play an important role in neuronal growth and development [64,75].

Microtubule Associated Proteins (MAPs)

In addition to tubulin, which is their basic building block, the neuronal microtubules also contain large quantities of minor components, the microtubule associated proteins (MAPs). There are three properties that the MAPs confer on microtubules that may be important viz: MTs are stabilised, lengthened and made more rigid in the presence of MAPs [70]. This is clearly an important contribution to the morphological stability and functional properties of microtubules.

Microtubules are formed in the cell by cycles of assembly and disassembly and are composed primarily of tubulin but also contain several MAPs which when added back to pure tubulin, promote assembly[76-78]. MAPs are therefore thought to regulate microtubule assembly in vivo. The original MAPs isolated were from mammalian

neurones and named according to the three major size classes of polypeptides as defined by molecular weight (MW). These were: MAP 1 (MW >250kDa), MAP 2 (MW approximately 200kDa) and tau (MW 50-70kDa)[76,79]. Recent advances have identified additional MAPs and refined the original classes. These are now described with emphasis on the main neuronal MAPs.

MAP1

On the basis of electophoretic, biochemical and immunological criteria MAP 1 was resolved into three separate proteins, MAP 1a, MAP 1b and MAP 1c. Within the nervous system MAP 1a (usually called MAP 1) is found in neuronal cell bodies, axons, dendrites and glial cells[80]. MAP 1b (sometimes called MAP 5), is expressed very early in development of the nervous system, suggesting a role in neuronal morphogenesis[64]. It continues to be expressed in all regions of the neurone but only attains high levels in areas of the adult brain where neuronal differentiation continues such as the hippocampus, retinal photoreceptors and olfactory system[64,81]. MAP 1c is the only neuronal MAP with an identified function. This MAP is the heavy chain of brain cytoplasmic dynein, a protein involved in intracellular transport[82]. It is not restricted to neurones.

MAP 2

Microtubule associated protein 2 (MAP 2) is a principal protein component of microtubules and is consistently most abundant in neuronal dendrites and attains only low levels or is undetectable, in axons[83]. As such it provides a useful marker of dendrites. It is a high molecular weight, heat stable protein doublet that promotes microtubule assembly in vitro [79,84] and may regulate microtubule interaction with other cytoskeletal elements[85,86]. MAP 2 is present only in neurones and is a component of the cross bridges between microtubules [87] and between microtubules and neurofilaments[88]. When purified it consists of three components [80]. These are:MAP 2a (MW 280kDa), MAP 2b (MW 270kDa) and MAP 2c (MW 70kDa)[79]. Peptide mapping and monoclonal antibody reactivity suggest that MAP 2a and MAP 2b are closely related and the term MAP 2 is used to refer to the high molecular weight form MAP 2a/b[89]. Alternate splicing of transcripts of a single gene gives rise to MAP 2 and MAP 2c[90]. All of these isoforms contain a cluster of imperfect amino acid sequences which are also found in tau and are thought to be the essential core of the microtubule binding domain of MAP 2[91-93]. These components are expressed differentially in that MAP 2 is present at low levels in the embryonic brain but becomes the major MAP in the adult brain. In contrast, MAP 2c is the major MAP in the embryonic brain but is expressed at very low levels in the adult brain, persisting mainly in areas of neuronal growth [90].

Tau

The microtubule associated protein, tau, plays an important role in the dynamics of microtubules. Tau is a phosphoprotein and is predominantly found in the axonal compartment of neurones where it is able to bind and stabilise MTs[94]. Purified tau from mammalian brain consists of four to six discrete isoforms ranging in molecular mass from 35-65kDa and differing by the presence or absence of two types of amino acid insertions[79,95]. This heterogeneity is generated, at least in part, by alternate splicing of a single gene[96]. All of these isoforms contain a cluster of imperfect amino acid sequences which are also found in MAP 2 and are thought to be the essential core of the microtubule binding domain of tau[91-93]. Phosphorylation of tau, at specific epitopes, has marked structural effects leading to a stiffening and elongation of the molecule associated with an increased stability of the microtubules[84]. Conversely, dephosphorylation inhibits this process and may lead to a decline in cytoskeletal integrity[97].

MAP3

MAP3 is a large protein found in various tissues. In the brain it occurs where there are high concentrations of neurofilament rich axons. Despite this association it does not appear to be stongly bound to neurofilaments[98].

MAP4

MAP4 comprises a family of MAPs distributed ubiquitously in tissues and across mammalian species. The function of MAP 4 is unknown but, in the brain, it is restricted to glial cells [99].

MAP 2 and tau : Suggested complementary roles in dendrites and axons.

From the above outline it is clear that MAPs have important functions in the maintenance of microtubule stability within neurones. It is useful to briefly consider MAP 2 and tau together in order to emphasise the complementary role these two distinct MAPs have.

MAP 2 and tau are two of the major and most studied microtubule associated proteins of the vertebrate nervous system. In neurones, immunohistochemistry shows complementary distributions, with tau being concentrated in axons [94] and MAP 2 being confined to dendrites [80]. Each protein consists of multiple isoforms that contain three or four homologous tandem repeats near the carboxy terminus which are thought to constitute microtubule binding domains [92]. Electron micrographs demonstrate these proteins as lateral projections that appear to cross-bridge adjacent microtubules. The spacing between adjacent microtubules in the presence of tau is about 20nm while that for MAP 2 is about 100nm[88]. This suggests that each protein consists of a common microtubule binding domain and a projection domain. Since microtubules are inherently unstable structures, the stabilising effect of tau and MAP 2

are likely to be important for the integrity of neuronal functions that depend on stable microtubules, such as fast axoplasmic transport.

Neurofilaments

Neurofilaments (NFs) or, intermediate filaments of the nervous system, appear in electron micrographs as solid rope like fibrils 8-12nm in diameter and are of variable length. They are present in most cell types. The primary type of NF is formed from three subunit polypeptides collectively called the neurolfilament triplet proteins [100], each coded by a separate gene[101]. The molecular weight (MW), as estimated by gel electrophoresis, for NF subunits vary from 200kDa for the high MW subunit (NF-H), 150 kDa for the middle subunit (NF-M) and 68kDa for the low MW subunit (NF-L)[71,101]. The 68kDa subunit is an assembly protein found predominantly in the neurofilament core, and the 150kDa and 200kDa subunits are crosslinking proteins found in the connecting branches [102]. All NF triplet proteins exhibit significant sequence homology in the amino terminal, alpha helical domain. The 150kDa and 200kDa subunits possess highly repeating lysine-serine-proline(LSP) sequences, which are heavily phosphorylated and contribute to anomalous electophoretic mobilities[103]. NFs exhibit an unusual degree of metabolic stability, which makes them well suited to maintaining neuronal morphology. They also play a critical role in determining axonal calibre and slow axoplasmic transport[98].

Microfilaments

Microfilaments are found throughout neurones and glia. Electron microscopy demonstrates that they consist of actin subunits arranged like two strings of pearls intertwined to form fibrils 4-6nm in diameter. They maintain cell shape and are well suited to resist deformation. The full extent of their function is unclear but, notably they mediate the interaction of the neurone with the surrounding extracellular matrix and neighbouring cells [98].

1.6 The Neuronal Cytoskeleton in Acute Brain Injury.

Derangements of the neuronal cytoskeleton are undoubtedly important pathological consequences of traumatic brain injury. Within the last few years laboratory studies have demonstrated that traumatic brain injury can produce more widespread alterations in the neuronal cytoskeleton than previously recognised. Initial investigations focused on the axonal cytoskeleton in diffuse axonal injury[8,37]. More contemporary studies have recognised that the cytoskeleton of dendrites and the neuronal cell body are also disrupted by the forces of traumatic injury[104,105]. The functional significance of the observed structural abnormalities remain unclear. However, it would seem logical that structural disorganisation of the cytoskeleton would result in functional correlates. As such, a greater understanding of the mechanisms, which contribute to disruption of the cytoskeleton, is essential for the future developments of therapeutic strategies which will aim to arrest the progression of changes in the cytoskeleton.

As noted previously, traumatic brain injury constitutes a wide range of both focal and diffuse pathologies[9]. It is therefore important to appreciate that all animal experimental models have limitations since only certain elements of this continuum can be investigated[106]. For ease of discussion it is convenient to consider the literature relevant to the role of the cytoskeleton in traumatic brain injury under two "anatomical "sub-headings.

The axonal cytoskeleton in traumatic brain injury

Despite the fact that it is well established that microtubules are the principal conduits for fast axoplasmic transport [98] and that reactive axonal swellings result from an impairment of this process,[8,37] scant attention has been applied to the study of microtubules within the axonal cytoskeleton after traumatic brain injury. Rather attention has been devoted to the study of the neurofilamentous components[20,107,108].
Based on the premise that the forces of traumatic injury can either mechanically or functionally disturb the cytoskeleton resulting in impaired axoplasmic transport, Povlishock and colleagues explored this issue using antibodies specific to the three neurofilament subunits[37,107,109,110]. As neurofilament accumulation is associated with reactive axonal change they reasoned that monoclonal antibodies directed at the various neurofilament subunits might provide insight into the events leading to reactive change. In this series of experiments animal models known to produce consistent axonal injury were employed [106].

Although some mechanistic insight was provided by all three antibodies the monoclonal antibody to the 68kDa subunit was the most useful. Specifically, within one hour of traumatic brain injury, a highly focal increase in the intra-axonal 68kDa subunit was detected, and this was most dramatic by two hours post injury[37,107,109]. Initially the 68kDa immunoreactive neurofilaments paralleled the longitudinal axis of the axon, but within the first two hours of injury, they began to withdraw from the axolemma and to display an abnormal alignment oblique to the

longitudinal axis of the axon. With increased survival the degree of disorganisation of the 68kDa neurofilaments increased and organelles accumulated at the periphery of the axon. This was followed over time by continued axonal expansion, lobulation and ultimately axonal disconnection with the formation of a reactive axonal swelling. Subsequent studies extended these findings to humans by demonstrating that a similar sequence of events occurred with a distinct progression of neurofilamentous changes as outlined above [20,108]. The implication from this series of reports is that the structural disorganisation of the axonal cytoskeleton plays an important role in the pathobiology of traumatic brain injury. However, the mechanism of neurofilament misalignment and how this translated to an impairment of axoplasmic transport was unclear.

A major problem in most of the experimental paradigms examined is the question of sampling. Although immunohistochemical and ultrastuctural abnormalities can be demonstrated within axons, we cannot be certain that we are sampling at an initiating site of injury. It could be that the most marked disturbances occur upstream or downstream from the actual initiating site, resulting in the investigator noting features which are only indirectly related to initiating events. This is an important consideration and must be borne in mind when comparing the findings from two different models.

In an attempt to overcome this problem Povlishock and co-workers utilised extracellular tracers on the basis that the site of tracer uptake

would mark the initiating site of injury[111,112]. Ultrastructural analysis revealed a complex and unexpected array of disturbances in the cytoskeleton. There appeared to be at least two different cytoskeletal responses to traumatc brain injury which were related to the severity level of the injurious insult. Specifically, following severe injury, in the cat model of fluid percussion injury, they detected a concomitant collapse in the cytoskeleton as evidenced by increased neurofilament packing, that is, a decreased inter-filament distance. This preceded the progressive, yet delayed, development of neurofilamentous disarray leading to the evolution of reactive axonal swelling as outlined in the previous reports from their laboratory. This was a new observation which they had not demonstrated previously. This failure was attributed to a problem of sampling methodology[111]. With more mild injuries a different cytoskeletal response was displayed. There was misalignment of the neurofilaments such that they adopted an abnormal helical pattern lying oblique to the longitudinal axis of the axon. In addition, analysis of coronal sections indicated that there was loss of microtubules in the reactive axons. Further investigations followed and demonstrated that these complex changes were neither model or species specific[112]. Using antibodies against the phosphorylated sidearms of the neurofilaments it was demonstrated that the observed compaction of the neurofilaments resulted from a loss of the sidearms following injury. A similar observation was made in lamprey spinal cord axotomy[113]. Through the novel use of antibodies specific for the neurofilament sidearm and rod domains these workers confirmed that the neurofilaments lost their side

arm projections allowing the unmasking of the rod domain in regions of cytoskeleton collapse and neurofilament compaction.

Subsequent quantitative analysis and characterisation of the ultrastructural changes occuring in the axonal cytoskeleton suggested that there were at least two distinct reactions following traumatic brain injury[114]. In severe injury, altered axolemmal permeability was associated with collapse of the cytoskeleton as evidenced by compaction of neurofilaments and loss of microtubules. In contrast, following mild injury, there was no evidence of altered axolemmal permeability but the neurofilaments were misaligned relative to the longitudinal axis of the axon. Both these groups progressed to reactive axonal swelling.

Extensive recent studies designed to investigate the histopathological correlates of cytoskeletal derangements demonstrated that there was little evidence of axonal involvement within 3 hours of traumatic brain injury[104]. These workers used the rat model of cortical impact injury and examined the cortical samples using quantitative Western blots. They detected a significant decrease in the levels of key neurofilament proteins (NFL and NFH) and concluded that this constituted evidence of disruption of the axonal cytoskeleton[115]. However, subsequent confocal microscopy on the same experimental material detected minimal axonal alterations within the cortex[104]. This suggested that preferential dendritic rather than axonal damage occurs within 3 hours post traumatic brain injury. In addition, minimal alterations in axonal NFL(68kDa) subunit immunoreactivity were observed in white matter tracts such as the corpus

callosum following injury and there was no evidence of reactive axonal swellings.

The dynamics of the microtubule component of the cytoskeleton are also affected by injurious insults. Surprisingly few studies have investigated this aspect of the cytoskeleton in traumatic injury. In the guinea pig optic nerve traction model of stretch injury, recent studies have provided evidence for the loss of microtubules within 15 minutes of injury[116]. Quantitative analysis demonstrated a significant decrease in the number of microtubules in the axoplasm of myelinated fibres and this was associated with an increased spacing of the neurofilaments, that is an increase in the inter-filament distance. Furthermore, these changes were most marked at the nodes of Ranvier suggesting that this specific region was most susceptible to stretch injury. In these nodes the axoplasm possessed a flocculent substructure between the remaining structurally recognisable microtubules and neurofilaments. This morphological appearance is consistent with degradation of substrate proteins.

The microtubule associated protein, tau, is altered in axons following ischaemic injury. Specifically, focal cerebral ischaemia results in the focal accumulations of tau, as detected by the Tau-1 antibody, in the white matter tracts of the ipsilateral caudate nucleus and corpus callosum in the rat. [130] These accumulations can be demonstrated within 2 hours of occlusion of the middle cerebral artery and appear as irregular immunostained profiles. Follow up studies also demonstrated alterations in tubulin and MAP 5 immunostaining in axons following focal cerebral

ischaemia in the rat[117]. The significance of these findings remains to be fully established.

The somato-dendritc cytoskeleton in traumatic brain injury

The first study to demonstrate the loss of a cytoskeletal protein following traumatic brain injury examined MAP 2 levels in hippocampal and cortical tissue [105]. In the rat model of fluid percussion injury they reported that moderate traumatic brain injury resulted in a significant decrease in the MAP2 protein level in the ipsilateral hippocampus compared to the contralateral side. This effect appeared to be regionally selective since the MAP 2 decrease did not occur in the cortex. More importantly, it suggested that cytoskeletal damage occurs in the somal and dendritic compartments following sub-lethal magnitudes of traumatic injury.

In more severe brain injury the levels of MAP 2 in the cortex were decreased. The cortical impact model produces severe focal cortical contusions. Within three hours of injury qualitative immunohistochemistry and Western blot analysis detected a substantial loss of MAP 2 in the cortex ipsilateral to the site of injury[104]. Moderate traumatic injury caused a reduction in cortical and hippocampal MAP 2 immunoreactivity within 10 minutes of injury and persisted for at least 7 days post injury[118]. Other investigators demonstrated that these disturbances in MAP 2 immunostaining were not model specific. Mild focal cortical trauma produced by the weight drop technique resulted in

decreased MAP 2 immunoreactivity in the rat cortex and hippocampus[119]. The loss of MAP 2 immunostaining was most marked at the perimeter of the lesion 1-3 days after injury. However, this study extended previous work by demonstrating that the reduction in cortical MAP 2 immunoreactivity was not observed 21 days after impact. This suggests that the pathological processes underlying the loss of MAP 2 may be reversible.

Quantitative analysis of the effects of severe traumatic brain injury on the levels of cytoskeletal proteins have added to the complexity of this topic. The rat model of severe lateral cortical impact injury results in an area of contusion in the ipsilateral cortex similar to those seen after severe human head injury. Quantitative Western blot analysis demonstrated that traumatic brain injury caused a significant decrease in the protein levels of two prominent neurofilament proteins, NF-L (68kDa) and NF-H (200kDa) within 3 hours of injury and this was also observed at all survival time points up to 2 weeks post injury [115].The decrease in the 68kDa neurofilament subunit was restricted to the site of contusions in the ipsilateral cortex with no decrease observed in the contralateral cortex or the hippocampus. A decrease in the protein level of the 200kDa neurofilament was detected in both cerebral cortices but not in the hippocampus. In contrast to earlier qualitative studies (1,6,7,22), these investigations suggested that the overall effect of traumatic brain injury on the level of the 68kDa neurofilament, throughout the cortex, was reduction in the level of the protein[115]. However, this decrease was associated with an increase in the presence of lower molecular weight

immunopositive bands. These protein bands may represent 68kDa neurofilament breakdown products produced by the action of proteases on the parent 68kDa neurofilament. This particular observation clearly complicates the interpretation of the immunohistochemical data which demonstrates a focal increase in 68kDa neurofilament subunit in reactive axonal swellings, following traumatically induced axonal injury [37,107-109]. An alternative explanation for this localised increase in 68kDa antigenicity may be that it represents the breakdown products of the NF-L subunit. Further support for the loss of neurofilament proteins following traumatic brain injury was provided by in-vitro studies on mixed septohippocampal cultures. Densitometric analysis of immunolabelled Western blots demonstrated that there was a 40% decrease in neurofilament immunoreacivity following potassium depolarisation as occurs in traumatic brain injury [120].

The morphological correlates of these disruptions in the cytoskeleton are of importance in determining the relative involvement of axonal and dendritic processes in the pathobiology of traumatic brain injury. Recently completed investigations used immunofluorescence and confocal microscopy to examine changes in the configuration of axons and dendrites within the cortex following traumatic brain injury[104,121]. In the same experimental model as used in the initial immunoblotting studies, the rat model of cortical impact injury, prominent alterations in the immunolabelling of NF-H(200kDa), MAP 2 and NF-L(68kDa) were detected in both the ipsilateral and contralateral cortical contusion sites[104]. Light microscopic studies of the NF-H subunit

immunofluorescence revealed a fragmented appearance of apical dendrites within pyramidal neurones layer 3 and 5, as well as a loss of fine dendritic arborisation within layer 1. Confocal microscopy detected varying degrees of NF-H subunit disassembly represented by a loss of linearity, associated with these areas of neurofilament fragmentation. A similar pattern of fragmentation was displayed in dendrites showing loss of MAP2 immunoreactivity. The NF-L neurofilament subunit (68kDa) was altered but not as dramatically as observed with the NF-H neurofilament subunit. The decrease in 68kDa immunolabelling after injury resulted in less fragmented dendrites. In conclusion, this study demonstrated that traumatic brain injury can produce significant cytoskeletal derangements in dendrites in the absence of marked axonal changes.

Ischaemic brain damage is by far the most common secondary insult in traumatically brain injured patients and is detectable in more than 80% of fatal cases[3,4]. Disturbances in the neuronal cytoskeleton may be involved in the evolution of ischaemic brain damage and alterations in the microtubule associated proteins may play an important role.

Morphological studies have demonstrated that the normal linear arrangement of the neuronal cytoskeleton is disrupted following even brief periods of cerebral ischaemia. Electron microscopy demonstrated prompt disintegration of microtubules within histologically defined ischaemic areas [122,123]. This rapid disassembly of microtubules was detected within 5 minutes of unilateral occlusion of the carotid artery in

gerbils. In addition, a differential vulnerability of the microtubule components to ischaemia was demonstrated. Specifically, microtubules in myelinated axons were more stable than those in dendrites suggesting that the structural characteristics of a neuronal process may effect it's susceptibility to injurious insults[122].

Numerous investigations have documented alterations in the components of the cytoskeleton following ischaemic insults of various types and durations. Several studies have indicated that MAP 2 is the most ischaemia sensitive component of the cytoskeleton. Even short periods of transient global ischaemia (5 minutes) resulted in a loss in MAP 2 immunoreactivity in the gerbil hippocampus[124]and a decrease in the cortical levels of MAP 2 was observed following 72 hours of permanent occlusion of the left middle cerebral artery in the rat[125]. These changes in immunoreactivity are consistent with ultrastructural studies that demonstrate alterations in dendritic structure after ischaemia [122]. However, no decrease in MAP 2 levels were detected following cardiac arrest for 30 minutes in the rat suggesting that reperfusion injury may have a role in the pathophysiology of cytoskeletal derangement[126]. Neurofilaments are also degraded in cerebral ischaemia [127].

The localisation and levels of the microtubule associated protein , tau also show changes following focal cerebral ischaemia. An early, variable and transient increase in perikaryal tau was detected following transient global ischaemia in the rat[128]. In the cat model, tau immunostaining was increased in a proportion of neurones within the ischaemic lesion[129]. In

the rat model there was increased tau immunostaining in axons and some neuronal cell bodies throughout the ipsilateral cortex and caudate nucleus compared to the corresponding contralateral regions[130]. Immunoblotting techniques detected a decrease in the cortical levels of the 200kDa neurofilament subunit (NF-H), MAP 2 and tubulin following focal cerebral ischaemia in the rat[125]. Other investigations demonstrated a decrease in immunolabelling of tubulin, MAP 1 and MAP 2 in the gerbil model of transient global ischaemia[131,132]. This multiplicity of disruption suggests that disturbances in the cytoskeleton may be a key feature in the pathogenesis of traumatic brain injury.

The biochemical mechanisms underlying these structural changes in the cytoskeleton are a subject of current investigation. In this regard the involvement of proteolytic enzymes and excitotoxic amino acids has attracted a great deal of research. In particular, the calcium activated neutral protease calpain is thought to play an important role in the mediation of traumatic brain injury.

1.7 The role of calpain in traumatically induced cytoskeletal injury

It is now becoming increasingly clear that traumatically induced brain damage cannot be attributed to a single factor, but that several mechanisms come into play. A multifactorial pathogenesis has implications for the pursuit of pharmacological intervention, since there is

probably no single therapeutic agent which will ameliorate brain damage of whatever cause.

A disturbance of ion homeostasis in traumatic brain injury triggers a variety of events which can ultimately lead to cellular dysfunction and neuronal damage[133]. It has been widely assumed for many years that neuronal cell damage is, at least in part, related to loss of cellular calcium homeostasis, with an ensuing calcium " overload" [133-135]. Sustained elevation of intracellular calcium results in the unrestrained activation of several calcium-sensitive events. In experimental models of traumatic brain injury, marked alterations in total tissue calcium levels and calcium fluxes occur in regions of the brain that exhibit subsequent cell loss [136,137]. It is suggested that cell calcium "overload" damages cells primarily by accelerating lipolysis and proteolysis, and by effecting protein phosphorylation through an effect on kinases and phosphatases [133,138]. In this series of events the proteolytic degradation of the cytoskeleton by the calcium activated protease, calpain, is assumed to play a key pathogenetic role. This is consistent with the hypothesis that unregulated proteolysis by calpain represents a final common pathway to neuronal cell death.

Calpain is a family of calcium-dependent, non-lysosomal, cysteine proteases. This enzyme family, also known as calcium-activated neutral protease (CANP), is present in most mammalian cells, normally in an inactive form. Calpain activation is believed to occur when intracellular free calcium levels surpass a certain threshold [139,140]. Two forms of

calpain, which differ in the level of calcium required for activation, have been identified in mammalian brain. Calpain I (or μM-calpain), activated in vitro by micromolar levels of ionic calcium, is located in neuronal cell bodies and is less abundant in axons and glia. In contrast, calpain II (or mM-calpain), is activated in vitro by millimolar levels of ionic calcium, and is most common in glia and axons [141-143]. This difference in cellular distribution may reflect a difference in physiological function between calpains I and II.

Calpain mediated proteolysis is an attractive candidate to participate in degenerative responses because it is activated by calcium and is capable of degrading several important intracellular proteins. Among the preferred substrates for calpain are cytoskeletal proteins and regulatory enzymes. Uncontrolled proteolysis of any, or all, of these proteins could have a severe impact on the structural and functional viability of neurones. In vitro studies demonstrated that MAP 2, tau, tubulin and neurofilaments are susceptible to calpain mediated proteolysis [144-149]. As outlined in the previous section, acute brain injury results in a general decrease in cytoskeletal proteins in a wide range of animal experimental studies. Overall these investigations suggest that calpain may play an important role in the pathological degradation of the cytoskeleton.

A recent report extended these findings by demonstrating that calpain activation was a feature of traumatic brain injury [150]. Specifically, fluid percussion injury in the rat resulted in prolonged calpain mediated breakdown of the cytoskeleton. Moreover, this study supported evidence

obtained from in vitro and in vivo work by suggesting that dendrites are the most susceptible component of the neuronal cytoskeleton to calpain [104,105,151].

1.8 The role of the axolemma in the initiation of traumatically induced axonal injury.

For many years it was assumed that the tensile forces of traumatic injury sheared axons at the time of injury causing them to retract and expel a ball of axoplasm, forming a reactive axonal swelling or retraction ball, the traditional histologic correlate of diffuse axonal injury[25-28,30]. More contemporary investigations have not supported this basic premise[15,23,37,108-110]. Rather, they have demonstrated that in the majority of cases of traumatically induced axonal injury, the process of axonal disconnection is not immediate but delayed for 4-24 hours depending on the severity of injury and the species examined. The traumatic insult elicits a focal axonal abnormality that leads to the impairment of axoplasmic transport with subsequent swelling of the axon [37]. This progresses to separation of the swollen axon into a proximal segment in continuity with the sustaining soma and a distal appendage which will undergo Wallerian degeneration. When, in the above described sequence of axonal change, axonal detachment does occur, the continued delivery of axoplasmic constituents via anterograde transport allows for the continued expansion of the proximal swollen axonal segment, which matures into a reactive axonal swelling or retraction ball of classic description[15,37].

Despite general agreement regarding the occurrence of secondary axotomy, that is axotomy occuring at some time after the initial traumatic event, there has been little consensus with regard to the pathogenesis of the initiating subcellular events involved in this process. The obvious question centres on precisely what subcellular event underlies the axon's propensity to fail with traumatic brain injury. In this regard, the role of the axolemma is a major topic of current investigation.

The axon membrane or axolemma consists of a bilipid membrane and an associated submembrane skeleton. This skeletal framework is essentially formed by the protein spectrin (also known as fodrin), arranged in a pentagonal or hexagonal meshwork lining the interior of the cell membrane, and actin, connecting via ankyrin and other components to transmembrane proteins. Transmembrane proteins, such as β1-integrin bind externally to the extracellular matrix and internally via the membrane skeleton to the cytoskeleton [152-154].

Historically, two hypotheses have attempted to explain the biochemical/biophysical mechanisms occurring in damaged axons after the application of tensile strain/stretch-injury as occurs in non-missile head injury. Adams and colleagues, proposed that focal pertubation of the axolemma was the crucial initiating event [7,15-17,155]. This was associated with increased permeability of the axolemma, allowing the influx of extracellular calcium. In the normal situation the intracellular concentration of free calcium is rigorously controlled and there is

normally a steep gradient of the order of 10,000:1 in the concentration of calcium between extracellular fluid and axoplasm. The elevation of intracellular calcium levels following stretch-injury may activate proteolytic enzymes, such as calpain, that cause dissolution and subsequent collapse of the cytoskeleton, disrupting axonal transport as reflected in the accumulation of membranous organelles and causing the pathological disturbances described above. On the other hand, Povlishock and colleagues proposed that the physical forces of injury directly perturbed the intra-axonal cytoskeleton, as against the axolemma, resulting in a series of structural changes causing its disorganisation to thereby elicit an impairment of axoplasmic transport [20,37,107,108]. The concept of an influx of calcium into axons after traumatic brain injury was criticised by Povlishock and colleagues for many years. Their main contention was that the sequence of cytoskeletal events which they observed in various experimental paradigms was inconsistent with a proteolytic process. Specifically, they did not detect any evidence of rapid dissolution of the cytoskeleton purportedly linked to calcium activated proteases.

Fortunately, recent studies have provided considerable insight into each of these mechanisms and their potential role in the genesis of the reactive axonal change associated with traumatic brain injury.

Observations of traumatically induced axonal abnormalities in the majority of experimental models of blunt head injury, without either myelin disruption or frank damage to the adjacent neural or glial

processes suggested that there was no evidence for direct tearing or shearing of axons following traumatic injury [109]. This was supported when freeze-fracture studies, conducted in the early phases of axonal injury, failed to demonstrate either immediate disruption of the internodal myelin or early loss of the glial-axonal junctions, suggesting that the myelin sheath itself is not immediately damaged by the forces of injury[156]. At this stage both the above groups, postulated that the forces of traumatic injury act first on the axolemma and/or the axoplasm while sparing the myelin sheath. Within this proposal, the mechanical forces of injury could stretch the axolemma to its biomechanical limits at which point it failed and initiated reactive axonal change.

However, in a series of subsequent investigations on both human [20,108] and animal [107,157] brain tissue, Povlishock and colleagues provided data to support an initiating role for the intra-axonal cytoskeleton. Their studies revealed a complex sequence of intra-axonal events which progressed to reactive axonal change. Specifically, using antibodies directed at the neurofilament component of the cytoskeleton they observed that there was focal accumulation of the intra-axonal 68 kDa subunit (NF-L) within one hour of injury and this increase was dramatic by two hours. With increasing time the neurofilaments became disorganised and misaligned relative to the longitudinal axis of the axon. There was no loss or dissolution of neurofilaments detected. Overall this data was inconsistent with neutral protease mediated degradation of the cytoskeleton. Instead the increase in the 68kDa neurofilament subunit supported the possibility of a traumatically induced rearrangement of the

neurofilament pool. Again no ultrastructural evidence of direct axolemmal disruption was detected in this series of experiments further refuting an initiating role for the axolemma. Infolding and distension of the axolemma was demonstrated and attributed to the ongoing reactive axonal change. These findings suggested that a direct mechanical effect upon the cytoskeleton of the axon cylinder was the pivotal event in the initiating pathogenesis of axonal injury .

Other approaches suggested that the axolemma was involved in the initiation of reactive axonal change. Maxwell et al (1991) used a nerve traction model to analyse the morphological changes within damaged axons in vivo [155]. They provided evidence that the initial site of damage after a non-disruptive stretch injury is the nodes of Ranvier, some of which develop "nodal blebs". These blebs are axolemma limited protrusions of the axoplasm into the perinodal space and are most numerous within 15 minutes of injury but less so at later intervals. The development of nodal blebs correlated with loss of the dense undercoating of the axolemma. In damaged axons with nodal blebs the neurofilaments were disorganised , deviated from the longitudinal axis of the axon and extended into the blebs. In contrast, the microtubules maintained their longitudinal arrangement and did not deviate into the bleb. Quantitative analysis of this material demonstrated that there was a significant loss of microtubules and an increased spacing of neurofilaments in the axoplasm of nodes with associated nodal blebs. The loss of microtubules would disrupt fast axoplasmic transport resulting in the focal accumulation of membranous organelles in adjacent paranodal

regions of the axon to form reactive axonal swellings. In addition, these workers provided the first cytochemical evidence to support the idea of calcium influx into stretch injured myelinated nerve fibres [116]. The oxalate/pyroantimonate technique for the localisation of calcium, demonstrated an increased content of pyroantimonate precipitate within nodal blebs which was detectable 15 minutes after a stretch injury. This correlated with a reduction in labelling for Ca-ATPase activity on the nodal axolemma[158]. The node of Ranvier is the specialised region of the axolemma in which there are localised groups of Na+ channels , ATPase driven pumps for calcium and a Na+/Ca2+ exchanger[159]. Therefore, loss of membrane pump activity in the nodal axolemma might provide a mechanism for influx of free calcium into nodes of Ranvier after traumatic injury. This evidence supports the hypothesis that the forces of traumatic injury result in a focal pertubation of the axolemma with the resultant influx of free calcium capable of activating neutral proteases.

Biophysical investigations have utilised novel techniques to analyse the axolemma in experimental conditions analagous to non-disruptive axonal injury. Following mild stretch-injury a series of constrictions and expansions occurs in sciatic nerve fibres from the adult rat. This form change is known as beading [160-162]. The transformation of the essentially cylindrical form of the normal nerve fibre to one that is beaded occurs rapidly, within 10-20 seconds on initiation of stretch. Initial experimental data suggested that beading is brought about by a mechanism related to the axolemma and/or the cytoskeleton [153]. The tensegrity mechanism suggested that stretch may engage the membrane

skeleton [163]. In this mechanotransduction model, the transmembrane protein β1-integrin binds to both the extracellular matrix and the cytoskeleton [153]. Tension placed on the matrix is signalled through β1-integrin to the cytoskeleton with resultant alteration in it's integrity and spatial arrangement. Evidence in favour of this hypothesis was the change in stretch resistance of the β1-integrins when cytoskeletal components were disrupted [164]. However, a recent study indicated that beading does not require an interconnected cytoskeleton as envisioned in the tensegrity model [36]. Moreover, these results also excluded a causal relation or a necessary participation of the cytoskeleton. The intriguing conclusion is that the axolemma is the initiating site of beading constriction [36]. The forces of stretch-injury are transmitted to the axolemma by transmembrane proteins resulting in beading constriction. It is worthy of speculation that beading may be part of the biological process that is described as reactive axonal change in the literature related to traumatically induced axonal injury.

Within the last three years the involvement of the axolemma in the pathogenesis of axonal injury has become established. The seminal observations of Maxwell et al (1993) demonstrated that severe traumatic brain injury was capable of direct renting of the axolemma following lateral acceleration head injury in non-human primates[165]. This study was the first to provide ultrastructural evidence which supported the concept of axonal shearing in traumatic injury to the brain. The loss of axolemmal integrity was associated with rapid dissolution of the axonal cytoskeleton. In the axons demonstrating axolemmal renting or

fragmentation, the fibrous/filamentous organisation of the cytoskeleton was replaced by a flocculent precipitate consistent with a rapid dissolution of the underlying cytoskeletal proteins. These changes were detected within minutes of injury but only occurred in a sub-population of fine calibre, thinly myelinated axons after severe injury. These morphological changes represented an acute response of axons to injury and was termed " primary axotomy" - defined as occurring within minutes of injury, in contrast to delayed secondary axotomy which developes over a period of hours. In the same experimental material there was no evidence of disruption of the axolemma one hour after injury. This suggests that the disrupted axonal membrane reseals within an hour of injury.

Following this publication, Povlishock and colleagues were forced to reconsider their central hypothesis which did not include a role for the axolemma in the initiation of traumatically induced axonal injury. On reflection, they suggested that direct renting of the axolemma may represent the most severe end of a spectrum of axolemmal disruption[111]. This was a major turning point, in that, for many years this research group had advocated that there was no evidence of direct alterations in the axolemma following injury in any of the numerous paradigms they had investigated. Moreover, they had argued that the pathobiology of traumatically induced axonal injury resulted from the direct impairment of axoplasmic transport due to the forces of injury disrupting the axonal cytoskeleton[37,107,108].

In a series of experiments designed to investigate this issue, Povlishock and colleagues employed the extracellular tracer, horseradish peroxidase, to determine if direct alterations in the axolemma were detectable in traumatic brain injuries of mild and moderate severity [111,112]. This novel approach was based on the principle that macromolecular tracers, such as horseradish peroxidase, are normally excluded from the axoplasm by an intact axolemma. Therefore, detection of intra-axonal peroxidase activity would constitute evidence of axolemmal disruption. Furthermore, the site of peroxidase activity would delineate the initial site of axonal pertubation, allowing insight into the initiating factors involved in the pathogenesis of secondary axotomy. Their findings demonstrated that the pathobiology of traumatically induced axonal injury was a heterogeneous and complex process involving multiple and varied initiating pathologies.

In particular, the severity of the traumatic injury determined subsequent events in the axolemma and the cytoskeleton resulting in a differential response to the insult. Specifically, following moderate injury, direct pertubations of the axolemma reflected in its altered permeability to macromolecules were detected [111]. This was associated with a rapid local compaction of axonal neurofilaments as evidenced by a decrease in the inter-filament distance. However, following mild traumatic brain injury no evidence of alterations in the axolemma were detected and a different set of cytoskeletal abnormalities, with misalignment and axonal swelling, were demonstrated. A differential response to injury had previously been described in a compression loading model of axonal injury [166]. With low tensile loading, axons assessed in vitro, show

axoplasmic disruption independent of any change in axolemmal integrity, entirely consistent with those changes described with mild traumatic brain injury. With more severe injuries, the same axons revealed axolemmal change that correlates with dramatic axoplasmic failure. Subsequent studies have extended these findings by demonstrating that these alterations in the axolemma and in the cytoskeleton are neither model[112] or species specific[113]. Furthermore, following the demonstration of traumatically induced alterations in axolemmal permeability they suggested that calcium may be involved in the initiation of cytoskeletal events. This apparent contradiction with former views stemmed from the fact that they posited that calcium was acting through previously unidentified mechanisms. Instead of activating proteolytic enzymes, calcium may act less dramatically to alter the neurofilament sidearms, causing the neurofilaments to collapse resulting in their increased packing density. Conceivably these neurofilament sidearms could be cleaved by calcium mediated processes that act on these heavily phosphorylated sidearms. The overall conclusion was that there appeared to be at least two initiating mechanisms in the pathobiology of reactive axonal change, one involving the axolemma and the other involving the cytoskeleton directly[111,112,114].

However, it must be emphasised that these important studies consistently detected another population of large calibre myelinated fibres that demonstrated the features of reactive axonal change without any evidence of axolemmal pertubation reflected in peroxidase passage from the extracellular to axoplasmic compartments [111,112]. This suggests that

alteration in axolemmal permeability to macromolecules, is not essential for the genesis of reactive axonal change. Alternatively, it also raises the possibility that, following injury, the axolemma may be permeable to smaller molecular species capable of evoking changes in the cytoskeleton. Macromolecules such as horseradish peroxidase may be too large to pass through smaller disruptions in the axolemma with the result that the axoplasm remained peroxidase negative despite the presence of membrane pertubation.

CHAPTER 2 - MATERIALS AND METHODS

2.1 ACUTE SUBDURAL HAEMATOMA IN THE RAT

Animals

All investigations were carried out on adult male Sprague-Dawley rats (Harlan Olac., Bicester, Oxon, U.K.) weighing 300-450g. The animals were maintained on a 12 hour light: dark cycle and allowed free access to food and water prior to experimentation. All animal experimentation was carried out under the appropriate Home Office licence.

General Surgical Preparation

The rat was placed in a Perspex chamber and anaesthetised using a mixture of 4% halothane, 30% oxygen and 70% nitrous oxide. The animal was removed from the chamber and placed supine. A tracheostomy was then perfomed and the animal was connected to a mechanical ventilator and maintained thereafter with a nitrous oxide/oxygen mixture (70%:30%) containing 0.5%-1% halothane. Polythene catheters (Portex: external diameter 0.96mm; internal diameter 0.58mm) were inserted into both femoral arteries , for continuous mean arterial blood pressure monitoring (P231D Gould Stratham, Model 2202) and blood gas analysis. The left femoral vein was cannulated for administration of fluids and the withdrawal of venous blood as required. The wounds were closed with nylon sutures. Body temperature was monitored throughout the operative procedure by a rectal thermometer,

and maintained normothermic (37 ± 0.5^{0}C) via a heating blanket controlled by the thermometer.

Production of Acute Subdural Haematoma in the rat

The anaesthetised rats were turned prone and mounted in a stereotactic frame (Kopf, Clarke Electromedical). A midline scalp incision was made, the skin retracted with a self retainer and the coronal and sagittal sutures identified. A burr hole 3mm in diameter was drilled 2mm to the left of the sagittal suture and 1mm posterior to the coronal suture. Using an operating microscope, the dura was incised and a blunt, pre-bent J-shaped needle was inserted into the subdural space with the curved tip pulled up against the overlying dura. Rapid-curing cyanoacrylate glue was used to seal the burr hole and secure the position of the needle. The subdural haematoma was created by the injection of 0.4ml of non-heparinised, freshly withdrawn autologous venous blood into the subdural space over a period of seven minutes. 0.4ml of saline was infused slowly via the femoral venous cannula. The needle was then cut and sealed with glue to prevent back flow. The scalp wound was sutured with nylon. Control animals had insertion of the needle only. Animals were randomly allocated to control or lesion groups and allowed to survive for 30 minutes, 2 hours or 4 hours.

Physiological Variables

The mean arterial blood pressure (MABP) was monitored continuously (P231D Gould Stratham, Model 2202) and maintained at levels above 90mmHg by adjusting the inspired halothane concentration if required. Arterial blood samples were taken before, 30 minutes after and at hourly intervals following insertion of the needle into the subdural space. These samples were used for the determination of the respiratory status (pO_2, pCO_2, and pH) by a blood gas analyser (238pH/Blood Gas System, Ciba Corning). Respiratory volume was adjusted to maintain pCO_2 between 34-42mmHg (normocapnia), and adequate arterial oxygenation (pO_2 >100mmHg) throughout. Body temperature was monitored by a rectal thermometer and the animals maintained normothermic via a heating blanket controlled by the thermometer. Extreme caution was taken to avoid any procedure (e.g. direct heating of the head) which could alter artifactually the gradient between deep body temperature and brain temperature.

Brain Tissue processing for histology and immunohistochemistry.

Transcardiac Perfusion

At the end of the procedure the halothane anaesthesia was deepened and the animal placed supine. A midline thoracotomy exposed the heart. A needle was inserted via the left ventricle into the ascending aorta and clamped in position with a surgical clip. The right atrium was incised and

ice-cold heparinised saline (5000 units/litre) was infused at a pressure equal to the mean arterial blood pressure until the effusate from the right atrium was clear of blood. 200mls of 4% paraformaldehyde in 50mM sodium phosphate buffer (PAM) was then infused at the same pressure. The animal was decapitated, the brain removed and post-fixed in 4% paraformaldehyde in phosphate buffered saline at 4^0 C for 48 hours. After this it was cryoprotected for 2-3 days in 30% sucrose in 50mM sodium phosphate buffer at 4^0C. Following this the brain was frozen in isopentane at -42^0 C for two minutes. 30μm coronal sections were cut in a cryostat and stored in cryoprotectant solution (30% glycerol/30% ehylene glycol in sodium phosphate buffer) at -20 ^{0} C.

Histology

Brain sections were mounted onto poly-lysine coated slides and stained with haemotoxylin and eosin as follows :

1. Air dry the sections.
2. Dehydrate in alcohol 70%-90%-100% each for two minutes.
3. Re-hydrate in alcohol 100%-90%-70% each for two minutes.
4. Wash in distilled water - 1 minute.
5. Haematoxylin - 5 minutes.
6. Wash in distilled water - 3 minutes.
7. Differentiate in acid alcohol (1% hydrochoric acid in alcohol).
8. Wash in distilled water - 3 minutes.
9. Wash in Scots tap water substitute- 2 minutes.

10. Wash in distilled water - 3 minutes.

11. Aqueous eosin -3 minutes.

12. Wash in distilled water - 2 minutes.

13. Dehydrate in alcohol 70%-90%-100% each for two minutes.

14. Histoclear - 4 minutes .

Immunohistochemistry

Free floating tissue sections were rinsed in 50mM phosphate buffered saline (PBS) and incubated in PBS containing 0.2% Triton X-100 for 30 minutes. Endogenous peroxidase activity was blocked by incubating in 3% hydrogen peroxide for 20 minutes.

Monoclonal antibody immunohistochemistry

Following two rinses in PBS the sections were incubated for one hour in a PBS blocking solution containing 10% normal horse serum and 1% bovine serum albumin. Incubation with the primary antibody (Tau-1, 1: 1000; MAP 2, 1:750; β-tubulin, 1:1000), was overnight at 4^0 C. After two rinses with PBS, primary antibody immunostaining was performed using a biotinyated horse anti-mouse secondary antibody (1:100) pre-adsorbed against rat serum for one hour (Vector Laboratories). Sections were then incubated with an ABC Vectastain complex for one hour and peroxidase activity was visualised with 3,3'-diaminobenzidine (Sigma) as the chromogen. Negative controls were included in each experiment where the primary antibody was omitted from the procedure. Sections

were then mounted onto poly-lysine slides, dehydrated, cleared and coverslipped in preparation for microscopic examination.

Polyclonal antibody immunohistochemistry

Following two rinses in PBS the sections were incubated for one hour in a PBS blocking solution containing 10% normal goat serum and 0.5% bovine serum albumin. Incubation with the primary antibody (1:5000), was overnight at 4^0C. After two rinses with PBS, primary antibody immunostaining was performed using a biotinyated goat anti-rabbit secondary antibody (1:100) pre-adsorbed against rat serum for one hour (Vector Laboratories). Sections were then incubated with an ABC Vectastain complex for one hour and peroxidase activity was visualised with 3,3'-diaminobenzidine (Sigma) as the chromogen. Negative controls were included in each experiment where the primary antibody was omitted from the procedure. Sections were then mounted onto poly-lysine slides, dehydrated, cleared and coverslipped in preparation for microscopic examination.

Description of the antibodies

The Tau-1 monoclonal antibody recognises a non-phosphorylated epitope on tau located between amino acids 192-204. Tau-1 binding to tau in axons is increased and, is only detected in neuronal perikarya and glia in normal rat brain sections, after dephosphorylation of the tissue [167]. The MAP 2 monoclonal antibody (clone HM-2; Sigma) reacts with all known

isoforms of MAP 2, namely MAP 2a, MAP 2b and MAP 2c. In normal adult brain it's cellular localisation is differentially distributed in dendrites and neuronal cell bodies. Monoclonal anti β-tubulin (clone no. 2-28-33; Sigma) localises β–tubulin in human and rat tissue providing a specific and useful tool in studying the intracellular distribution of tubulin and the static and dynamic aspects of the cytoskeleton. The antibody (Ab38) used to detect the breakdown products of spectrin specifically generated by calpain proteolysis was provided by Smith Kline and Beecham Inc. (Harlow, England). The antibody recognises the COOH-terminus of the NH_2-terminal spectrin fragment. This antibody shows minimal reactivity with intact spectrin or with spectrin breakdown products generated by proteases other than calpain. The production and characterisation of each of the antibodies has been described in detail previously (Tau-1: [94,167], MAP 2: [80], β– tubulin : [168,169] and Ab38 [170]).

In preliminary studies, serial dilutions of all antibodies were performed to determine the optimum concentration of antibody to be used in the definitive investigations.

Western blots

Preparation of tissue homogenate samples

At 30 minutes or 4hr following the placement of the needle (sham operated) or subdural haematoma induction (lesion), the animals were decapitated. The brain was rapidly removed and the area of cortex underlying the haematoma and corresponding contralateral area of cortex dissected out on ice and frozen in liquid nitrogen. Tissue homogenates were prepared by homogenising the frozen tissue with a Polytron in ice-cold 5mM HEPES buffer pH8 containing 0.32 M sucrose, 5mM benzamidine, 2mM β-mercaptoethanol, 3mM EGTA, 0.5mM MgSO4, 10mm sodium vanadate, 0.1mM phenylmethylsulfonylflouride, 2μg/ml leupeptin, 5μg/ml pepstatin A, 2μg/ml aprotinin. The homogenate was centrifuged at 10 000g for 15minutes at 4°C and the supernatant carefully removed. The protein content of the supernatant was determined according to the method of Lowry et al. (1951) [171]. The protein was adjusted to a protein concentration of 1mg/ml. Samples were then solubilised with buffer composed of 0.1M sucrose, 3% sodium dodecyl sulphate (SDS), 2mMNa_2EDTA, 60mMTris, pH6.9 and 1% β-mercaptoethanol, 0.05% bromophenol blue. Prior to loading on to the gel, samples were heated to 37°C for 10minutes.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and protein transfer

40μg protein from ipsilateral and contralateral cortex of sham-operated and acute subdural haematoma animals were separated by SDS-PAGE (5-20% gradient gel for Tau-1 and β-tubulin; 5-15% gradient gel for MAP2). The gels were run overnight at a constant current of 6mA per gel using an electrophoresis buffer composed of 190mMglycine, 25mM Tris and 0.1%SDS. After electrophoresis, the proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, 0.45μm) at 80V for 3hours in buffer composed of 190mMglycine, 25mMTris and 20%methanol. The extent of transfer and equity of protein loading on the gel were checked by staining the gel in a solution containing 0.05% comassie brilliant blue R, 50%methanol and 10%acetic acid and destaining the gel in the same solution minus the comassie brilliant blue R.

Detection of protein bands

After transfer the nitrocellulose blots were incubated in Tris-buffered saline, TBS (20mMTris-HCl pH7.5, 0.5M sodium chloride, 0.2%Tween-20, TBS-T) containing 3%non-fat dried milk for 2hr at room temperature to block non-specific binding sites. The blots were then washed three times for ten minutes in TBS-T and incubated overnight at 4°C with primary antibodies diluted in TBS-T (Tau-1 1:1000, MAP 2 1:750, β–tubulin 1:500). Blots were then washed as before and incubated with

biotinylated anti-mouse (1:500 in TBS-T, Vector labs.) for 1hr at room temperature. Following three washes in TBS-T, blots were then incubated with streptavidin-alkaline phosphatase (1:1500 in blocking buffer, Amersham) for 1hour at room temperature. The blots were washed and developed using an alkaline phosphatase substrate kit (Bio-Rad).

Measurement of protein bands : Relative optical density.

The resultant protein bands were measured as a relative optical density value using an image analyser (MCID) connected to a camera and corrected for background. In each case five arbitary points were measured within the selected immunoreactive band. The relative optical density values from the ipsilateral and contralateral cortices of injured and control animals were compared using Student's paired t-test.

Brain Tissue processing for transmission electron microscopy (T.E.M.)

Transcardiac Perfusion

At the end of the procedure the inspired halothane was increased to 3% and the animal placed supine. A midline thoracotomy exposed the heart. A needle was inserted via the left ventricle into the ascending aorta and clamped in position with a surgical clip. The right atrium was incised and ice-cold heparinised saline (5000 units/litre) was infused at a pressure equal to the mean arterial blood pressure until the effusate from the right atrium was clear of blood. At this point 250 mls of 2.5% glutaraldehyde

in a 0.2M Pipes (piperazine N-N'-bis-2-ethanol sulphonic acid) buffer (pH 7.6) was infused over 60 minutes. The animal was then decapitated, the brain dissected out and post fixed in 2.5% glutaraldehyde in a 0.2M Pipes buffer (pH 7.6) for 48hours at 4^0 C. Following this stage, the cerebral hemispheres were detached from the brain stem and cerebellum and cut into 6mm thick coronal slices and stored in the same fixative.

Transmission Electron Microscopy

Three to five 1-mm^3 blocks of tissue were removed from the cerebral cortex under the acute subdural haematoma and the ipsilateral corpus callosum. Control tissue (sham operated) and naive (perfusion fixation only) was also prepared. Blocks were osmicated in 1% osmium tetroxide in 0.2M Pipes buffer for 1 hour. Rinsed three times in 0.2M Pipes buffer and then dehydrated in alcohols and propylene oxide as follows :

1. 70% alcohol for 20 minutes.
2. 90% alcohol for 20 minutes.
3. 100% alcohol for 20 minutes.
4. Rinsed three times in Propylene oxide for 15 minutes each rinse.

Following dehydration the specimens were embedded in Araldite resin using epoxypropane as an intermediary. Transverse semithin sections were cut from all blocks using a glass knife. Serial thin sections were cut on a diamond ultramicrotome from one randomly selected block from each animal and stained in 12.5% methanolic uranyl acetate and lead

citrate for ultrastructural examination on a Philips 301 transmission electron microscope.

2.2 ACUTE SUBDURAL HAEMATOMA IN HUMAN POST MORTEM TISSUE

Post Mortem Brain Tissue

All cases of fatal head injury had been managed by the Department of Neurosurgery, Institute of Neurological Sciences, Southern General Hospital, Glasgow and ethical committee approval had been granted for the use of this tissue in these studies. Subjects sustaining an acute subdural haematoma were identified from autopsy examination. Control patients had no known history of neurological or neuropsychiatric impairment. All brains in both groups were free from gross tissue abnormalities such as tumours and infarcts. The charateristics of control and acute subdural haematoma brains are listed in Tables 1 and 2.

Full autopsies were performed on each case. Brains were fixed in 10% formol saline for at least three weeks before dissection. Cerebral hemispheres were cut in the coronal plane, the cerebellum at right angles to the folia, and the brain stem horizontally. Standard blocks of tissue were cut from these slices. Comprehensive studies were carried out in each case including assessments of the amount of contusional injury, the severity of any diffuse axonal injury, the severity of any ischaemic brain

damage and whether the intracranial pressure had been high during life as a result of a supratentorial expanding lesion. A similar number of controls matched for age and gender, were also studied.

All autopsies and gross dissection of the brains were performed by the Department of Neuropathology, Institute of Neurological Sciences, Southern General Hospital, Glasgow.

Table 1

Clinical and neuropathological details of fatally head injured patients with an acute subdural haematoma.

Case No.	Age	Sex	Survival	Post mortem delay	Cause of death	Intracranial haematoma	Ischaemic brain damage
H 33	51	M	12.5d	67h	raised ICP	Left ASDH (E)	Extensive
H 35	62	M	27h	17h	raised ICP	Left ASDH (NE)	Extensive
H 21	41	M	17h	42h	raised ICP	Right ASDH(NE)	Minimal
H 34	21	M	216d	67h	raised ICP	Left ASDH (E)	Severe
H 25	52	M	96h	120h	raised ICP	Left ASDH (E)	Extensive
H 27	47	M	60h	69h	raised ICP	Left ASDH (E)	Extensive

Abbreviations used : ASDH: acute subdural haematoma E: evacuated, N: not evacuated, ICP : intracranial pressure

Table 2

Clinical details of neurological control patients.

Case No.	Age	Sex	Survival	Post mortem delay	Cause of death	Head Injury or intracranial haematoma	Other major pathologies
N8	64	F	0h	31h	I.H.D.	No	None
N6	68	F	4h	22h	I.H.D	No	None
N4	52	M	0h	63h	I.H.D	No	None
N12	29	F	0h	48h	P.T.E.	No	None
N1	77	F	0h	42h	I.H.D.	No	None
N5	80	F	0h	62h	R.A.A.A.	No	None
N7	71	M	0h	57h	I.H.D	No	None
N11	59	M	0h	45h	M.I.	No	None
N10	62	M	0h	54h	P.T.E.	No	None

Abbreviations used : I.H.D. : ischaemic heart disease,
P.T.E. : pulmonary thromboembolism,
R.A.A.A. : ruptured abdominal aortic aneurysm.

Processing of brain tissue for histology and immunohistochemistry

The blocks of brain were post fixed in 4% paraformaldehye (PAM) in a sodium phosphate buffer at 4^0C for 4 days . After 24 hours the PAM was changed and any membranes or blood vessels adherent to the brain tissue removed. The blocks were then transferred to 30% sucrose in 50mM sodium phosphate buffer at 4^0 C and cryoprotected for 5 days. Solutions were changed as necessary. Following this the block was frozen in isopentane at -42^0C for two minutes. 30μm coronal sections from the superior sagittal blocks were cut in a cryostat, mounted onto polylysine slides and stored at -80^0C.

Histology

Human brain tissue sections were stained with haemotoxylin and eosin as follows :

1. Air dry the sections.
2. Dehydrate in alcohol 70%-90%-100% each for two minutes.
3. Re-hydrate in alcohol 100%-90%-70% each for two minutes.
4. Wash in distilled water - 1 minute.
5. Haematoxylin - 5 minutes.
6. Wash in distilled water - 3 minutes.
7. Differentiate in acid alcohol (1% hydrochoric acid in alcohol).
8. Wash in distilled water - 3 minutes.

9. Wash in Scots tap water substitute- 2 minutes.
10. Wash in distilled water - 3 minutes.
11. Aqueous eosin -3 minutes.
12. Wash in distilled water - 2 minutes.
13. Dehydrate in alcohol 70%-90%-100% each for two minutes.
14. Histoclear - 4 minutes .

Immunohistochemistry

The sections were rinsed twice in 50mM phosphate buffered saline (PBS) and incubated in PBS containing 0.2% Triton X-100 for 30 minutes. Endogenous peroxidase activity was blocked by incubating in 3% hydrogen peroxide in methanol for 20 minutes. Following two rinses in PBS the sections were incubated for one hour in a PBS blocking solution containing 10% normal horse serum and 1% bovine serum albumin (BSA). Incubation with the primary antibody (Tau-1 , 1: 750; MAP 2, 1:750; β-tubulin, 1:750) was overnight at 4^0 C. After two rinses with PBS, primary antibody immunostaining was performed using a biotinyated horse anti-mouse secondary antibody (1:100) pre-adsorbed against rat serum for one hour (Vector Laboratories). Sections were then incubated with an ABC Vectastain complex for one hour and peroxidase activity was visualised with 3,3'-diaminobenzidine (Sigma) as the chromogen. Negative controls were included in each experiment where the primary antibody was omitted from the procedure. Sections were then dehydrated, cleared and coverslipped in preparation for microscopic examination.

2.3 AXOLEMMAL PERMEABILITY FOLLOWING TRAUMATIC BRAIN INJURY

Animals

All investigations were carried out on adult male Sprague-Dawley rats (Hilltop Lab Animals Inc., Scottsdale, PA) weighing 350-400g. All animals were housed in individual cages in a climate controlled room and allowed intake of water and rat chow, ad libitum. All protocols for injury and use of animals were approved by the Institutional Animal Care and Use Committee.

Surgical Preparation

The rat was placed in a Perspex chamber and anaesthetised using a mixture of 4% halothane, 30% oxygen and 70% nitrous oxide. The animal was removed from the chamber and placed supine. A tracheostomy was then perfomed and the animal was connected to a mechanical ventilator and maintained thereafter with a nitrous oxide/oxygen mixture (70%:30%) containing 0.5%-1% halothane. Body temperature was monitored throughout the surgical procedure by a rectal thermometer, and maintained normothermic (37±0.5 ^{0}C) via a heating blanket controlled by the thermometer.

Cannulation of the cisterna magna and withdrawal of cerebro-spinal fluid (CSF).

The anaesthetised rat was placed prone and attached to a stereotactic frame (Kopf, Clarke Electromedical). The rats head was positioned in the frame to provide optimal flexion of the cervical spine without compromising the airway. A 1.5cm midline incision was made from the occiput to the posterior tubercle of the atlas and the atlanto-occipital membrane identified, and directly visualised, with a combination of blunt and sharp dissection. A 25 guage spinal needle was attached to an arm of the stereotactic frame and positioned superior to the atlanto-occipital membrane. The needle was advanced slowly penetrating the atlanto-occipital membrane. The cisterna magna was tapped and 0.05cc of cerebro-spinal fluid(CSF) was slowly withdrawn into a tuberculin syringe. This CSF was mixed with 5mg of microperoxidase (Sigma Chemical Co., St.Louis, MO, USA) or 5mg of horseradish peroxidase (Sigma Chemical Co., St.Louis, MO, USA) and the mixture was slowly reinfused into the cisterna magna from where the tracer could diffuse through the CSF compartment to reach the extracellular space. The needle was withdrawn and the wound closed with nylon sutures. The animal was removed from the stereotactic frame and placed supine on the heating blanket. The extracellular tracer was allowed to circulate for thirty minutes. During this time the animal was prepared for transfer to the injury device. Animals were randomly allocated to control or lesion groups.

Production of the impact acceleration closed head injury in the rat.

A midline scalp incision was performed and the periosteum covering the vertex reflected. A stream of air was used to keep the area dry. A stainless steel disc, 1cm in diameter and 3mm thick, was fixed to the central portion of the skull vault between the coronal and lambdoid sutures with dental acrylic. The adhesive was left to dry for five minutes. The animal was disconnected from the respirator, transferred to the injury device and placed in a prone position on a foam bed of known spring constant (Type E bed manufactured by Foam to Size, Inc., Ashland, Virginia) contained within a Plexiglass frame. Two belts secure the rat to the foam bed. The lower end of the vertical section of the plexiglass tube was positioned directly above the stainless steel disc. The injury was delivered by dropping the brass weight (450grammes) from 2 metres through the vertical section of the plexiglass tube onto the metallic disc. Rebound impact was prevented by sliding the foam bed containing the animal away from the tube immediately following the initial impact. The animal was then re-connected to the ventilator. No animal was disconnected from the ventilator for more than one minute. Post injury observations were made for convulsions, haemorrhage or the presence of a skull fracture. Control animals were exposed to mock injury with the same temporal course of anaesthesia. The metallic disc was removed, the scalp sutured and the animal allowed to survive for 5, 15, or 60 minutes post injury.

Brain Tissue Processing for histochemistry and electron microscopy

Transcardiac Perfusion

At the end of the procedure the halothane was increased to 3% and the animal placed supine. A midline thoracotomy exposed the heart. A needle was inserted via the left ventricle into the ascending aorta and clamped in position with a surgical clip. The right atrium was incised and ice-cold heparinised saline (5000 units/litre) was infused at 90mmHg until the effusate from the right atrium was clear of blood. 700mls of fixative containing 2.5% glutaraldehyde and 2% paraformaldehyde in a sodium phosphate buffer was then infused at the same pressure over a period of one hour. The animal was decapitated and the brain removed taking care to preserve the cervical spinal cord. Next, the brainstems were blocked sagittally and transferred to the stage of a vibratome, where 50μm serial sections were obtained. Alternate serial sections were prepared for the histochemical visualisation of the microperoxidase or processing for electron microscopy. All sections were stored in a sodium phosphate buffer at 4^0C.

Histochemistry : The Cobalt-Glucose Oxidase Reaction

Sections selected for peroxidase reaction product visualisation were processed using a cobalt-glucose oxidase reaction. The vibratomed sections were rinsed three times in a Tris-buffered saline (TRIS) pH 7.6 and then reacted with 0.5% cobalt chloride in Tris-buffered saline for 10

minutes. Then three rinses in Tris-buffered saline and two rinses in 0.1M sodium phosphate buffer. The sections were then incubated twice for 1 hour at 37^0C with 0.05% diaminobenzidine, 0.2% B-D glucose, 0.04% ammonium chloride, and 0.00041% glucose oxidase type II in a 0.1M sodium phosphate buffer solution and rinsed three times for 5 minutes in 0.1M sodium phosphate buffer. Sections were mounted on glass slides, dehydrated and cleared with alcohol and xylene for light microscopic evaluation.

Transmission Electron Microscopy

The alternate serial sections selected for electron microscopy were osmicated in 1% Osmium Tetroxide in a phosphate buffer (0.1M Sorensen buffer, pH 7.2-7.3) for one hour. Rinsed three times in 0.1M sodium phosphate buffer and then dehydrated in alcohols and propylene oxide as follows :

1. 30%, 40%, 50%, 60%, 70% alcohol for 5 minutes in each.
2. 80%, 95%, 95%, 100%, 100% alcohol for 10 minutes each.
3. Rinsed twice in Propylene oxide for 10 minutes each rinse.
4. Rinse once in 100% alcohol/ propylene oxide (50:50 mix) for 5 minutes.

The tissue sections were then placed on an agitator overnight in a 50:50 mixture of propylene oxide and eponate 12. Following this, the solution was changed and the sections were placed in 100% eponate 12 and

agitated for 4 hours. The sections were then flat embedded in Medcast resin on plastic slides (Thomas Scientific Co.) and incubated overnight at 60 °C. The next day the plastic slides were separated and the sections further incubated at 60 °C for 24-30 hours. These flat embedded materials were screened at the light microscopic level and compared with those sections cleared for light microscopy.

Regions demonstrating extracellular peroxidase or intraaxonal peroxidase influx or both were identified in the cleared sections, and comparable regions of the flat embedded materials examined for the presence of the peroxidase reaction product. Once those regions demonstrating peroxidase activity were identified, they were cut out and mounted on Medcast resin blocks. Thick sections of 1.5μm were cut on an LKB 2128 ultramicrotome with glass knives. Thin sections were cut with a diamond knife (Diatome), picked up on Formvar-coated slotted grids, and stained in 5% uranyl acetate in 50% methanol and 0.3% lead citrate for ultrastructural examination on a JEOL 1200 electron microscope.

Quantitative analysis of peroxidase containing axons

Both the horseradish peroxidase (HRP) and the microperoxidase (MP) slides were examined under a microscope (Nikon Inc., x10). Based upon the finding that peroxidase flooded axons occurred mainly in the lateral ponto-medullary and cervico-medullary regions of the brain stem, the lateral ponto-medullary junction was selected for data analysis. In this region, five semi-serial 50μm sections from each animal were randomly

selected and detailed analysis was carried out to determine the number of HRP and MP containing axons per unit area. The counting and area measurement were performed with the MCID M4 imaging program (Imaging Research Inc., St. Catharines, Ontario, Canada) using a Nikon BH-2 microscope (40x with a 2.5x intermediate lens). After the microscopic image was digitalised (Dage MTI Inc., Michigan City, IN.), the flooded axons were counted and marked with a circle overlay on the image to prevent duplicate counting. The area in which the flooded axons were counted corresponded to the area covered by the monitor, encompassing 50,000 μm^2. Once the values were obtained, the means and standard deviation was determined.

CHAPTER 3 - RESULTS

3.1 ALTERATIONS IN THE NEURONAL CYTOSKELETON FOLLOWING ACUTE SUBDURAL HAEMATOMA IN THE RAT.

3.1.1 Physiological variables

At all time points examined there were no significant differences between the sham operated and subdural haematoma groups with respect to mean arterial blood pressure (MABP), arterial blood gas status or rectal temperature (Tables 3a-c). The production of a subdural haematoma caused an increase in MABP immediately following the infusion of blood (Cushing response). This was a consistent finding and in all cases the MABP returned to pre-infusion levels within 15 minutes.

3.1.2 Gross Neuropathology.

In all animals the haematoma was contained within the subdural space ipsilateral to the side of injection. It usually extended over much of the lateral aspect of the hemisphere, its thickest point measuring some 2-3mm in the posterior/parietal region. As a result of the mass effect of this haematoma there was deformation of the underlying cerebral hemisphere in all animals, the surface of which usually had an obvious concave contour.

Table 3a

Physiological variables during the period of survival after production of an acute subdural haematoma in the rat : 30 minutes survival animals.

Sham operated group.

Time after insertion of needle	Mean arterial blood pressure (mmHg)	Temperature	pO2 (mmHg)	pC02 (mmHg)	pH
0	102±9	36.8± 0.3	156 ± 5	41.5 ± 1.3	7.42 ± 0.02
30 mins.	105±10	36.7± 0.1	162 ±9	42.0 ± 0.6	7.41 ± 0.03

Data are presented as mean ± SEM (n=7). Data represents values at the time of insertion of the needle into the subdural space and at thirty minutes.

Subdural haematoma group.

Time after production of ASDH	Mean arterial blood pressure (mmHg)	Temperature	pO2 (mmHg)	pC02 (mmHg)	pH
0	104±11	36.7 ± 0.3	147 ± 7	41.2 ± 1.7	7.39± 0.01
30 mins	106±14	36.9 ± 0.2	150 ± 5	40.8 ± 1.1	7.44 ± 0.02

Data are presented as mean ± SEM (n=11). Data represents values at the time of induction of acute subdural haematoma (ASDH) and at thirty minutes.

Table 3b

Physiological variables during the period of survival after production of an acute subdural haematoma in the rat : 2 hour survival animals.

Sham operated group.

Time after insertion of needle (hours).	Mean Arterial Blood Pressure (mm Hg)	Temperature	pO2 (mmHg)	pC02 (mmHg)	pH
0	100± 8	36.9± 0.2	146 ± 5	42.5 ±1.3	7.41 ± 0.01
0.5	107± 4	36.7± 0.1	162 ±7	42.3 ± 0.6	7.43 ± 0.02
1	105± 10	36.9± 0.1	162 ± 8	39.9 ±1.3	7.44 ± 0.01
2	95± 4	37.0± 0.1	156 ±8	38.9 ± 0.7	7.41 ± 0.02

Data are presented as mean ± SEM (n=2). Data represents values at the time of insertion of the needle into the subdural space and at intervals thereafter.

Subdural haematoma group.

Time after production of ASDH (hours).	Mean Arterial Blood pressure (mmHg)	Temperature	pO2 (mmHg)	pC02 (mmHg)	pH
0	100±6	36.7 ± 0.3	147 ± 7	41.2 ± 1.7	7.39± 0.01
0.5	102±12	36.9 ± 0.2	150 ± 5	40.8 ± 1.1	7.44 ± 0.02
1	101±8	37.2± 0.1	157± 5	42.5± 0.9	7.38± 0.01
2	98±5	36.9 ± 0.1	155 ± 6	41.8 ± 1.0	7.41 ± 0.01

Data are presented as mean ± SEM (n=6). Data represents values at the time of induction of acute subdural haematoma (ASDH) and at intervals thereafter.

Table 3c

Physiological variables during the period of survival after production of an acute subdural haematoma in the rat : 4 hour survival animals.

Sham operated group.

Time after insertion of needle (hours).	Mean Arterial Blood Pressure (mm Hg)	Temperature (°C)	pO2 (mmHg)	pC02 (mmHg)	pH
0	105± 08	36.8± 0.1	136 ± 5	43.5 ±1.3	7.44 ± 0.01
0.5	108± 10	36.9± 0.2	152 ±10	42.3 ± 0.9	7.45 ± 0.02
1	106± 07	36.9± 0.1	165 ± 8	38.9 ±1.2	7.43 ± 0.01
2	98± 10	37.0± 0.1	166 ±8	38.5 ± 0.8	7.42 ± 0.02
3	101± 11	37.0± 0.1	169 ± 7	40.1 ± 1.0	7.41 ± 0.03
4	100± 05	37.0 ± 0.2	171 ± 6	41.2 ± 0.9	7.42 ± 0.01

Data are presented as mean ± SEM (n=9). Data represents values at the time of insertion of the needle into the subdural space and at intervals thereafter.

Subdural haematoma group.

Time after production of ASDH (hours).	Mean Arterial Blood Pressure (mm Hg)	Temperature	pO2 (mmHg)	pC02 (mmHg)	pH
0	102± 11	36.7 ± 0.2	137 ± 7	41.2 ± 1.5	7.43 ± 0.01
0.5	107± 5	36.9 ± 0.1	160 ± 5	39.8 ± 1.1	7.45 ± 0.02
1	102± 7	37.0 ± 0.1	160 ± 6	38.8 ± 1.0	7.44 ± 0.01
2	98± 4	36.9 ± 0.1	150 ± 5	40.5 ± 0.9	7.41 ± 0.01
3	94± 8	37.0 ± 0.2	156 ± 4	40.5 ± 1.0	7.42 ± 0.01
4	95± 9	36.9 ± 0.1	165 ± 5	41.0 ± 0.9	7.42 ± 0.02

Data are presented as mean ± SEM (n=15). Data represents values at the time of induction of acute subdural haematoma (ASDH) and at intervals thereafter.

Removal of the subdural haematomas revealed a cortical surface that was usually free of blood. However, there were one or two instances of small amounts of subarachnoid haemorrage and in many instances the meningeal vessels immediately beneath the subdural haematoma were engorged, in contrast to the remainder of the specimen which was essentially bloodless as a result of the perfusion fixation procedure.

Coronal sections of these specimens also revealed that the meningeal and intracortical vessels deep to the haematoma, were invariably engorged. This gave a pink appearance in contrast to the remainder of the specimen which was blanched white.

3.1.3 Neurohistopathology.

With the exception of the tissue immediately beneath the haematoma the brains were well perfused and fixed and free of the histological artefacts, " dark cell and hydropic cell change" [172]. In contrast, the cortex beneath the haematoma was engorged and in a few instances a small amount of bleeding had occured into the cortex : this occured most often at the site of surgical procedure.

The histological appearances seen at 30 minutes, 2 hours and 4 hours were similar although more advanced with the longer survival times (Figure 3). The principal changes were those of a volume of tissue immediately beneath the subdural haematoma that was undergoing sequential changes consistent with early infarction.

Figure 3

Ischaemic brain damage after induction of an acute subdural haematoma in the rat.

Coronal sections stained with haematoxylin and eosin 30 minutes and 4 hours after production of an acute subdural haematoma in the rat. Note the region of pallor in the cortex underlying the haematoma. In addition, note the mechanical effect the haematoma has on the ipsilateral corpus callosum.

30min H+E

4h H+E

The margins of the affected tissue were evident at 30 minutes, as a band of tissue that was pale in the H&E stained sections as a consequence of loss of eosinophilia of neurones and vascuolation of the neuropil. Such changes extended from the superficial layers to the junction between grey and white matter and were present to an equal extent at all the margins. Medially, the margin of the lesion represented the "boundary" between the distributions of the middle and anterior cerebral arteries and laterally it was thought to represent the "boundary" between the distributions of the middle and posterior cerebral arteries.

At 30 minutes the neurones at the margin of the lesion were contracted, rather triangular in shape, and more basophilic in staining than usual, the appearances being those of the ischaemic cell process (Figure 4). These changes were in marked contrast to those seen in the adjacent normal tissue and indeed within the centre of the lesion, the neuronal staining properties and the histological appearance of which were entirely normal. After 2 hours the changes had evolved to the extent that the boundary was now more obvious with clear separation between affected and normal tissue. Again the boundary was sharp, measuring no more the 50-100μm in width. At the boundary the neuronal changes had developed to the extent that some of them now demonstrated the features of the ischaemic cell process with incrustation formation. The vacuolation of the neuropil was more pronounced and minimal changes were seen in the astrocytes and microglia.

Figure 4

The histopathological features of the ischaemic cell process after production of an acute subdural haematoma in the rat.

Micrograph of the rat cortex stained with haematoxylin and eosin 4 hours after production of an acute subdural haematoma. The cortex demonstrates features of the ischaemic cell process with early infarction, vacuolation of the neuropil and swelling of astrocytes.

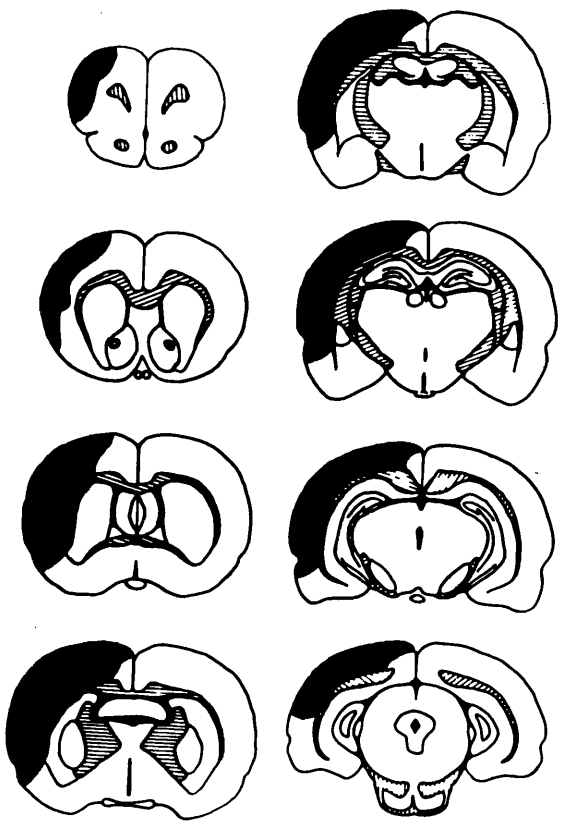
By 4 hours the lesion had developed further with better definition of its boundaries. The ischaemic cell process at the margins of the lesion was also better developed. Although changes within the centre of the lesion were still minimal there was some variation in the intensity of staining, suggesting that early ischaemic changes were also beginning to develop within the centre of the lesion.

At none of the time points examined were abnormalities detected other than in the cortex immediately beneath the haematoma either within the ipsilateral or contralateral hemisphere. The extent and distribution of ischaemic brain damage following production of an acute subdural haematoma in the rat is indicated in Figure 5.

Figure 5

The extent and distribution of ischaemic brain damage at eight predetermined stereotactic planes through the rat brain.

Reproduced with the permission of Mr R.Bullock and colleagues (Miller et al., 1990)



3.1.4 Immunohistochemical analysis of the microtubule component of the neuronal cytoskeleton following acute subdural haematoma in the rat

In total 24 animals were processed for immunohistochemistry. At each time point (30 minutes, 2 hours and 4 hours) 6 animals underwent induction of an acute subdural haematoma and 2 animals were sham operated only.

Tau -1

In control animals (n= 2) the distribution of Tau-1 immunostaining was the same as has been previously described being predominantly localised to axons with minimal staining in the neuronal perikarya and glia [94,167].

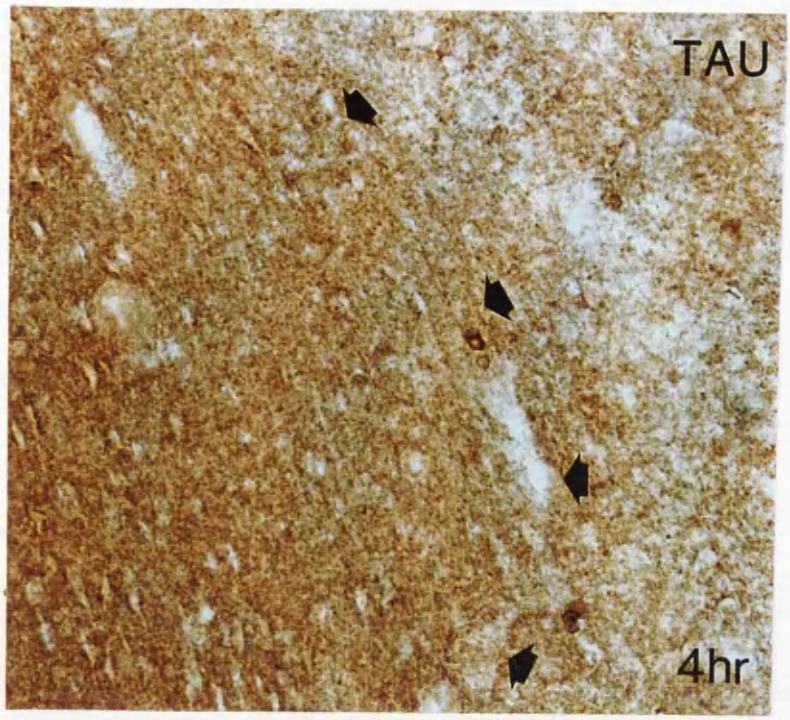
In all animals examined 4 hours after induction of a subdural haematoma (n=6) there was an increase in the intensity of Tau -1 immunostaining in the neuropil of the ipsilateral cerebral cortex compared to the contralateral cortex (Figure 6). A clear line of demarcation, which corresponded to the histologically defined boundary of ischaemic damage, was present. The enhanced immunolabelling of the neuropil was restricted to tissue within this region. A similar effect was detected 2 hours after production of an acute subdural haematoma although the increase in immunoreactivity of the neuropil was less pronounced than at 4 hours. No clear alteration in neuropil immunostaining was present in any of the 30 minutes post subdural haematoma(n=6) group. The neuropil of the contralateral

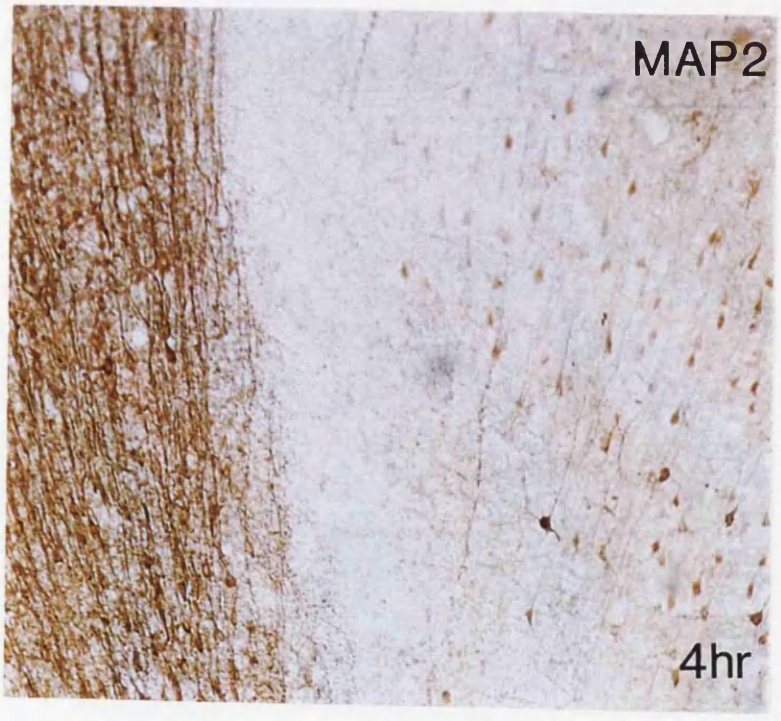
cortex appeared similar to sham operated cortex throughout the post injury period, with no changes in Tau-1 immunoreactivity detected.

Figure 6

Immunostaining of Tau-1 and MAP 2 in cerebral cortex 4 hours after production of an acute subdural haematoma.

There is an increase in Tau-1 immunolabelling of the neuropil within the histologically defined ischaemic territory. A clear line of demarcation (arrows) indicates adjacent histologically non-ischaemic cortex underlying the haematoma. Note the pronounced loss of MAP 2 immunoreactivity within the ischaemic region and the well defined boundary this forms with the adjacent histologically non-ischaemic brain.


TAU
4hr

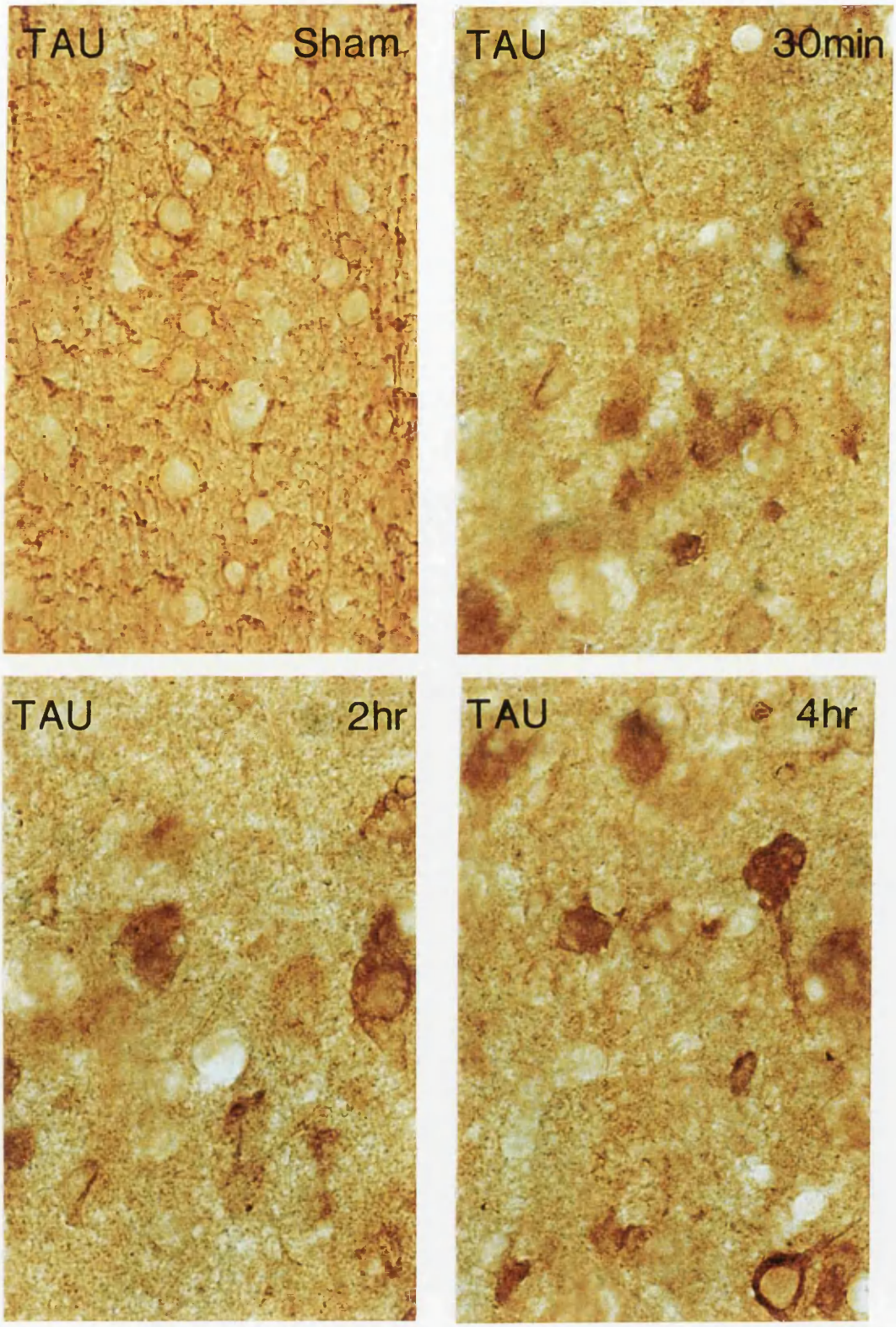

MAP2
4hr

In all animals and, at every time point examined, characteristic cellular changes in Tau-1 immunorectivity were identified within the ipsilateral cortex of animals subjected to subdural haematoma compared to the contralateral cortex. Specifically, Tau-1 immunoreactivity was increased in a proportion of neuronal perikarya within the ischaemic region (Figure 7). Although isolated Tau-1 immunopositive perikarya could be identified throughout the ipsilateral cortex, these cells were most common at the boundary between histologically normal tissue and ischaemic tissue. There was no apparent laminar distribution of these cells within the cerebral cortex. Quantitative analysis was not performed but there was the distinct impression that the number of Tau-1 reactive neurones was decreased in the 4 hour group of animals compared to the 2 hour group. This decline correlated with the observed increase in the number of neurones demonstrating abnormal morphology in the corresponding histological sections.

Figure 7

Immunostaining of Tau-1 in the cerebral cortex after induction of an acute subdural haematoma.

Immunostaining of Tau-1 in cerebral cortex in a sham operated rat (SHAM) and at 30 minutes, 2 hours and 4 hours after production of an acute subdural haematoma. Note the increase in Tau-1 immunostaining in neuronal perikarya and glial cells in the cortex after acute subdural haematoma.


TAU
Sham
TAU
30min
TAU
2hr
TAU
4hr

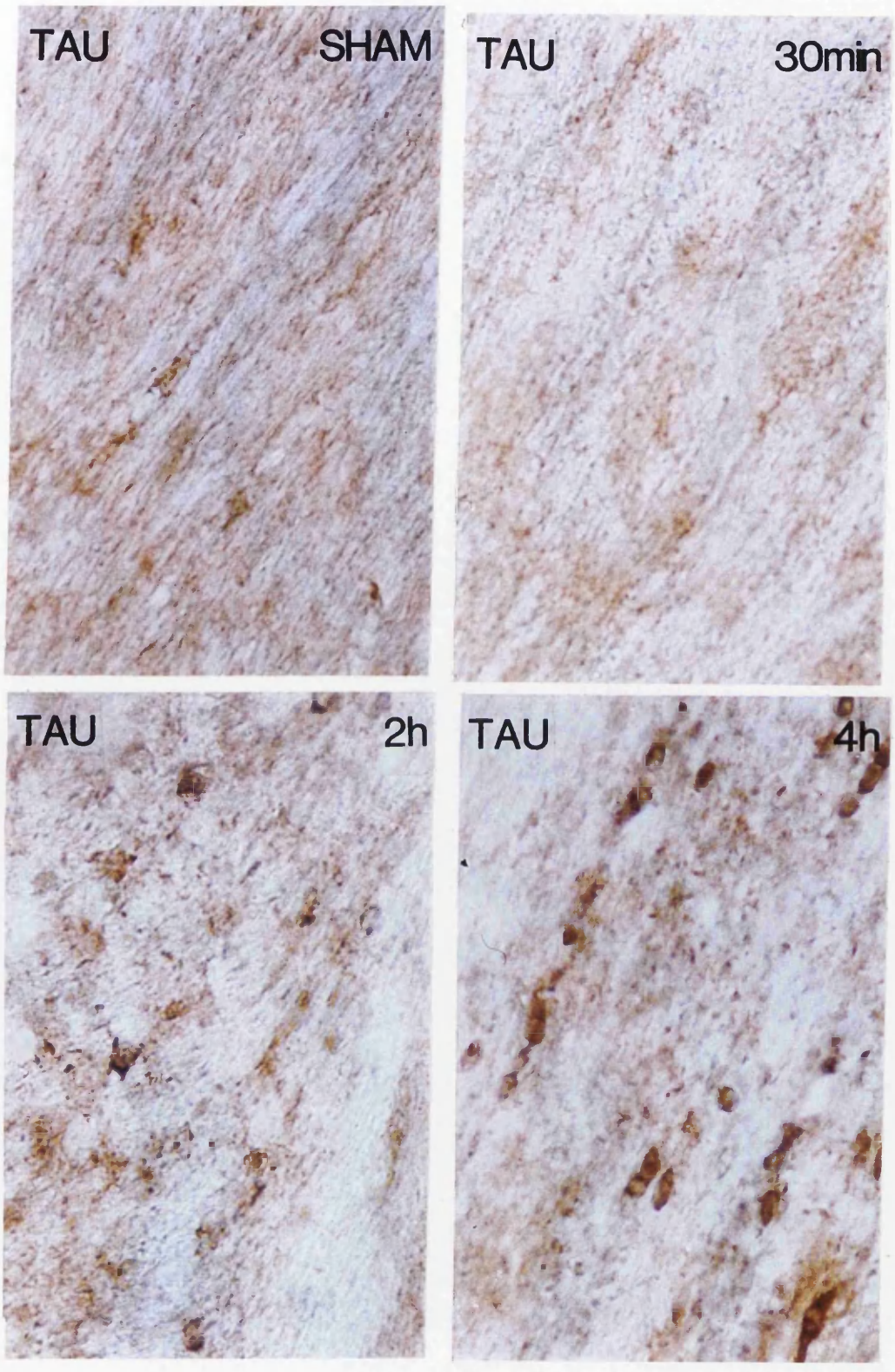
As early as 30 minutes post injury the ipsilateral white matter tracts demonstrated disturbances in Tau-1 immunoreactivity compared to the contralateral white matter tracts (Figure 8). These changes progressed with time being most apparent in the 4 hour group of animals. Typically, the intensity of Tau -1 immunostaining was increased in the axons of the corpus callosum adjacent to the ischaemic lesion. The altered immunoreactivity appeared irregular with a granular effect and was most concentrated towards the midline of the brain. This appearance was in marked contrast to the smooth, regular distribution of Tau-1 immunostaining identified in the white matter tracts from both sham operated and contralateral subdural haematoma sections .

Numerous Tau -1 positive glial cells were detected, scattered throughout the injured hemisphere. This feature was present as early as 30 minutes following production of the subdural haematoma. The Tau-1 reactive glial cells were typically small round cells arranged in a linear pattern and were predominantly located in the white matter (Figure 8).

Figure 8

Immunostaining of Tau-1 in myelinated fibre tracts in the corpus callosum after induction of an acute subdural haematoma.

Immunostaining of Tau-1 in myelinated fibre tracts in the corpus callosum in a sham operated rat (SHAM) and at 30 minutes, 2 hours and 4 hours after production of an acute subdural haematoma. Note the progressive disruption in the pattern of Tau-1 immunostaining after acute subdural haematoma.


TAU
SHAM
TAU
30min
TAU
2h
TAU
4h

Microtubule associated protein 2 (MAP 2)

In control animals (n=2) the distribution of MAP 2 immunostaining was the same as has been previously described [83]. MAP 2 was restricted to neurones, being preferentially associated with dendritic processes and not detected in axons. The dendrites were arranged in slender elongations radiating from the cerebral cortex and were more heavily stained than the neuronal cell bodies.

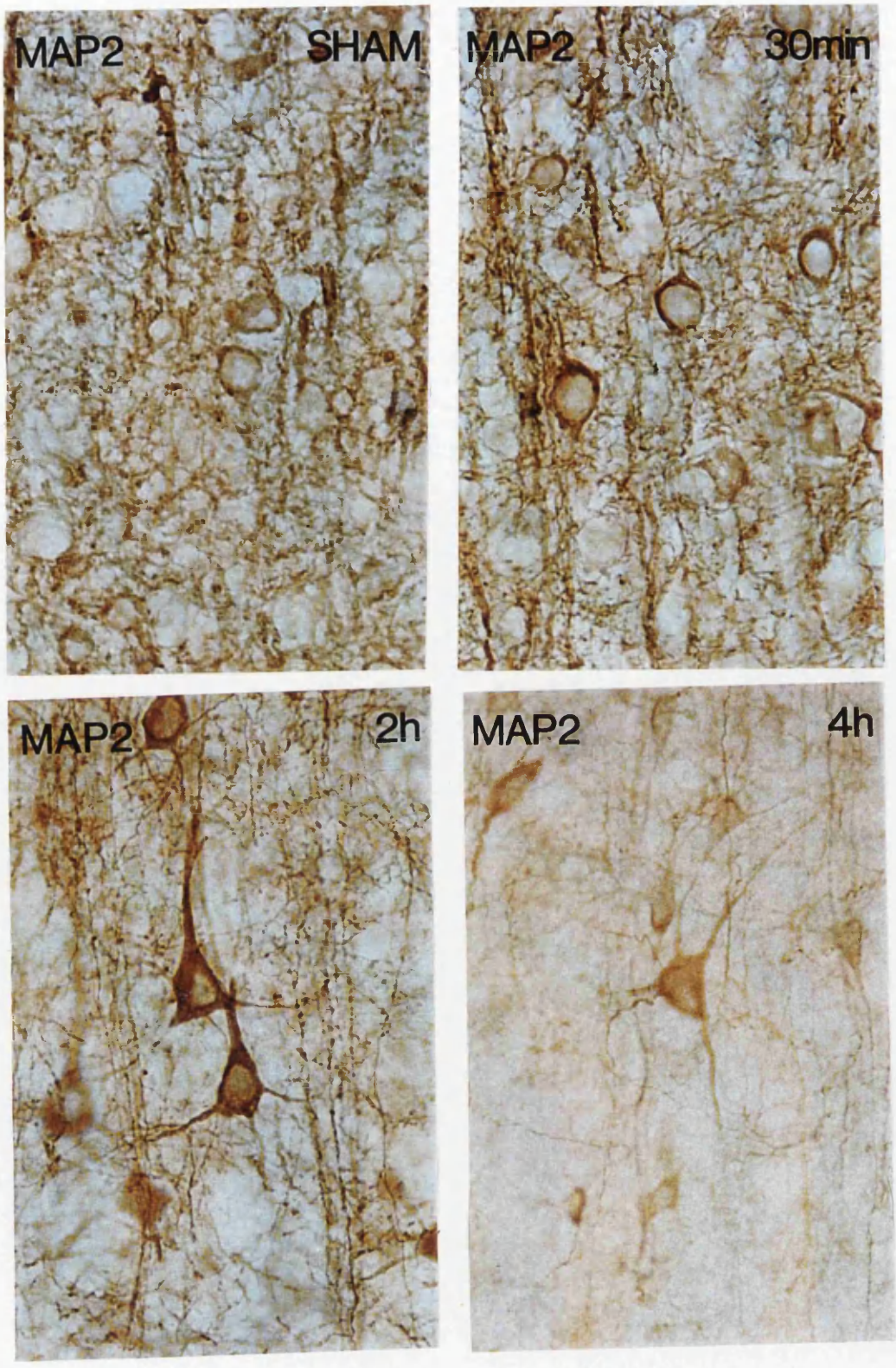
In all of the 4 hour subdural haematoma animals (n=6) there was a reduction in MAP 2 immunostaining in the cortex beneath the haematoma compared to the contralateral cortex. The area of decreased immunolabelling was well delineated with distinct margins and normal MAP 2 immunolabelling was maintained lateral to the affected area in the ipsilateral cortex (Figure 6). The histologically defined ischaemic lesion approximated to the MAP 2 immunonegative cortex. Within this area there was prominent loss of dendritic processes in comparison to the sham operated animals (Figure 9). Nevertheless, several densely stained, distorted dendrites were usually detectable and the occassional neuronal perikaryon was strongly immunoreactive. A similar, although less advanced, appearance was present in the cortex of animals examined 2 hours after production of an acute subdural haematoma (n=6). However in this group, the region of immunonegativity was less uniform than after 4 hours and there was patchy preservation of MAP 2 immunostaining within the defined margins of the ischaemic lesion (Figure 9) . In contrast, 30 minutes post injury (n=6) the distribution and localisation of

Figure 9

Immunostaining of MAP 2 in the cerebral cortex after induction of an acute subdural haematoma.

Immunostaining of MAP 2 in cerebral cortex in a sham operated rat (SHAM) and at 30 minutes, 2 hours and 4 hours after production of an acute subdural haematoma. Note the progressive loss of MAP 2 immunostaining in the cortex after acute subdural haematoma. In addition, certain neuronal perikarya are MAP 2 immunopositive with prominent MAP 2 immunolabelled dendritic processes.

MAP 2, as detected by immunohistochemistry, was not effected by the subdural haematoma with no obvious alterations in the intensity of the cortical MAP 2 immunostaining detected at this time point.

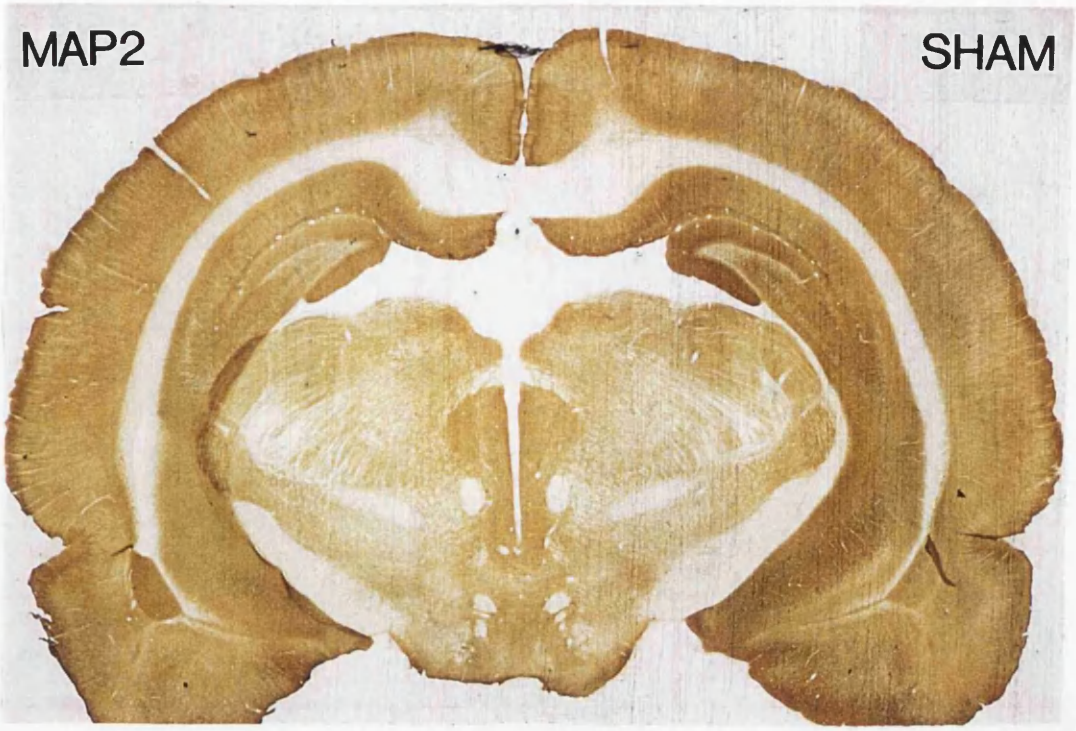

MAP2
SHAM
MAP2
30min
MAP2
2h
MAP2
4h

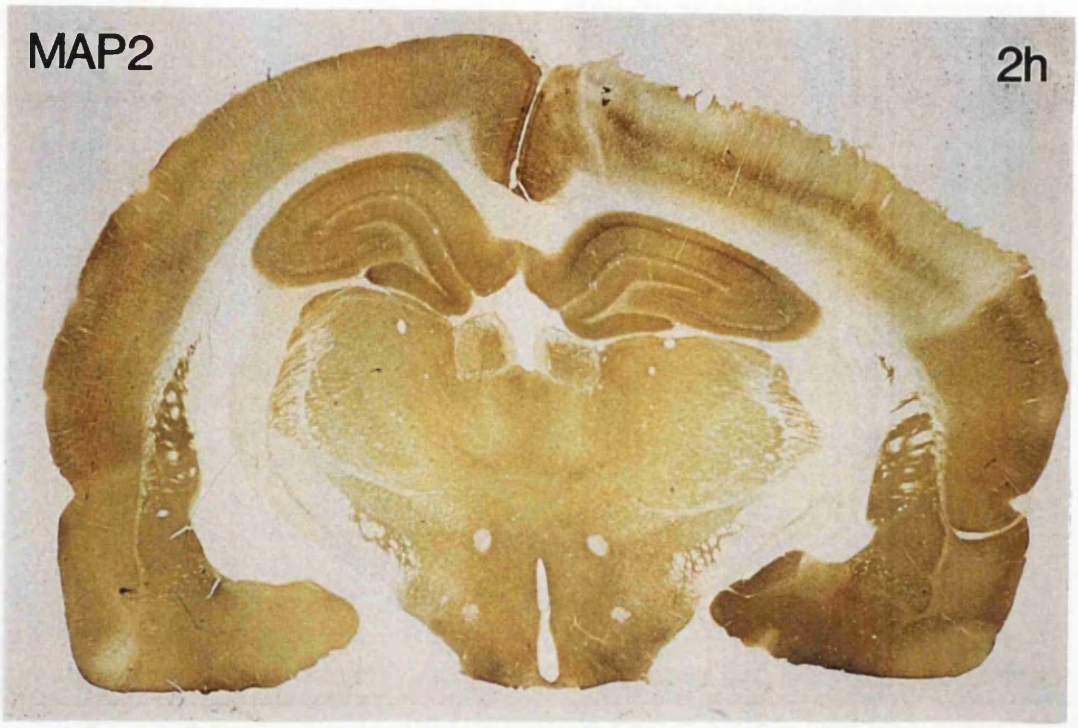
Of interest, the overall decrease in ipsilateral cortical MAP 2 immunostaining did not effect all cortical layers to the same extent (Figures 10 &11). Certain neuronal perikarya within the centre of the ischaemic lesion exhibited positive immunostaining with prominent cell bodies and apical dendrites. These neurones were restricted to cortical layers 3 and 5. This effect was most pronounced in animals allowed to survive for 2 hours after induction of acute subdural haematoma. In these cases, there was a well defined bi-laminar arrangement of MAP 2 immunopositive pyramidal neurones with a distinct band of decreased immunostaining separating between them. 4 hours post injury the bi-laminar arrangement was not detected and there were fewer MAP 2 immunopositive neurones identified in the cortex (Figure 10). The remaining MAP 2 labelled pyramidal neurones were localised to a single layer of the cortex(layer 3). No clear cellular alteration in MAP 2 distribution was detected by immunohistochemistry 30 minutes after the injury .

Figure 10

Immunostaining of MAP 2 in the cerebral cortex after induction of an acute subdural haematoma.

Immunostaining of MAP 2 in cerebral cortex in a sham operated rat (SHAM) and at 2 hours and 4 hours after production of an acute subdural haematoma (see following page). Note the loss of MAP 2 immunoreactivity within the cortex underlying the subdural haematoma. This micrograph demonstrates that the loss of MAP 2 immunolabelling is not uniform throughout the cortex following induction of an acute subdural haematoma.


MAP2
SHAM


MAP2
2h

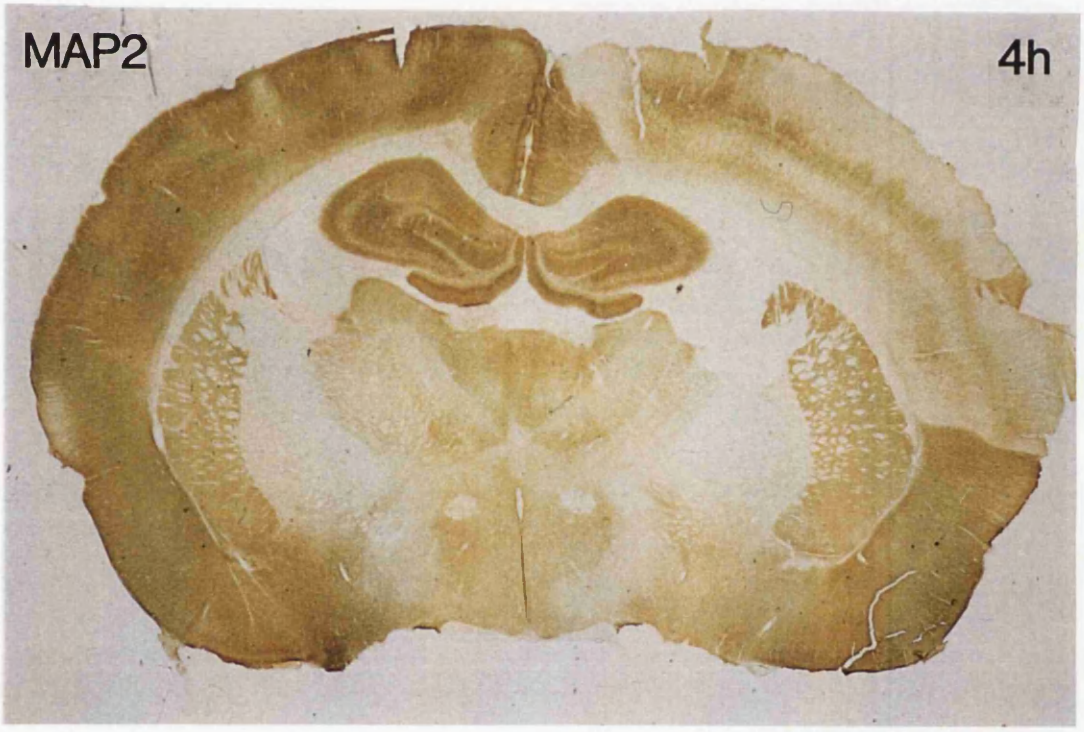
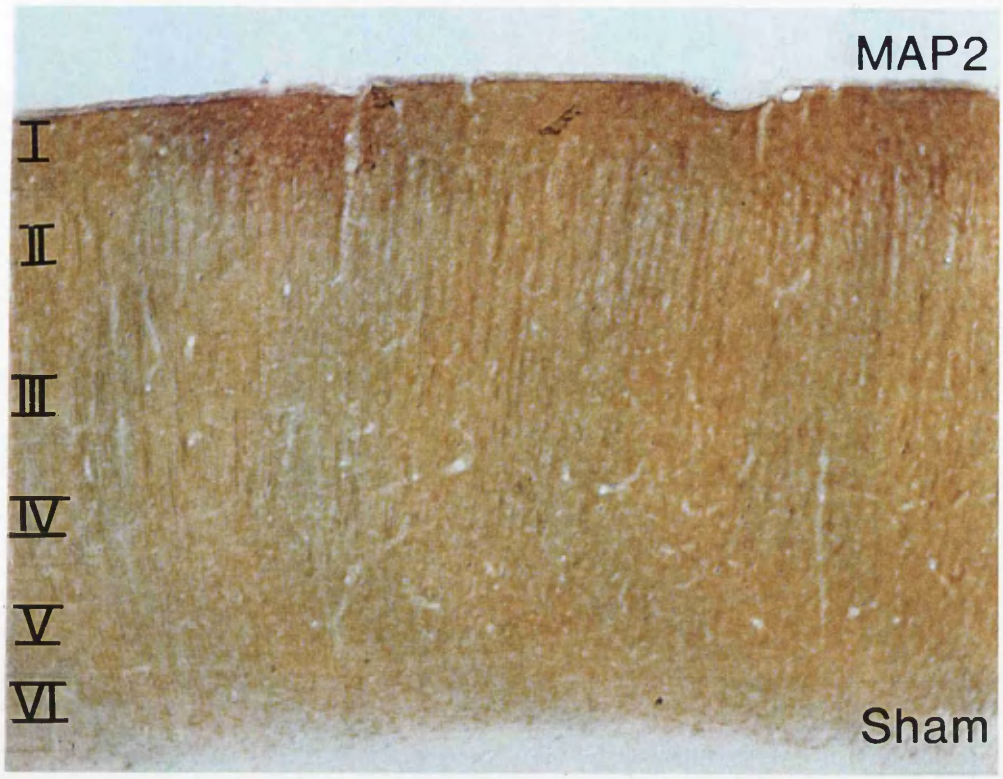
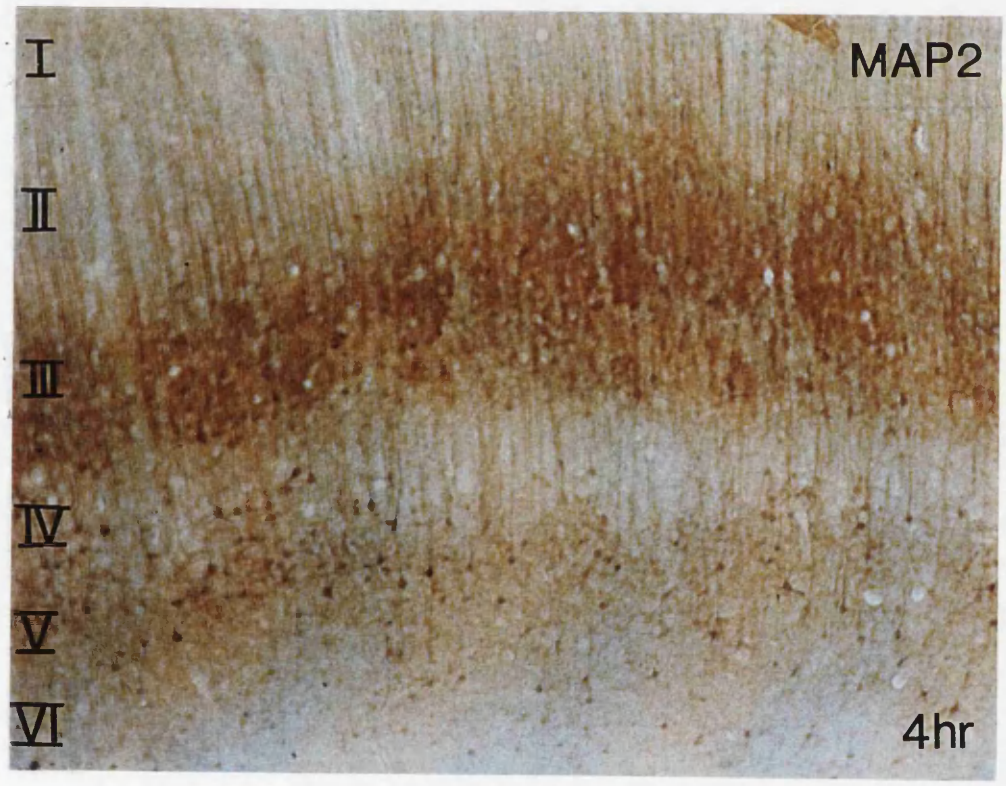

MAP2
4h

Figure 11

Laminar pattern of MAP 2 immunostaining following acute subdural haematoma.

Immunostaining of MAP 2 in cerebral cortex in a sham operated rat (SHAM) and 4 hours after production of an acute subdural haematoma. The loss of MAP 2 immunolabelling is not uniform throughout the layers of the cortex (I-VI). There is some preservation of MAP 2 immunoreactivity within cortical layers III and V.


MAP2
I
II
III
IV
V
VI
Sham


I
MAP2
II
III
IV
V
VI
4hr

No alterations in MAP 2 immunostaining were detected in the white matter tracts of the corpus callosum, at any of the post injury points examined. In all cases the control distribution of MAP 2 persisted with no immunoreactivity detected in axonal pathways (Figure 12).

Figure 12

Immunostaining of MAP 2 in myelinated fibre tracts in the corpus callosum after induction of an acute subdural haematoma.

Immunostaining of MAP 2 in myelinated fibre tracts in the corpus callosum in a sham operated rat (SHAM) and at 4 hours after production of an acute subdural haematoma. Note that there are no detectable alterations in MAP 2 immunostaining after acute subdural haematoma.


MAP2
Sham


MAP2
4hr

β-tubulin

In control animals (n=2) the immunohistochemical distribution and subcellular localisation of β–tubulin was the same as has been described previously. In rat brain, β-tubulin is present throughout and can be detected in neurones, glia and astrocytes [173].

At all time points examined following induction of an acute subdural haematoma, the intensity of β–tubulin immunoreactivity of the neuropil was unaltered in the ipsilateral cortex compared to the contralateral cortex. No defined margin correlating to the region of histologically determined ischaemic brain damage was detected. However, certain neuronal perikarya within the centre of the ischaemic area were β–tubulin immunoreactive (Figure 13). These immunopositive cells were detected as early as 30 minutes post injury and their number increased progressively, becoming most prominent in the 4 hour survival group. They were localised to a single layer of the ipsilateral cortex (layer 3) with characteristic bandlike laminar distribution (Figure 14). Morphologically these cells were pyramidal neurones with densely stained cell bodies and long, straight neuronal processes radiating from the soma. This localised area of cellular immunoreactivity was not detected in any of the sham operated animals or in the contralateral cortex of the animals subjected to subdural haematoma.

Figure 13

Immunostaining of β–tubulin in the cerebral cortex after induction of an acute subdural haematoma.

Immunostaining of β-tubulin in cerebral cortex in a sham operated rat (SHAM) and at 30 minutes, 2 hours and 4 hours after production of an acute subdural haematoma. Note the increased somato-dendritic β-tubulin immunoreactivity within the cortex after acute subdural haematoma.

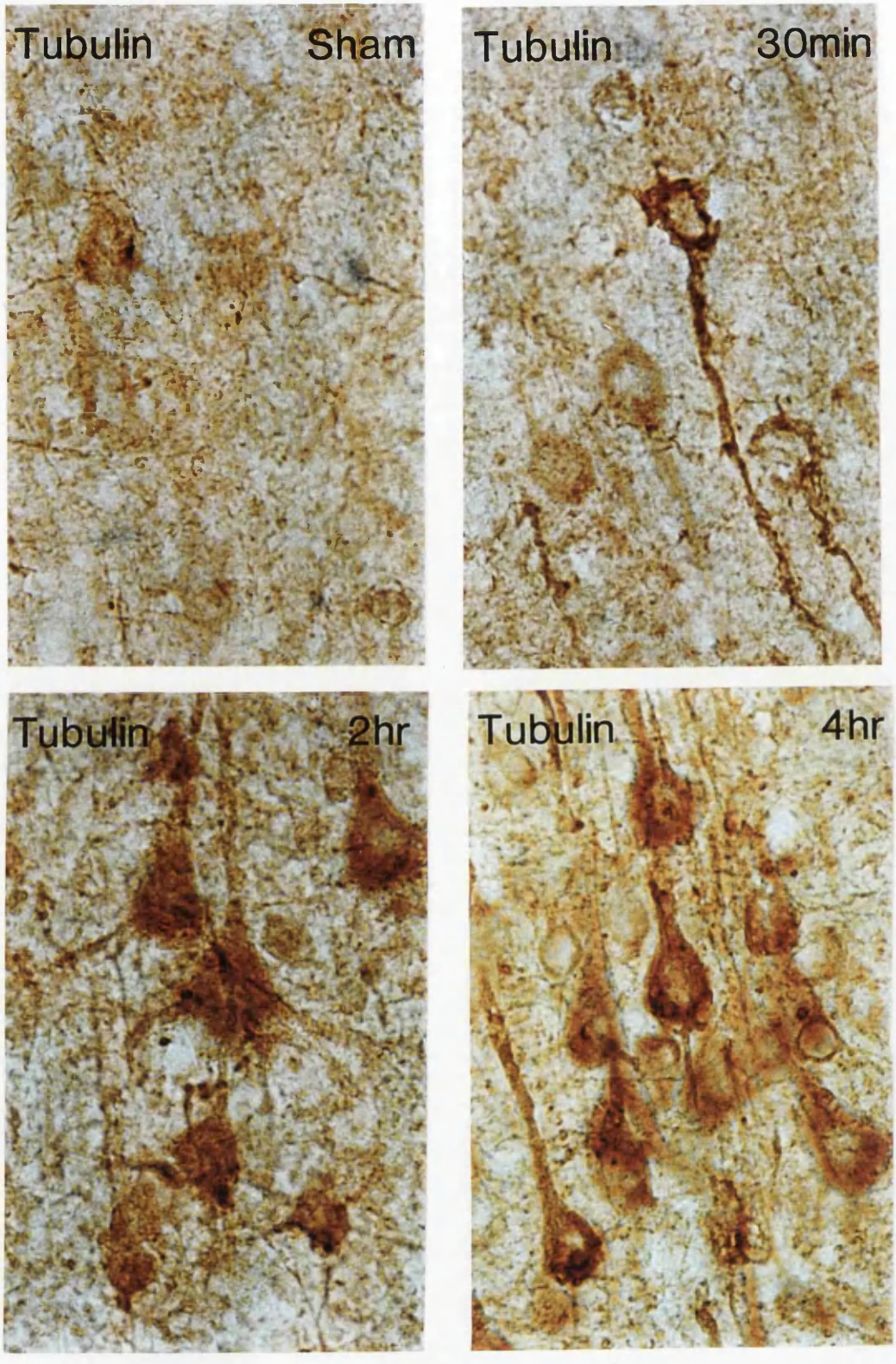
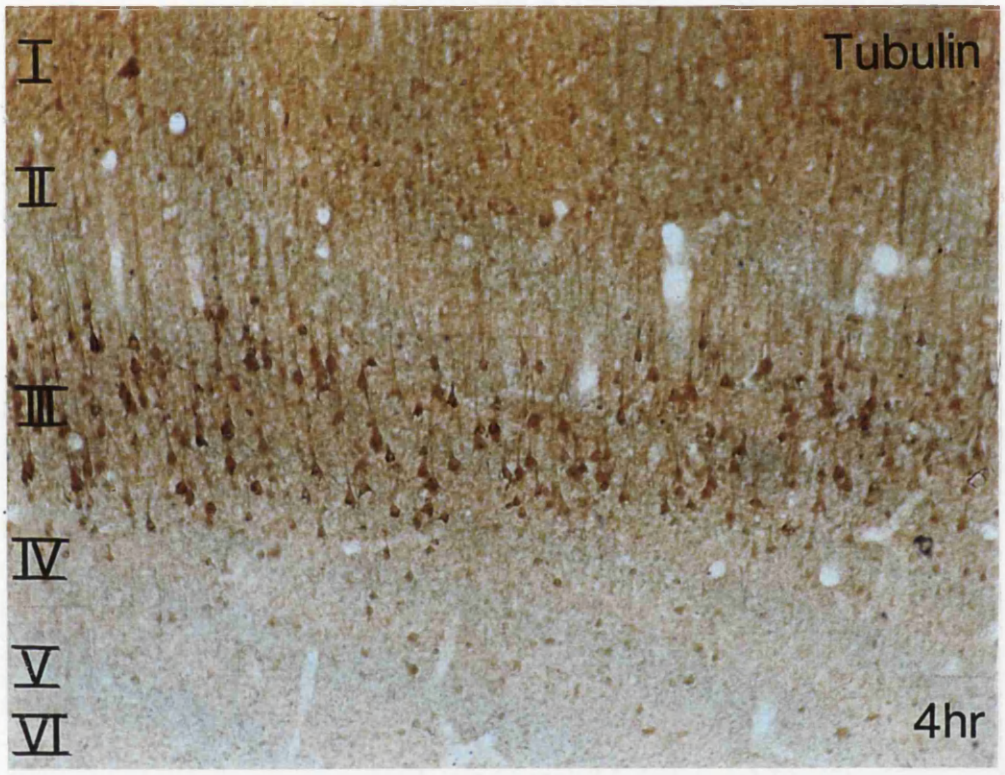

Tubulin
Sham
Tubulin
30min
Tubulin
2hr
Tubulin
4hr

Figure 14

Laminar pattern of β-tubulin immunostaining following acute subdural haematoma.

Immunostaining of β-tubulin in cerebral cortex 4 hours after production of an acute subdural haematoma. Note that β-tubulin immunolabelling is not uniform throughout the layers of the cortex (I-VI). There is pronounced β-tubulin immunoreactivity within cortical layer III but less immunolabelling in all other layers of the cortex.

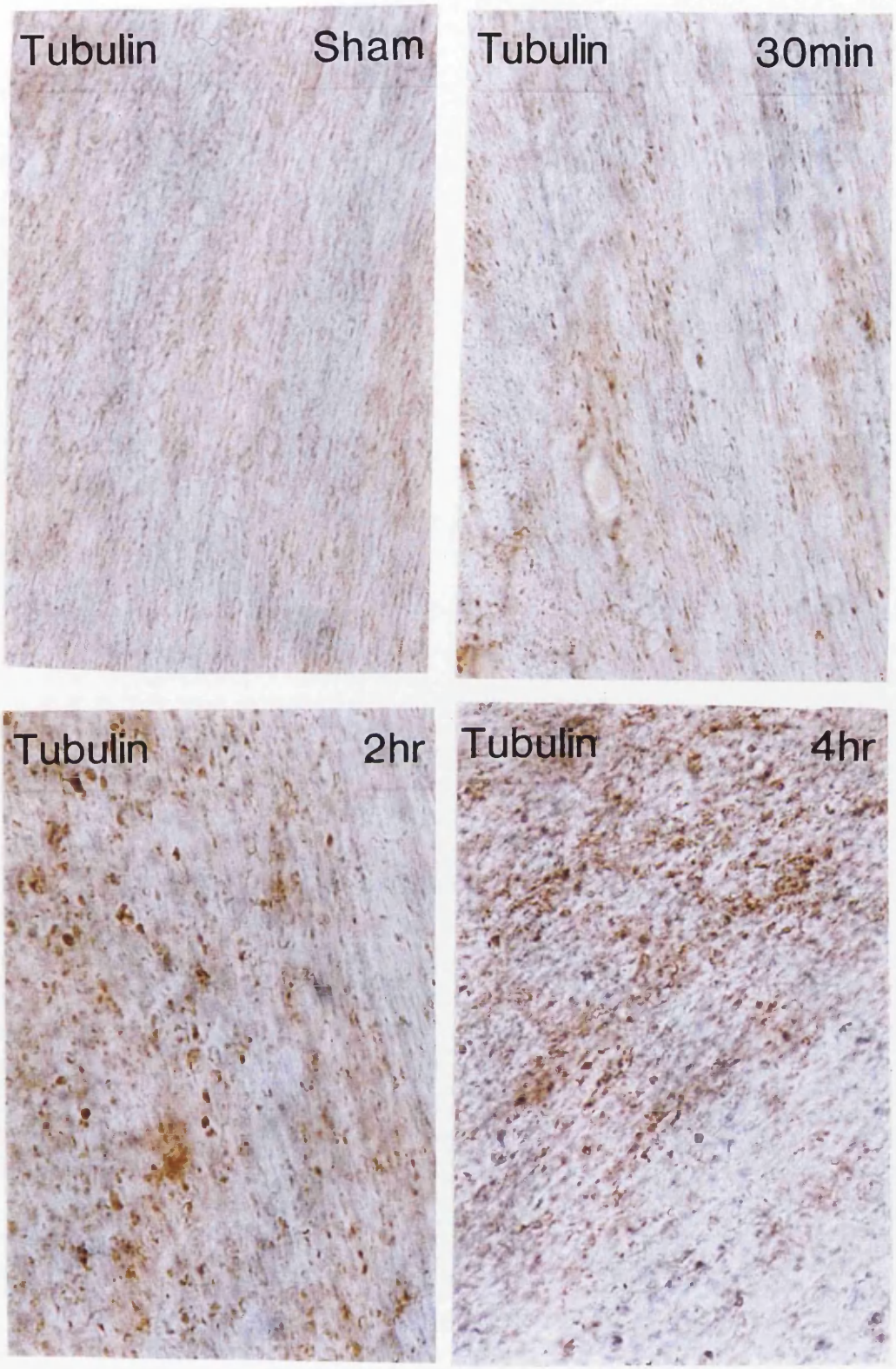

Tubulin
I
II
III
IV
V
VI
4hr

In sham operated animals, β–tubulin immunostaining of the white matter tracts of the corpus callosum indicated a smooth, regular pattern with no areas of focal accumulation . Following subdural haematoma the pattern of immunostaining in the contralateral corpus callosum appeared to be the same as sham operated tissue with no clear differences detected. In contrast, after production of an acute subdural haematoma, the ipsilateral corpus callosum demonstrated marked alterations in β–tubulin immunostaining with numerous, irregular, granular profiles forming in the white matter adjacent to the cortical lesion. This disruption in β–tubulin immunoreactivity was most pronounced in the 4 hour injury group of animals but was detectable 30 minutes after production of acute subdural haematoma. It appeared that the immunostained profiles were confined to the axons adjacent to the lesion and did not extend throughout the white matter tracts of the corpus callosum (Figure 15).

Figure 15

Immunostaining of β–tubulin in myelinated fibre tracts in the corpus callosum after induction of an acute subdural haematoma.

Immunostaining of β-tubulin in myelinated fibre tracts in the corpus callosum in a sham operated rat (SHAM) and at 30 minutes, 2 hours and 4 hours after production of an acute subdural haematoma. Note the progressive disruption in the pattern of β-tubulin immunostaining after acute subdural haematoma.


Tubulin
Sham
Tubulin
30min
Tubulin
2hr
Tubulin
4hr

3.1.5 Western blot analysis of the cerebral cortex following acute subdural haematoma in the rat

In total 20 animals were processed for Western blotting analysis at survival times of 30 minutes or 4 hours. In each group five animals had induction of an acute subdural haematoma and five animals were sham operated.

Tau

In normal adult rat brain Tau-1 recognises five major protein bands corresponding to molecular weights between 52-68 kDa.[94] The electrophoretic profile of tau was altered in the ipsilateral cortex compared to the contralateral cortex of animals subjected to an acute subdural haematoma. Within this subgroup, four major protein bands were observed instead of the five protein bands detected in the controls (sham operated and injured contralateral cortex). These protein bands did not correspond exactly to the normal electrophoretic profile of tau. The higher molecular weight bands (bands 1 and 2) were decreased following injury and a protein band, which was not detected in control samples, appeared within the range of bands 2 and 3. Further, following subdural haematoma induction the profile of band 4 changed and shifted to a position of higher molecular weight. These changes were present in animals examined at the 30 minute time point but were more pronounced in the 4 hour group of injured animals. The electrophoretic profile of tau was not altered in the ipsilateral cortex compared to the contralateral

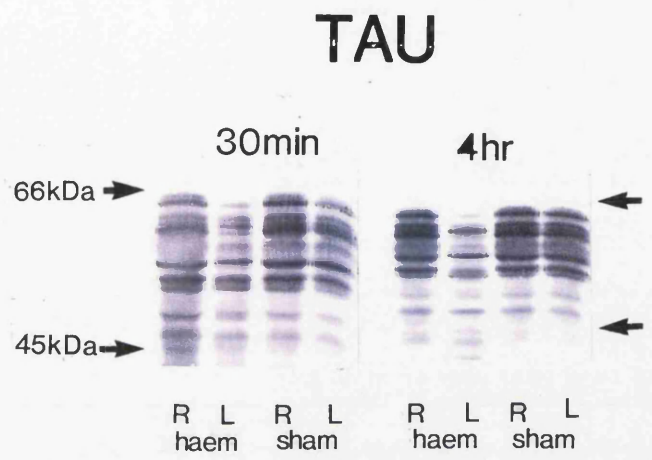


Figure 16

Western Blot analysis of cortical Tau following acute subdural haematoma.

Western Blot analysis of Tau in the left (L) and right (R) cerebral hemispheres of sham operated rats (sham) and at 30 minutes or 4 hours after production of an acute subdural haematoma (haem). The positions of 45kDa and 66kDa molecular weight markers are indicated. Note the shift in the electrophoretic profile and the reduction in the level of Tau in the left cerebral hemisphere at both 30 mins and 4 hrs following haematoma induction compared to the contralateral (R) cerebral hemisphere and both hemispheres of the sham operated animals.

cortex of sham operated animals. The contralateral cortex of injured animals showed an electrophoretic profile of tau approximating those of the sham operated group (Figure 16).

Measurement of tau levels as relative optical density revealed that there was a statistically significant reduction in the level of tau , in the ipsilateral cortex, compared to the contralateral cortex at both 30 minutes (20 %, $p < 0.05$) and 4 hours (43 %, $p < 0.05$) following subdural haematoma (Figure 17).

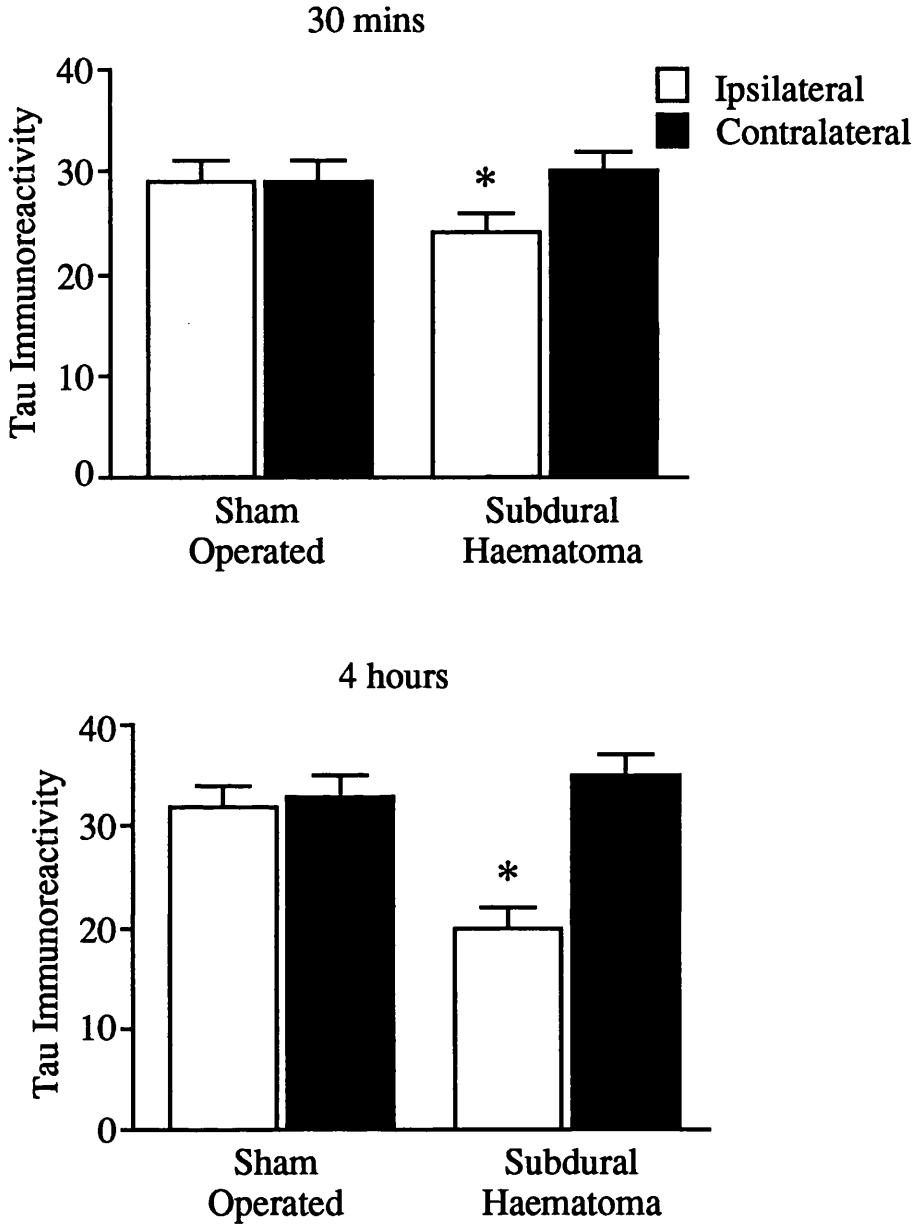


Figure 17

Tau immunoreactivity in the cortex following acute subdural haematoma.

Tau immunoreactivity in the cerebral cortex in sham operated rats and at 30 minutes and 4 hours after production of an acute subdural haematoma. Data are expressed as mean ± SEM and represent arbitary densitometric units. A significant decrease (*$p<0.05$) in Tau immunoreactivity is demonstrated in the ipsilateral hemisphere at both time points after production of an acute subdural haematoma. n=5 animals per group.

MAP 2

In normal adult rat brain MAP 2 recognises one major protein band, corresponding to the isoforms MAP 2a and MAP 2b, with a molecular weight of approximately 280 kDa . The electrophoretic profile of MAP 2 was not altered by the production of an acute subdural haematoma (Figure 18).

Measurement of MAP 2 protein levels as relative optical density revealed that there was a statistically significant reduction in the level of MAP 2, in the ipsilateral cortex, compared to the contralateral cortex at both 30 minutes (53 %, $p < 0.05$) and 4 hours (76 %, $p < 0.05$). The level of MAP 2 in the ipsilateral compared to the contralateral cortex in sham operated animals was not significantly altered at 30 minutes or 4 hours (Figure 19).

Figure 18

Western Blot analysis of cortical MAP 2 following acute subdural haematoma.

Western Blot analysis of MAP 2 in the left (L) and right (R) cerebral hemispheres of sham operated rats (sham) and at 30 minutes or 4 hours after production of an acute subdural haematoma (haem). The position of the 280 kDa molecular weight marker is indicated. Note the reduction in MAP 2 levels in the left (L) cerebral hemisphere at both 30 mins and 4 hrs following haematoma induction compared to the contralateral (R) cerebral hemisphere and both hemispheres of the sham operated animals.

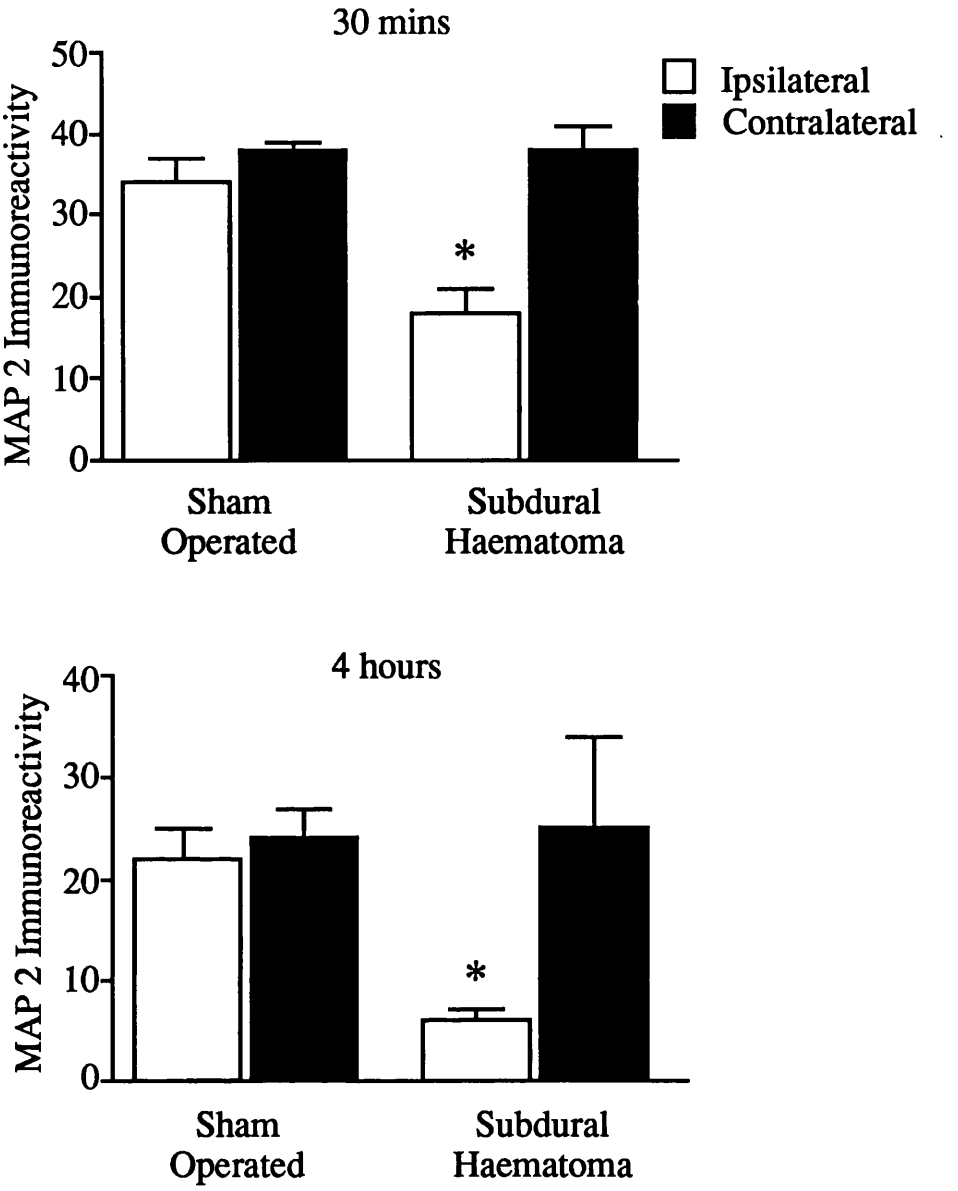


Figure 19

MAP 2 immunoreactivity in the cortex following acute subdural haematoma.

MAP 2 immunoreactivity in the cerebral cortex in sham operated rats and at 30 minutes and 4 hours after production of an acute subdural haematoma. Data are expressed as mean ± SEM and represent arbitary densitometric units. A significant decrease (*$p<0.05$) in MAP 2 immunoreactivity is demonstrated in the ipsilateral hemisphere at both time points after production of an acute subdural haematoma. n=5 animals per group.

Tubulin

In normal adult rat brain the monoclonal anti-tubulin antibody used in this study recognises a dimer of approximately 50kDa which corresponds to α–tubulin and β–tubulin . No alteration in the electrophoretic profile of tubulin was detected following the production of an subdural haematoma (Figure 20) . The distribution of protein bands approximated those of the sham operated samples (ipsilateral and contralateral cortex).

Relative optical density measurements demonstrated that the duration of time the animal was subjected to an acute subdural haematoma altered the level of tubulin detected. 30 minutes after subdural haematoma there was no significant difference in the level of tubulin in the ipsilateral cortex compared to the contralateral cortex. In contrast, in the 4 hour group, both isoforms of tubulin were significantly reduced in the ipsilateral cortex compared to the contralateral cortex, after subdural haematoma (54% reduction for upper band and 58% reduction for the lower band: $p < 0.05$). See Figure 21.

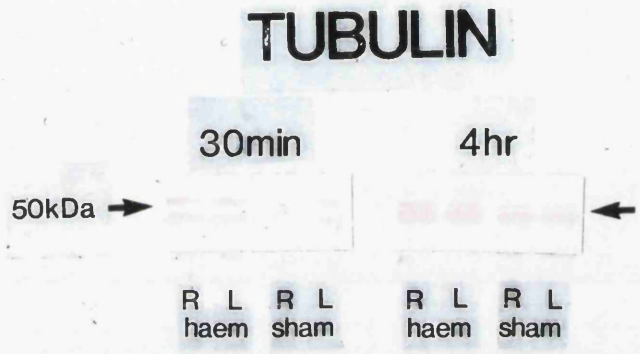


Figure 20

Western Blot analysis of cortical tubulin following acute subdural haematoma.

Western Blot analysis of tubulin in the left (L) and right (R) cerebral hemispheres of sham operated rats (sham) and at 30 minutes or 4 hours after production of an acute subdural haematoma (haem). The position of the 50 kDa molecular weight marker is indicated. Note the reduction in tubulin levels in the left (L) cerebral hemisphere at 4 hrs following haematoma induction compared to the contralateral (R) cerebral hemisphere and both hemispheres of the sham operated animals.

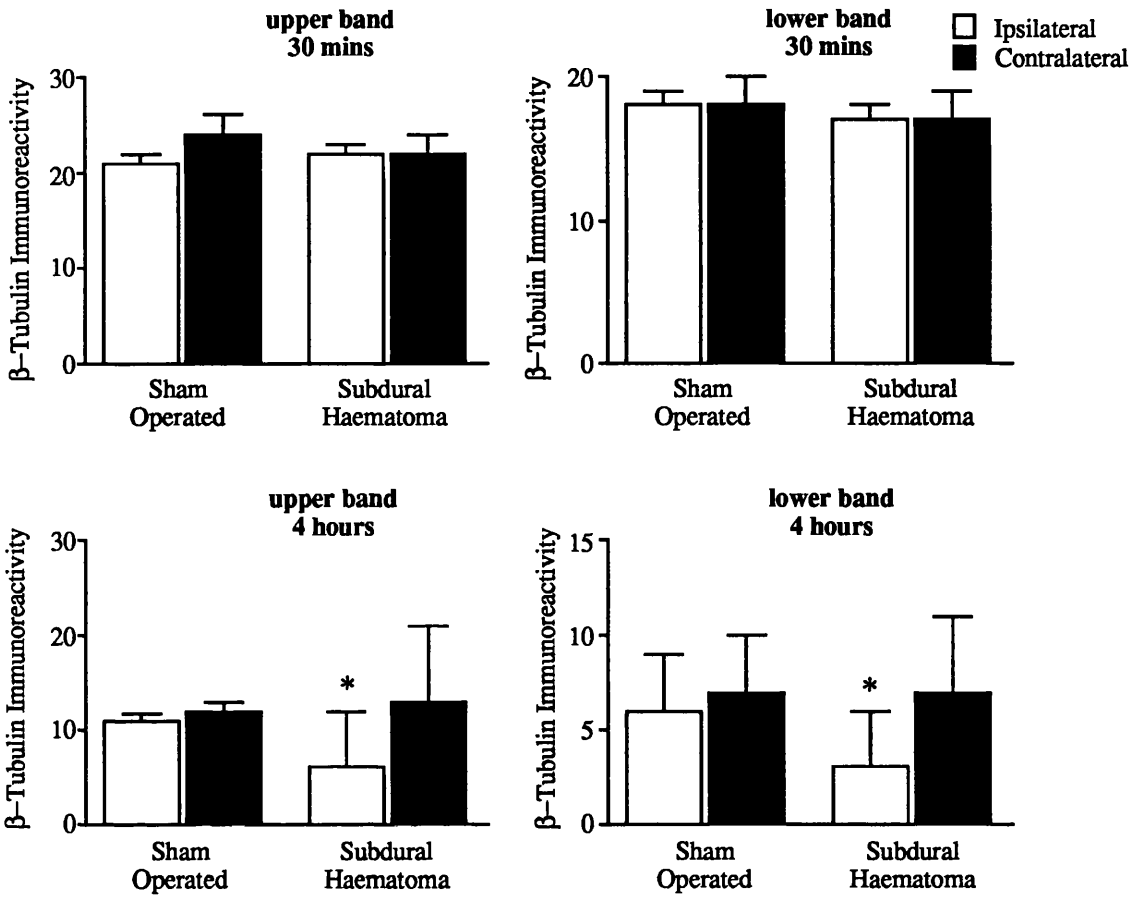


Figure 21

β-tubulin immunoreactivity in the cortex following acute subdural haematoma.

β-tubulin immunoreactivity (upper and lower bands as outlined in Western blot analysis) in the cerebral cortex in sham operated rats and at 30 minutes and 4 hours after production of an acute subdural haematoma. Data are expressed as mean ± SEM and represent arbitary densitometric units. There is no significant difference in b-tubulin immunoreactivity in any of the samples examined 30 minutes after production of an acute subdural haematoma. A significant decrease (* $p<0.05$) in β-tubulin immunoreactivity is demonstrated in the ipsilateral hemisphere 4 hours after production of an acute subdural haematoma. n=5 animals per group.

3.1.6 Transmission Electron Microscopy examination of the neuronal cytoskeleton following acute subdural haematoma in the rat.

In total seven animals were processed for transmission electron microscopy. Four animals were subjected to an acute subdural haematoma and killed by perfusion fixation after 4 hours. Two animals were controls (sham-operated) and had insertion of the needle only. A single animal was perfusion fixed to provide a naive control.

Control animals

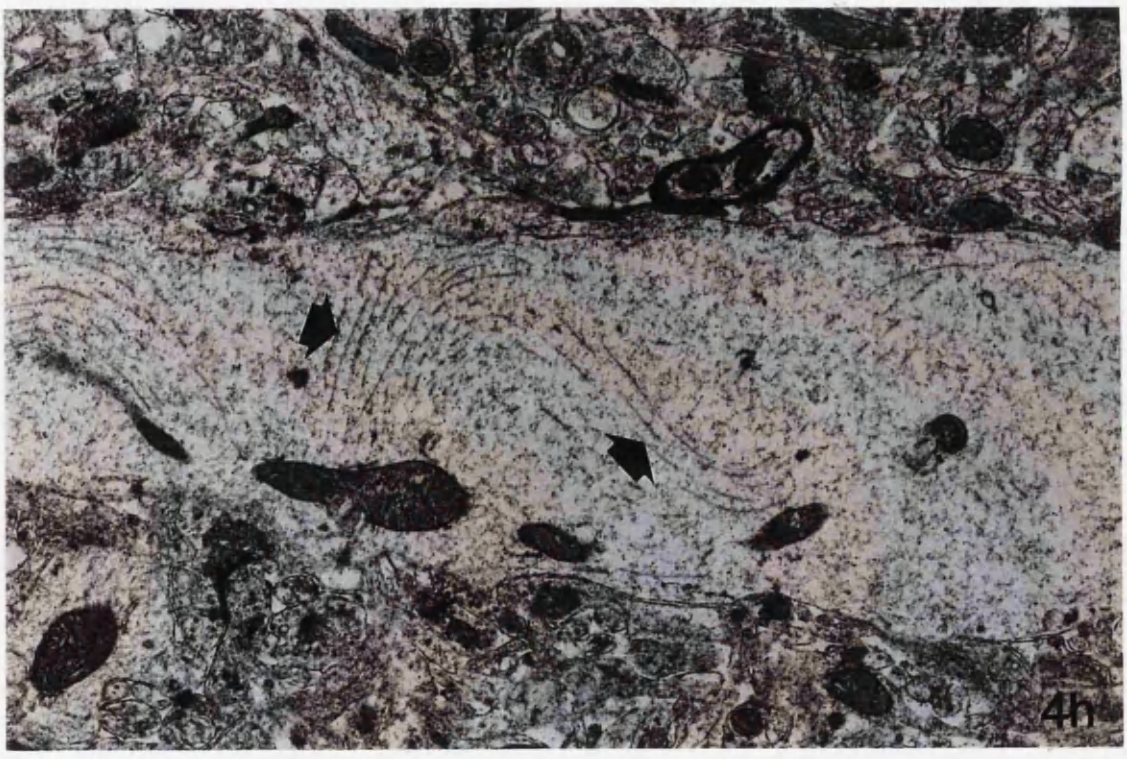
The sections from the control (sham operated and naive) animals had an entirely normal ultrastructure (Figures 22 and 23). Within the cerebral cortex, the dendritic cytoskeleton was linearly organised with microtubules and neurofilaments disposed along the longitudinal axis of the dendrite. The nodal, paranodal and internodal axoplasm had a discrete cytoskeleton with microtubules and neurofilaments that formed linear arrays parallel to the longitudinal axis. An occassional membranous organelle, either smooth endoplasmic reticulum or mitochondrion, occurred in the axoplasm but these were always very low in number. The internodal myelin sheath was uniform with closely apposed lamellae. The internal aspect of the myelin sheath and the external surface of the axolemma were always in close relation.

Figure 22

Ultrastructural analysis of the dendritic cytoskeleton.

A longitudinal thin section of a dendrite in the cerebral cortex from a sham operated animal (SHAM) and from an experimental animal 4 hours after placement of the haematoma. In the sham operated cortex cytoskeletal elements are linearly arranged along the long axis of the dendrite where microtubules are evenly spaced. In the experimental animal, microtubules within the dendritic cytoskeleton now follow a helical disposition (arrowheads) rather than being linearly organised. However, microtubules still lie in parallel within this abnormal pattern.


SHAM


4h

Experimental animals

A variety of structural abnormalities were observed following production of an acute subdural haematoma. Specifically the cytoskeleton was disrupted with resultant alterations in its three dimensional configuration.

In the ipsilateral cerebral cortex the dendritic cytoskeleton was also altered. Ultrastructural analysis demonstrated misalignment of the dendritic microtubules with the formation of an abnormal helically orientated pattern rather than the longitudinal arrangement identified in the control tissue. The microtubules remained parallel within this abnormal pattern (Figure 22).

In transverse sections of myelinated axons in the corpus callosum there were reduced numbers of microtubules and a decreased spacing between neurofilaments . Microtubules were absent within the axoplasm. The neurofilaments appeared compacted, that is, there was a reduced spacing between neurofilaments and they tended to lie in the centre of the injured axon. No obvious misalignment of the neurofilaments was detected in this protocol. Electron microscopy demonstrated dissociation of adjacent lamellae the myelin sheath and the occurrence of peri-axonal spaces associated with a reduction in axonal transverse diameter. In some axons the axoplasm consisted of a diffuse, flocculent precipitate, while in others there was a central core of neurofilaments. Aggregates of membranous profiles, resembling smooth endoplasmic reticulum, were identified in the axoplasm. The mitochondria displayed a disrupted organisation of their

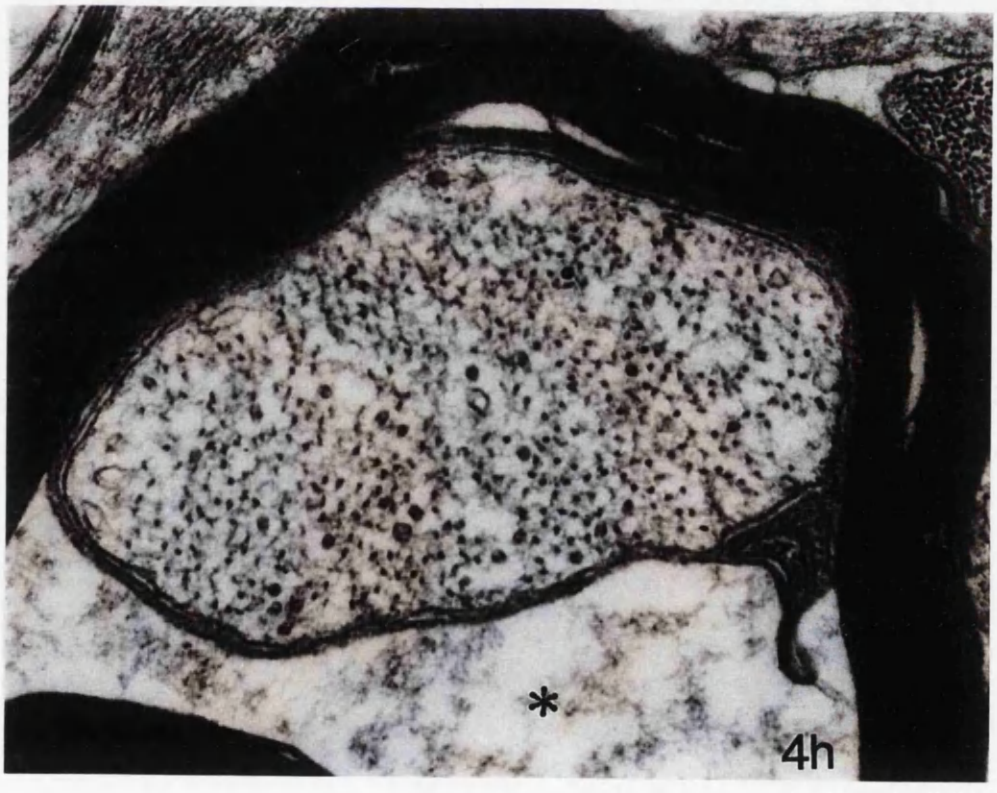
cristae. Typically these abnormal features occurred in a field containing axons demonstrating normal morphology (Figure 23).

Figure 23

Ultrastructural analysis of the axonal cytoskeleton.

A transverse section of a myelinated fibre from the corpus callosum of a sham operated animal (SHAM) and from an experimental animal 4 hours after placement of the haematoma. The axonal cytoskeleton consists of regularly spaced neurofilaments and microtubules all arranged parallel to the long axis of the axon. In the experimental animal, there are no microtubules present and the neurofilaments have a reduced spacing, or are compacted, tending to lie in the centre of the axon. Note the presence of peri-axonal spaces (*) in the myelinated fibres after production of an acute subdural haematoma.


SHAM


*
4h

3.2 Immunohistochemical analysis of calpain mediated spectrin breakdown following acute subdural haematoma in the rat.

In total 24 animals were processed for immunohistochemistry. At each time point (30 minutes, 2 hours and 4 hours) 6 animals underwent induction of an acute subdural haematoma and 2 animals were sham operated only.

Immunohistochemical analysis confirmed that the ipsilateral cortex is the main site of calpain mediated breakdown of spectrin following acute subdural haematoma (ASDH) in the rat. Spectrin breakdown products (BDPs) were confined within the histologically defined ischaemic lesion. No immunoreactivity was detected in the contralateral cortex of animals subjected to induction of an ASDH. In sham operated animals there was increased immunoreactivity demonstrated in the cortex directly beneath the site of insertion of the needle. Spectrin BDPs immunostaining was only observed in axons examined later than two hours after induction of an acute subdural haematoma.

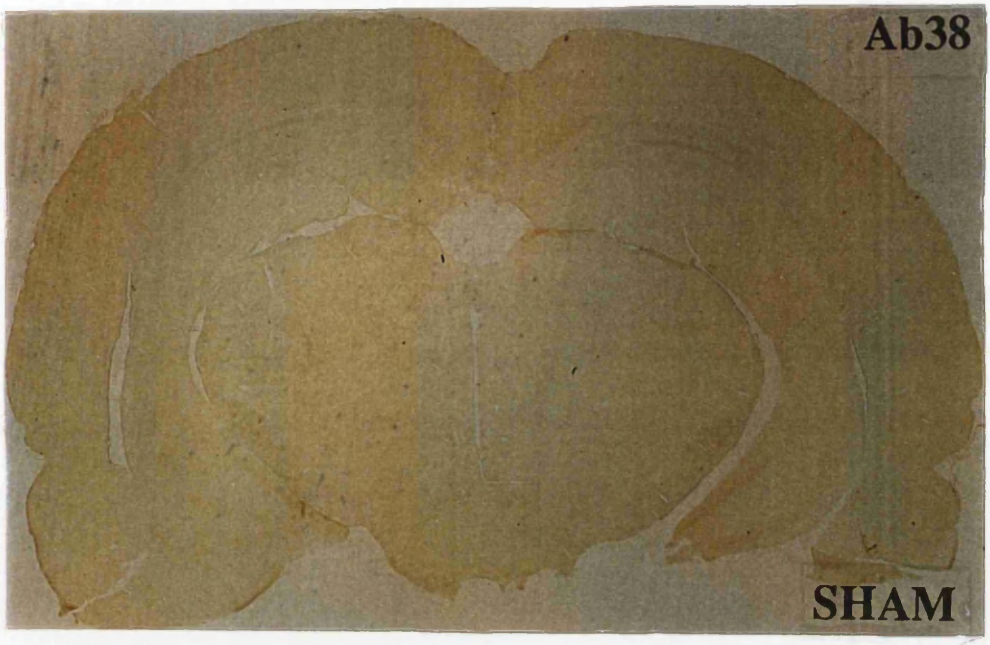
Spectrin breakdown occurred over a large area of the cortex underlying the haematoma (Figure 24). Although haematoxylin and eosin histology revealed only early ischaemic cell process in the cortex at 30 minutes following ASDH, calpain mediated spectrin BDPs were present in cortical dendrites in the region that proceeded to exhibit the most marked ischaemic cell process changes. Within the cortex, the distribution of spectrin BDP immunoreactivity did not change with time. In all cases the

area of immunoreactivity correlated with the histologically defined ischaemic area. However, 2 hours after induction of an ASDH, the intensity of the immunoreactivity had increased and this persisted, unchanged, in the 4 hour group of animals.

Figure 24

Calpain mediated spectrin breakdown in the cerebral cortex after induction of an acute subdural haematoma.

Immunostaining of calpain mediated breakdown products (BDPs) of spectrin in cerebral cortex in a sham operated rat (SHAM) and at 30 minutes, 2 hours and 4 hours after production of an acute subdural haematoma. Note the progressive increase in calpain mediated spectrin breakdown in the cortex underlying the haematoma.


Ab38
SHAM


Ab38
30min


Ab38
2hours

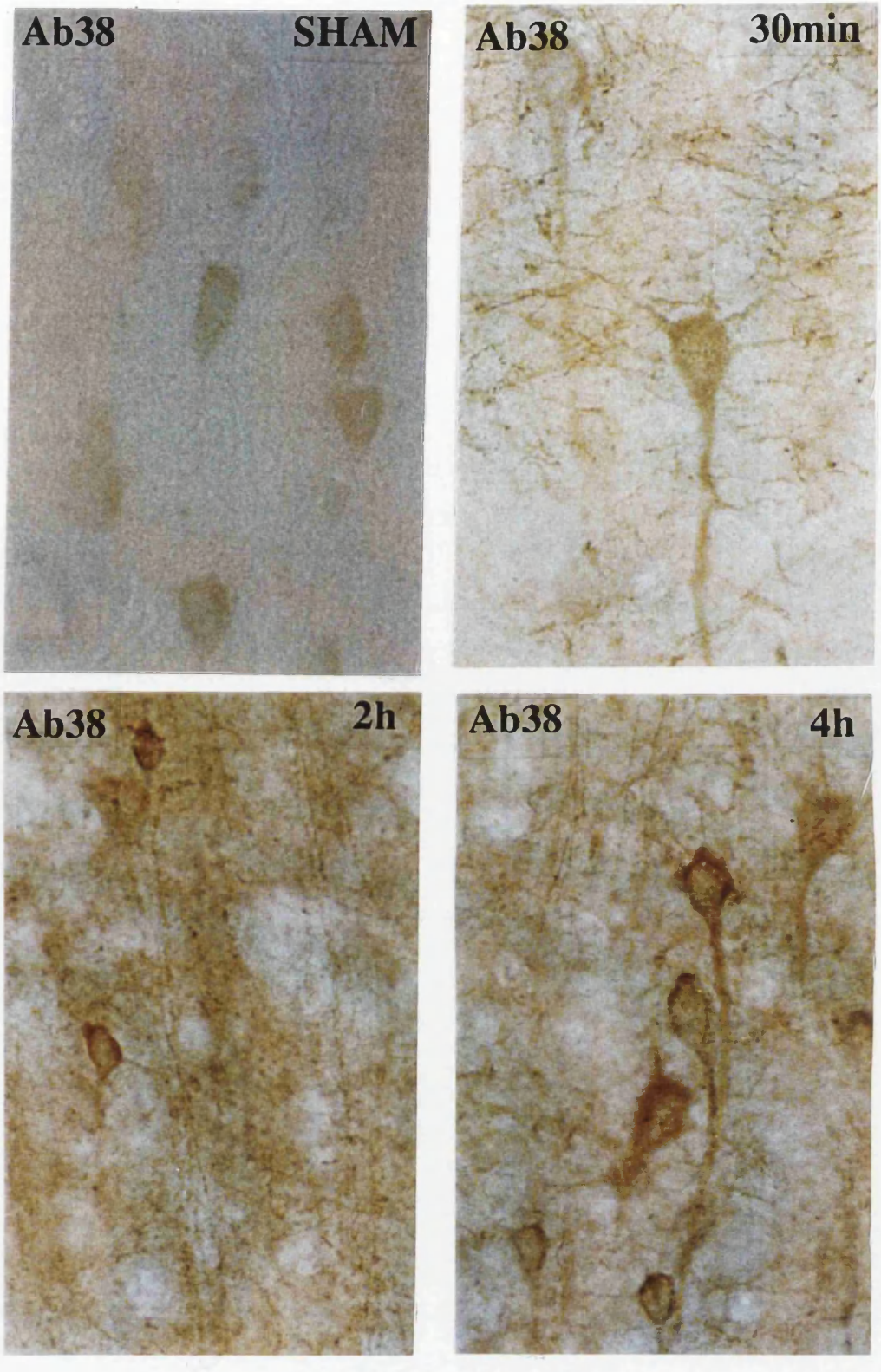

Ab38
4hours

Neuronal perikarya containing spectrin BDPs were detected in the ipsilateral cortex 30 minutes after induction of an ASDH (Figure 25). This was a consistent finding displayed in all animals. These neurones became more prominent in the 2 and 4 hour group of animals. Examination of the adjacent H&E tissue sections revealed that these neurones were confined to cortical layer 4. Immunoreactive dendrites were identified in all layers of the cortex and demonstrated various degrees of fragmentation. In the 30 minute survival animals the spectrin BDP immunolabelled dendrites were in continuity but appeared irregular and distorted. By 2 hours these dendrites were fragmented, that is they were not continuous, and contained areas of immunonegativity suggesting that the whole length of the dendrite was not intact. Immunoreactive dendrites increased in both number and in the severity of fragmentation in the 4 hour group of animals (Figure 25).

Figure 25

Calpain mediated spectrin breakdown in neuronal perikarya and dendrites following acute subdural haematoma.

Immunostaining of calpain mediated breakdown products (BDPs) of spectrin in cerebral cortex in a sham operated rat (SHAM) and at 30 minutes, 2 hours and 4 hours after production of an acute subdural haematoma. Note the progressive increase in calpain mediated spectrin breakdown. Proteolytic fragments of spectrin are primarily localised in dendrites and neuronal perikarya.

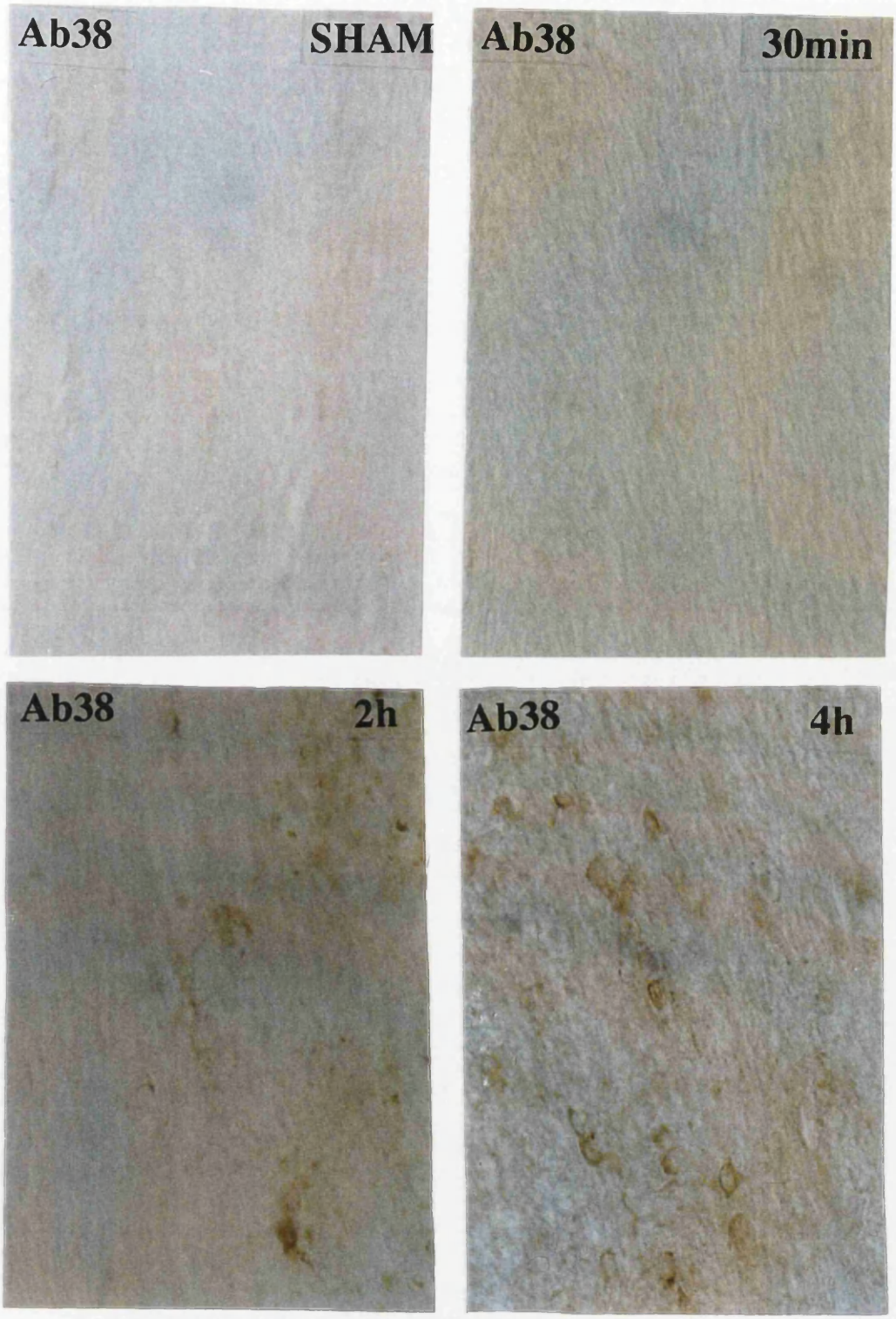

Ab38
SHAM
Ab38
30min
Ab38
2h
Ab38
4h

In all animals , spectrin BDP immunoreactivity was observed along the interface with the subcortical white matter. The adjacent cortical tissue was labelled in a punctate fashion and there was a well defined boundary with the subjacent white matter. There was no definite evidence of calpain mediated spectrin BDPs immunoreactivity detected in the white matter of any of the animals examined 30 minutes after production of an acute subdural haematoma. In contrast, at both 2 hours and 4 hours after induction of the acute subdural haematoma, calpain mediated spectrin BDPs immunoreactivity was demonstrated in axons underlying the haematoma (Figure 26). The immunoreactivity had an irregular punctate appearance. In addition, immunopositive oligodendrocytes were detected within the white matter of the 4 hour group of injured animals only.

Figure 26

Calpain mediated spectrin breakdown in myelinated fibre tracts in the corpus callosum following acute subdural haematoma.

Immunostaining of calpain mediated breakdown products (BDPs) of spectrin in myelinated fibre tracts in the corpus callosum in a sham operated rat (SHAM) and at 30 minutes, 2 hours and 4 hours after production of an acute subdural haematoma. Note the presence of increased levels of calpain mediated spectrin breakdown immunoreactivity in the 2 hour and 4 hour group of experimental animals.


Ab38
SHAM
Ab38
30min
Ab38
2h
Ab38
4h

3.3 Alterations in the neuronal cytoskeleton following acute subdural haematoma in humans

3.3.1 Neurohistopathology

The presence of an acute subdural haematoma resulted in an area of ischaemic brain damage in the ipsilateral superior parietal cortex. The histologically defined ischaemic tissue, as detected by haemotoxylin and eosin, was pale compared to the control brain tissue (Figure 27).

Microscopy of the haemotoxylin and eosin, stained cryostat sections taken from the superior parietal lobule reveals both cortex and related white matter. Scattered throughout the deeper layers of the cortex are a number of contracted neurones, the nuclei of which show condensed basophilic chromatin and the cytoplasm of which is eosinophilic having lost its' Nissl substance. Many of these neurones are associated with swelling and vacuoles in related astrocytes and a number of incrustations are seen. Apart from this swelling, reactive cellular changes are not seen in either glia or blood vessels. There is some accentuation of these changes within layers 5 and 6 but in some areas there are effected neurones in layers 4, 5 and 6. The superficial layers appear to be spared. The related white matter is normal. There is no evidence of vascular occlusion or of infiltration by neurophils.

Figure 27

The histopathological features of the ischaemic cell process after an acute subdural haematoma in humans.

Micrograph of the left superior parietal cortex from a patient sustaining an acute subdural haematoma. The cortex demonstrates features of the ischaemic cell process with contracted neurones containing condensed nuclear basophilic chromatin and eosinophillic cytoplasm.

3.3.2 Immunohistochemical analysis of the microtubule component of the neuronal cytoskeleton following acute subdural haematoma in human post mortem tissue

Tau -1

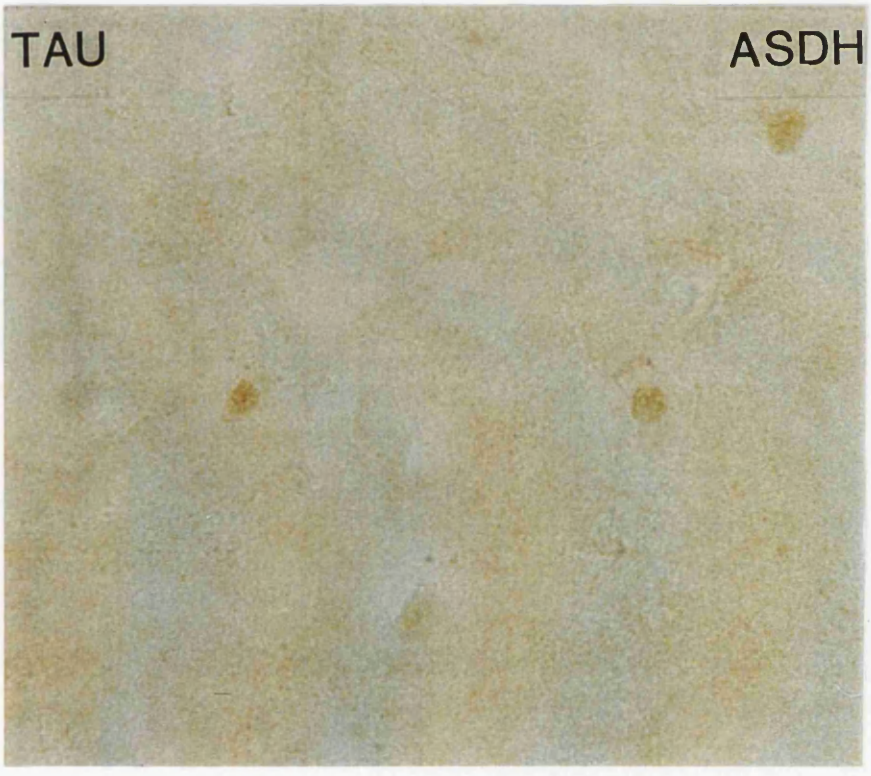
The degree of immunolabelling was very faint in all brain sections processed for Tau-1 immunhistochemistry. In control cases the distribution of tau, as detected by the Tau-1 antibody, was the same as has been described previously being preferentially localised in axons and not detectable in the neuronal cell bodies. However, the presence of an acute subdural haematoma resulted in clear alterations in the localisation of Tau-1 within the ischaemic superior parietal cortex (Figure 28). Tau-1 immunostaining was decreased in the neuropil of all the cases with a subdural haematoma. There was no correlation with this observation and either the post mortem delay or survival time after injury. Within the ischaemic lesion Tau-1 immunoreactivity was faintly increased in some scattered neuronal perikarya. However, these were few in number and difficult to detect due to the intense loss of neuropil staining. No alterations Tau-1 immunoreactivity in the white matter were detected in any of the cases examined .

Figure 28

Immunostaining of Tau-1 in the human cerebral cortex after an acute subdural haematoma.

Immunostaining of Tau-1 in the human cerebral cortex in a neurological control patient (control) and in a patient sustaining an acute subdural haematoma (ASDH). Note that Tau-1 immunolabelling is faint in both sections. However, there is the suggestion of an increase in Tau-1 immunoreactivity in certain neuronal perikarya following acute subdural haematoma.


TAU
Control


TAU
ASDH

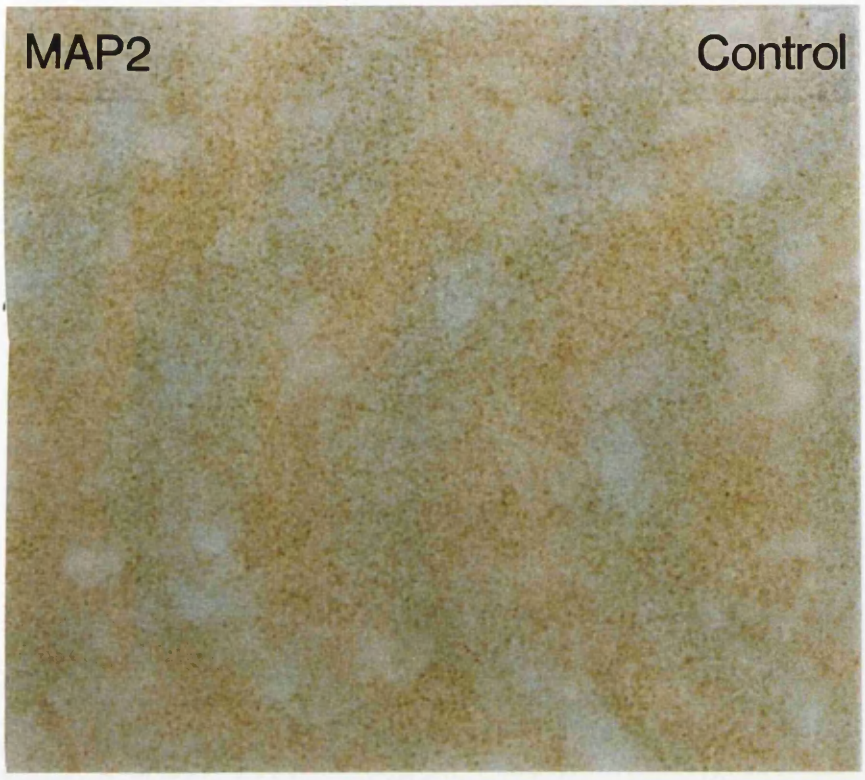
Microtubule associated protein 2 (MAP 2)

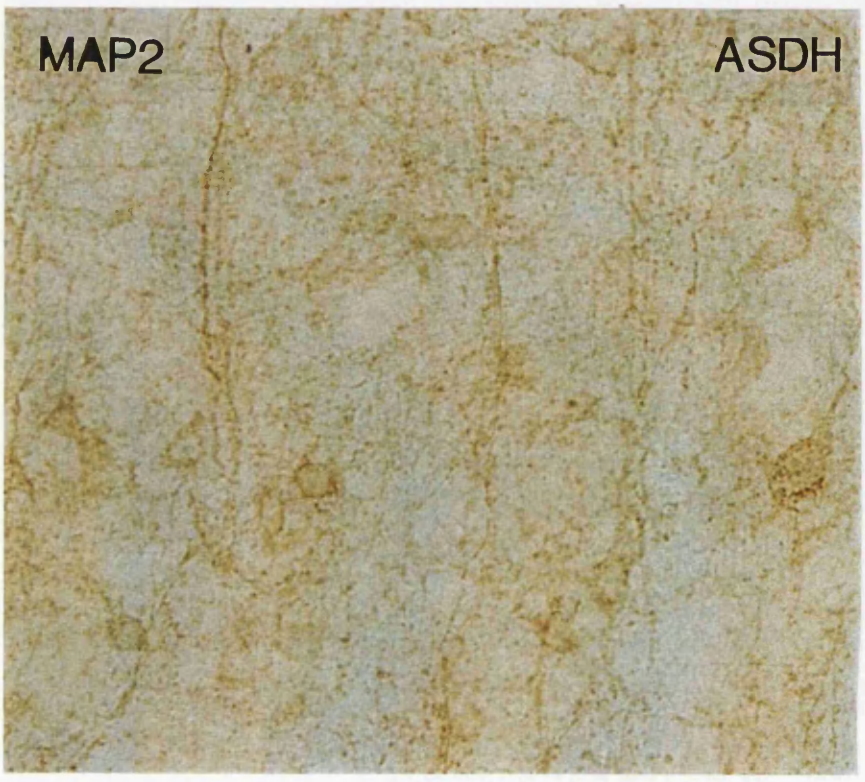
In the control cases MAP 2 was distributed as has been previously described being preferentially localised to the somato-dendritic compartment. There were alterations in MAP 2 immunostaining in the superior parietal cortex after an acute subdural haematoma (Figure 29). In these cases, there was a pronounced loss of MAP 2 immunoreactivity within the histologically defined ischaemic area in the superior parietal cortex. Although there was some variability in the extent of the changes detected, all cases examined revealed a decreased labelling of dendrites and minimal staining of neuronal cell bodies within the ischaemic area. The neuropil was less intensely stained than in the control cortex and there were no alterations in MAP 2 immunostaining of axons which remained immunonegative. These findings were the same irrespective of the survival time or post mortem delay.

Figure 29

Immunostaining of MAP 2 in the human cerebral cortex after an acute subdural haematoma.

Immunostaining of MAP 2 in the human cerebral cortex in a neurological control patient (control) and in a patient sustaining an acute subdural haematoma (ASDH). Note the decrease in MAP 2 immunostaining following acute subdural haematoma. In addition, certain neuronal perikarya are MAP 2 immunopositive with fragmented MAP 2 immunolabelled dendritic processes.


MAP2
Control


MAP2
ASDH

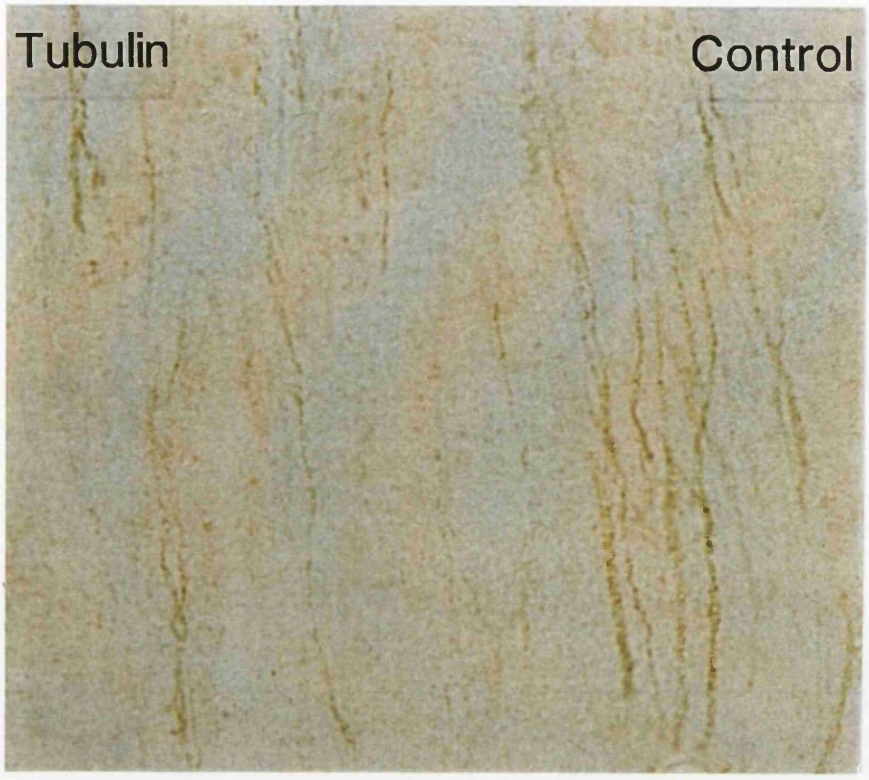
β–tubulin

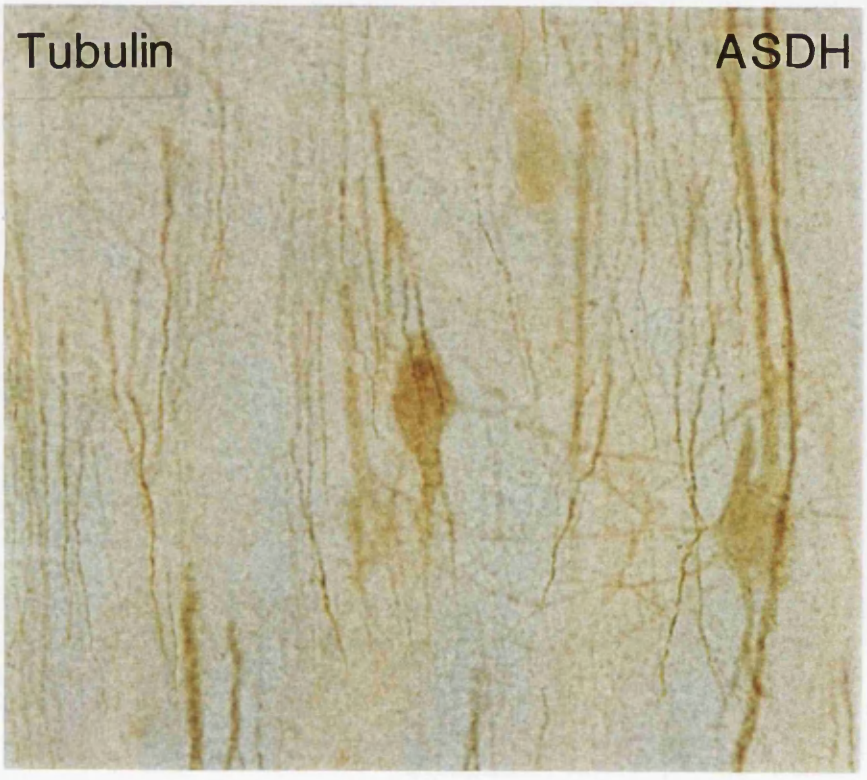
In the control cases β-tubulin was widely distributed being detectable in dendrites, axons and in a few neuronal cell bodies. There were numerous fine, slender, immunopositive dendrites detected throughout the cortex. These processes had a regular arrangement, radiating from the cortical surface. In the cases with an acute subdural haematoma there were marked alterations in this pattern (Figure 30). Within the histologically defined ischaemic area the regular appearance of β-tubulin immunostaining was disrupted and multiple, irregular, immunopositive profiles were detected. There was also an increased immunostaining of neuronal perikarya within the ischaemic area but these were scattered and did not form a laminar arrangement. Other elongated neuronal processes were more intensely stained than the control cases but were distorted and mishapen. The impression was formed that this range of disruption may result from differences in the plane of section of the tissue relative to the direction of the neuronal processes such that they are detected at different angles of projection. No alterations in the subcortical white matter were detected in any of the cases examined.

Figure 30

Immunostaining of β-tubulin in the human cerebral cortex after an acute subdural haematoma.

Immunostaining of β-tubulin in the human cerebral cortex in a neurological control patient (control) and in a patient sustaining an acute subdural haematoma (ASDH). Note the increased somato-dendritic β-tubulin immunoreactivity within the cortex after an acute subdural haematoma.


Tubulin
Control


Tubulin
ASDH

3.4 Alterations in axolemmal permeability following traumatic brain injury

In total 24 animals were used in this study . At each time point examined (5 minutes, 15 minutes and 1 hour) six animals were subjected to traumatic brain injury and two animals were sham operated only.

3.4.1 Clinical Observations

All animals survived the trauma. Immediately after injury all rats had a period of apnea which lasted for 10 to 20 seconds and was associated, in the majority of animals, with a generalised convulsion that lasted 15-30 seconds. These convulsions occurred only once and always immediately after trauma. In addition all injured rats developed a decortication flexion deformity in their forelimbs. No skull fractures were detected in any of the animals.

3.4.2 Neuropathology Examination

All brains were considered to be well perfusion fixed as evidenced by good neuronal morphology, the absence of intravascular blood and the lack of cytological artefacts such as " dark cells" or " hydropic cells" 172.

3.4.3. Gross Neuropathology

In injured animals there was significant sub-arachnoid haemorrhage occupying the basal cisterns and cisterna magna. The blood extended to the sub-arachnoid spaces over the cerebral hemispheres. There was no focal brain lesion after injury. Petechial haemorrhages were frequently observed in the brain stem and occassionally in the mid-brain.

3.4.4. Neurohistopathology

Injured neurones were common in the supraventricular areas of the cerebral cortex, especially the central portion under the injury site. In sections stained with haematoxylin and eosin, shrunken neurones associated with perineuronal vacuolation were identified in animals surviving 1 hour after injury. It was observed that the density of damaged neurones decreased towards the periphery of the lesion.

3.4.5 Control animals

Light microscopy

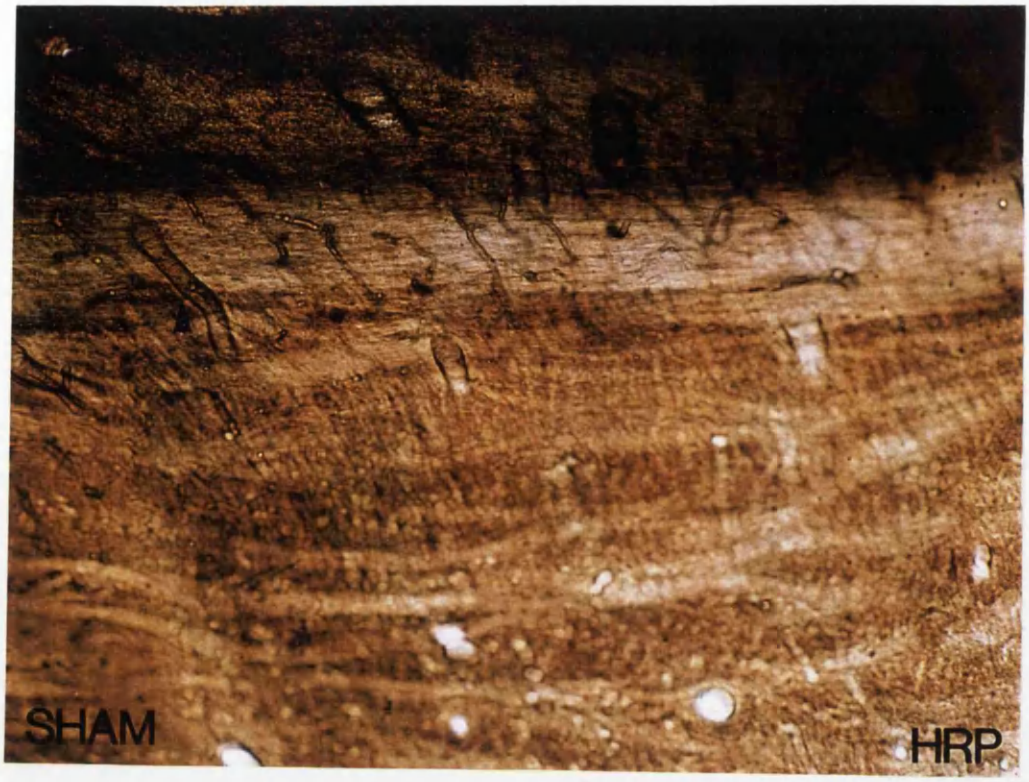
The cleared sections reacted for visualisation of peroxidase activity displayed tracer diffusion throughout the basal cisterns. From here, the tracer could be seen diffusing directly into the extracellular compartment of the ventral brainstem, and along the perivascular sleeves (Figure 31). In all cases, the tracer penetrated 1-2 mm into the brainstem parenchyma,

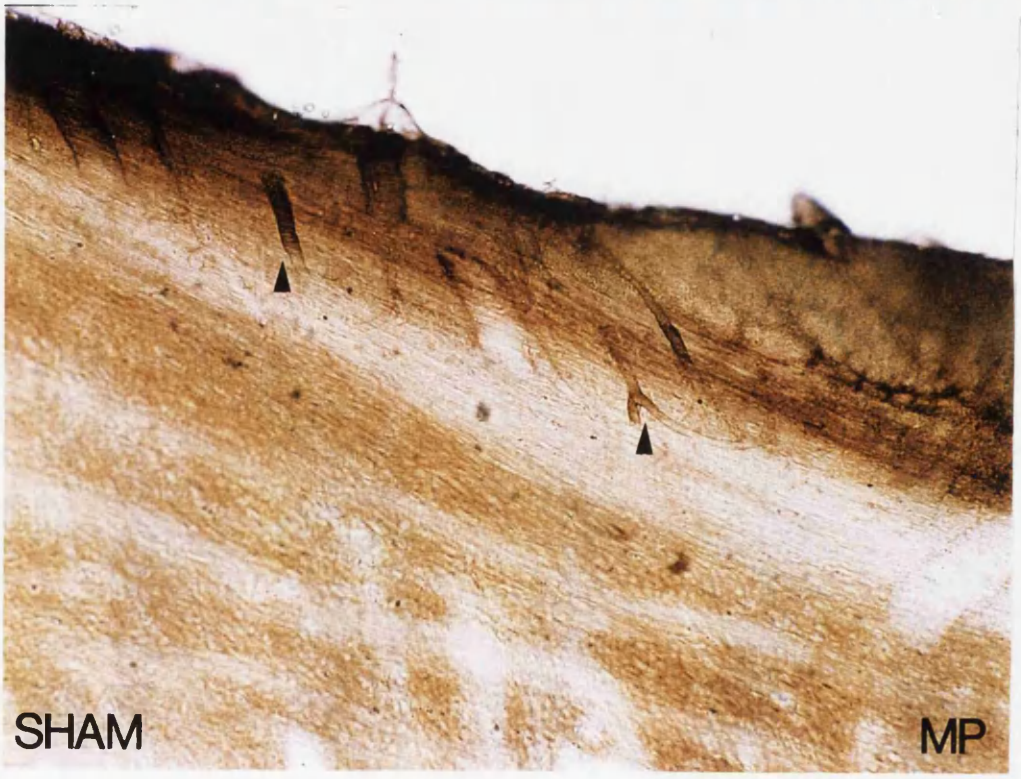
encompassing some of the fibre systems known to undergo reactive change in this animal model, such as the corticospinal tract. Light microscopic examination revealed that the peroxidase reaction product was confined to the extracellular space, where it was conspicuous in the axonal nodal regions as well as the perivascular spaces. No intra-axonal peroxidase activity was detected in any of the control animals.

Figure 31

Axolemmal permeability following traumatic brain injury.

Light micrographs of cleared sections from the ventral pontomedullary junction of sham operated animals reveals the passage of peroxidase through the interstices of the brain parenchyma. Note that horseradish peroxidase (HRP) and microperoxidase (MP) both pass through the perivascular sleeves (arrowheads) and are confined to the extracellular space. After 1 hour of diffusion no signs of intraaxonal peroxidase uptake are evident.


SHAM
HRP


SHAM
MP

Electron microscopy

Electron microscopic examination of this material revealed that the peroxidase reaction product was confined to the extracellular compartment, with all axons demonstrating normal ultrastructural detail. The morphological appearance of these axons were similar to those described in Figure 23 . Specifically, there was a normal regular arrangement of the axonal cytoskeleton with microtubules and neurofilaments arranged parallel to the longitudinal axis of the axon.

3.4.6. Experimental animals

Light microscopy

All animals subjected to an impact acceleration closed head injury, in the presence of extracellular microperoxidase (MP) or horseradish peroxidase (HRP) demonstrated focal peroxidase uptake at two sites within the ventral brainstem (Figures 32, 33, 34). Specifically, scattered axons within the pontomedullary and cervicomedullary junctions displayed focal inudation with the once extracellularly confined peroxidase. Such axons could be seen in relation to other fibres which demonstrated no evidence of peroxidase uptake, consistent with the diffuse nature of the injury. These peroxidase containing axons were seen at all survival times including those animals assessed within 5 minutes of the traumatic injury. Despite the presence of their peroxidase content, these axons appeared otherwise unaltered at the light microscopy level, with the exception that they all displayed microvacuolation scattered throughout the peroxidase laden axoplasm . Although intra-axonal peroxidase activity could be detected with either tracer it was readily apparent that the horseradish peroxidase was detected in more axons than the microperoxidase. Quantitative analysis confirmed this impression.

Figure 32

Axolemmal permeability following traumatic brain injury.

Light micrographs of cleared sections from the ventral pontomedullary junction of animals examined 5 minutes after induction of traumatic brain injury. Intra-axonal peroxidase is demonstrated with horseradish peroxidase (HRP) and microperoxidase (MP). Note that these peroxidase containing axons are visualised in a field where other fibres show no evidence of peroxidase influx. In addition, there is a greater uptake of HRP compared to MP.


5min
HRP

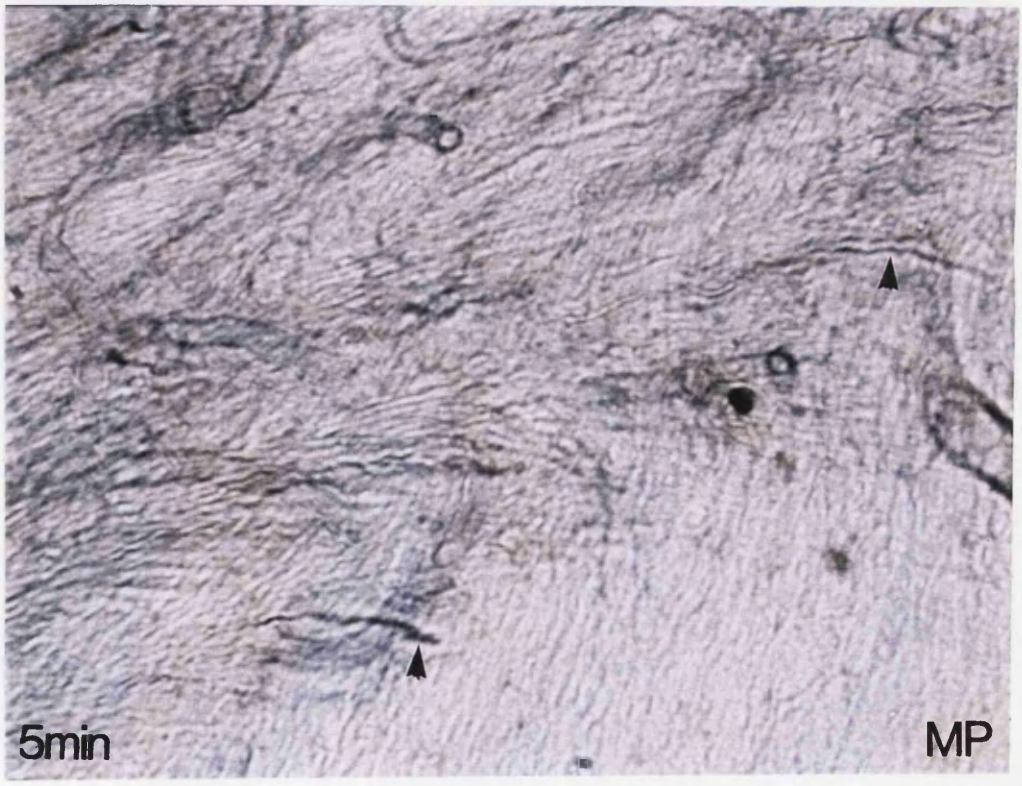
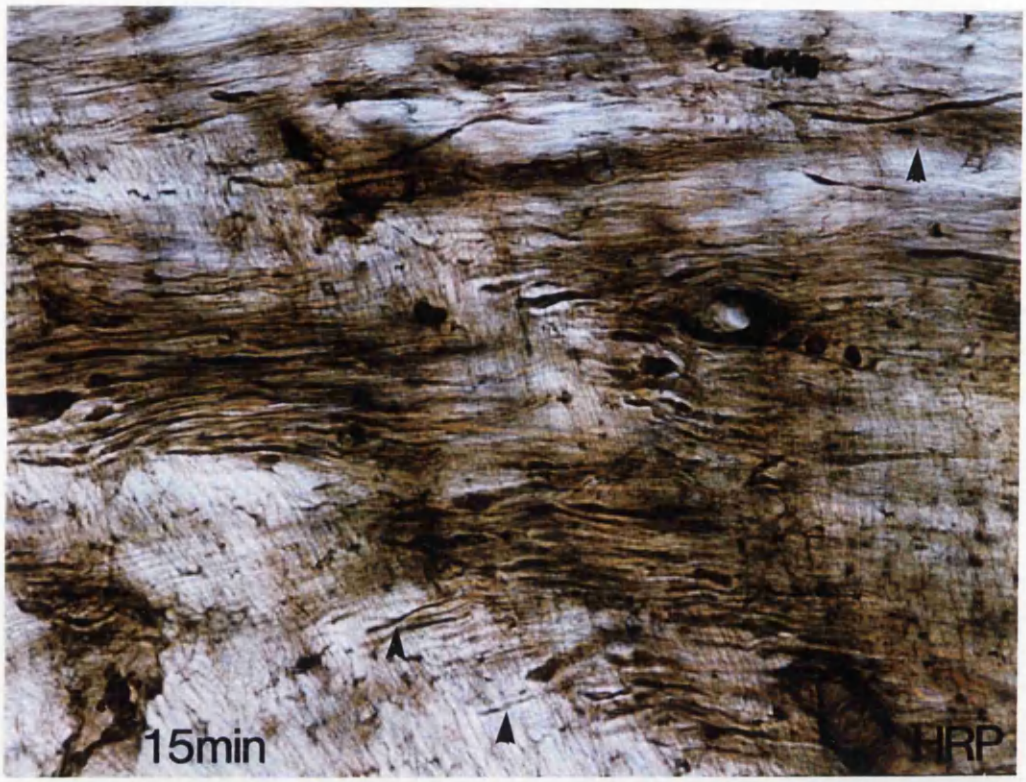

5min
MP

Figure 33

Axolemmal permeability following traumatic brain injury.

Light micrographs of cleared sections from the ventral pontomedullary junction of animals examined 15 minutes after induction of traumatic brain injury. The findings detected are similar to the group of animals examined 5 minutes after injury. Intra-axonal peroxidase is demonstrated with horseradish peroxidase (HRP) and microperoxidase (MP). Note that these peroxidase containing axons are visualised in a field where other fibres show no evidence of peroxidase influx. In addition, HRP demonstrates a greater number of flooded axons compared to MP.


15min
HRP

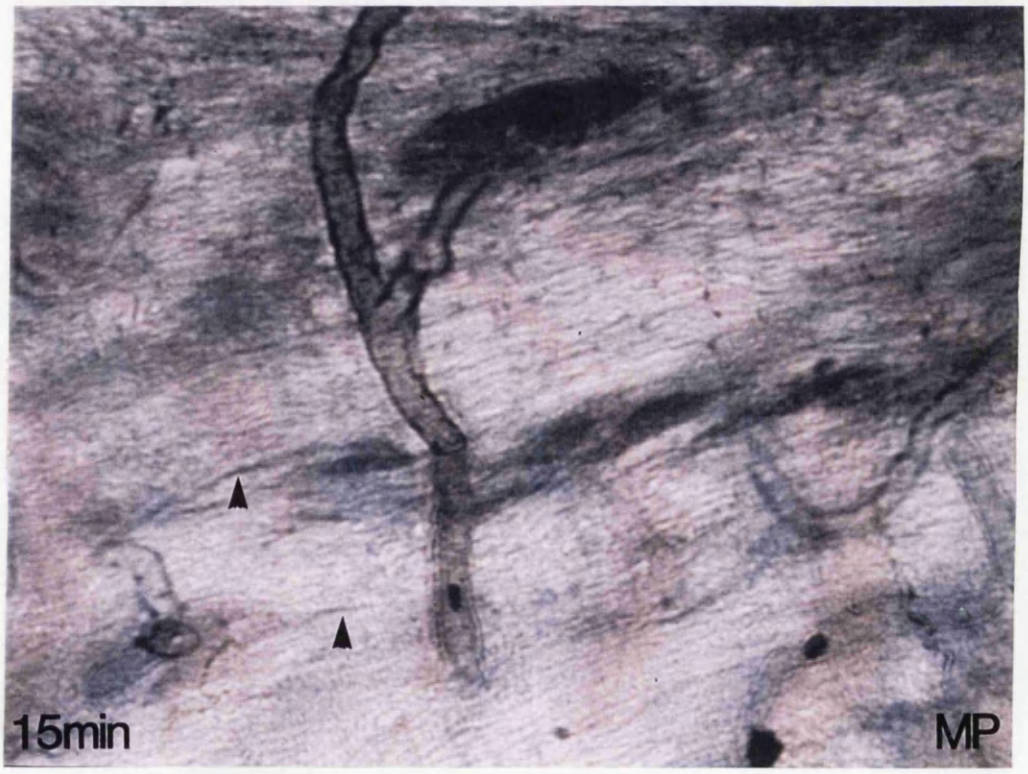
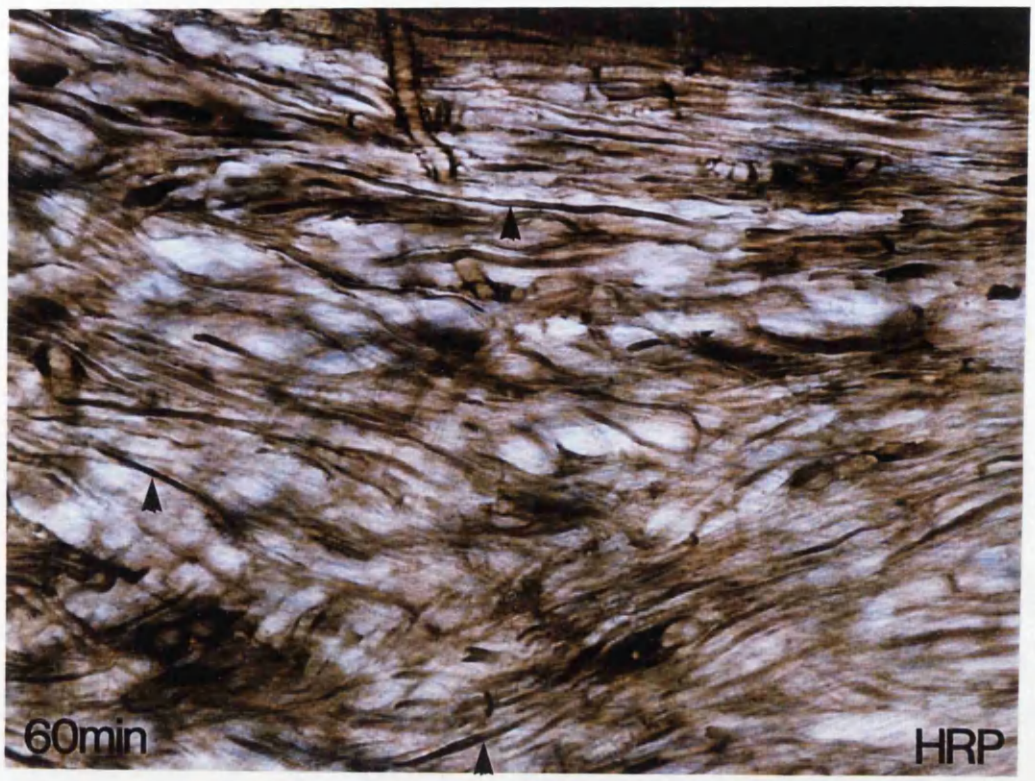
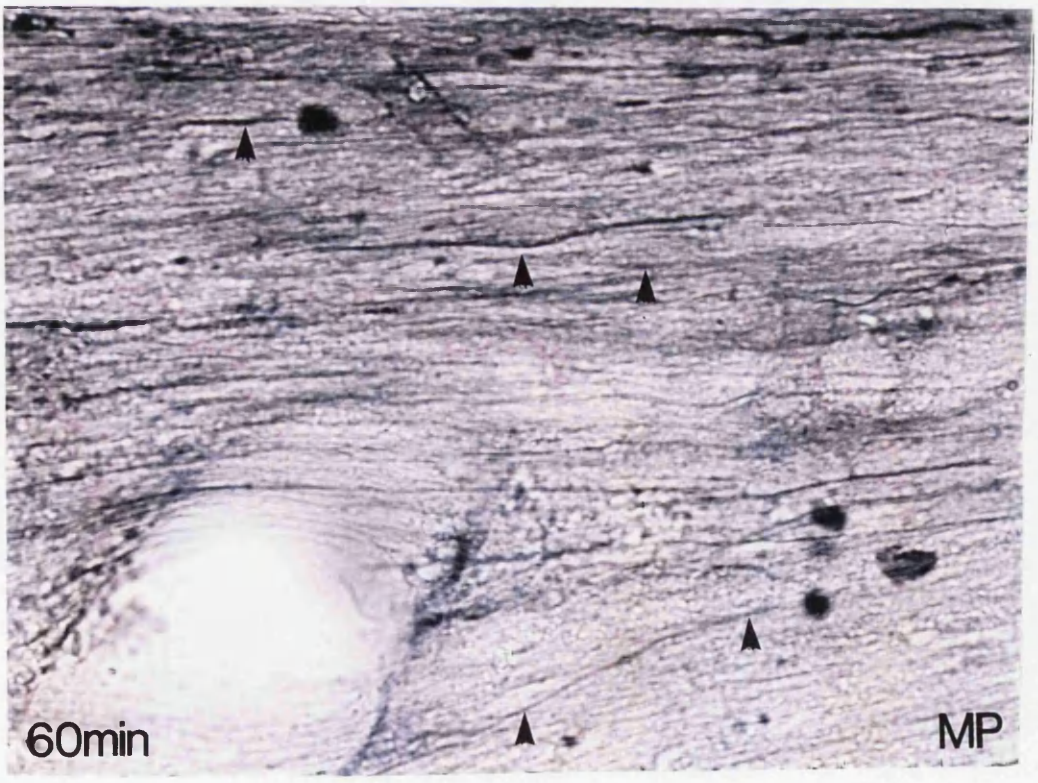

15min
MP

Figure 34

Axolemmal permeability following traumatic brain injury.

Light micrographs of cleared sections from the ventral pontomedullary junction of animals examined 60 minutes after induction of traumatic brain injury. The findings detected are similar to the group of animals examined 5 and 15 minutes minutes after injury. Intra-axonal peroxidase is demonstrated with horseradish peroxidase (HRP) and microperoxidase (MP). Note that these peroxidase containing axons are visualised in a field where other fibres show no evidence of peroxidase influx. In addition, HRP demonstrates a greater number of flooded axons compared to MP.


60min
HRP


60min
MP

Electron microscopy

Ultrastructural analysis of the peroxidase-containing axons revealed a distinct set of morphological alterations (Figure 35). These were the same irrespective of what tracer had been used. The peroxidase reaction was confined to a limited segment of the axon cylinder where it dispersed throughout the axoplasm . Within 5 minutes of the traumatic event, the peroxidase tracer could be seen in the axoplasm where it was identified in nodal, paranodal and internodal regions. In these foci of tracer influx, the related neurofilament network appeared dense, with the suggestion that the neurofilaments were now more tightly compacted than in the control tissue. This alteration was demonstrated at all survival times examined (up to 1 hour post injury) and did not change or progress during this period.

Local mitochondrial abnormalities were most dramatic at these foci of peroxidase activity. The mitochondria appeared swollen with disrupted cristae. These abnormal features seen at the electron microscopic level, correlated with the microvacuolation seen with light microscopy. Typically, these injured axons occurred in a field containing other fibres with no detectable intra-axonal peroxidase reaction product. No evidence of direct axolemmal renting was observed in any of the animals in this series.

Figure 35

Ultrastructural detection of microperoxidase flooded axons following traumatic brain injury.

Electron micrographs from the ventral pontomedullary junction of animals examined 5 minutes after induction of traumatic brain injury. They demonstrate a microperoxidase (MP) flooded axon. The peroxidase containing axon (arrows) is seen in a field demonstrating other unaltered axonal profiles. Note that the peroxidase containing axon cylinder displays a densely packed filamentous network and the mitochondria appear dilated.


MP
5min
RMFP16-1
7231 80 0kV X2500 2μm


MP
5min
RMFP16-1
7236 80 0kV X10K 500nm

3.4.7 Quantatative analysis

As noted above, homologous 50,000μm^2 regions from the pontomedullary and cervicomedullary junctions were selected and subjected to quantatative analysis to determine the number of HRP or MP containing axons per unit area. At all survival times statistically fewer flooded axons were detected with microperoxidase as the tracer than in the corresponding sections utilising horseradish peroxidase (Figure 36).

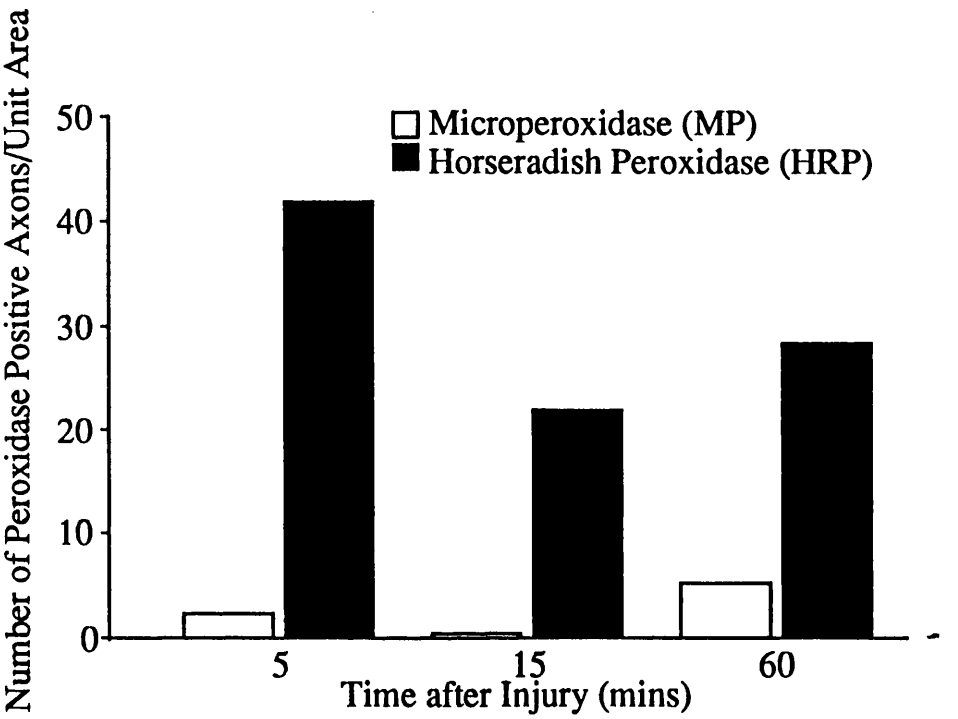


Figure 36

Comparison of horseradish peroxidase and microperoxidase in the detection of alterations in axolemmal permeability following traumatic brain injury.

CHAPTER 4 - DISCUSSION

4.1 ALTERATIONS IN THE NEURONAL CYTOSKELETON FOLLOWING ACUTE SUBDURAL HAEMATOMA IN THE RAT.

The role of the cytoskeleton in traumatic brain injury is a controversial issue. There is no doubt that cytoskeletal abnormalities are a pathological feature of numerous neurological disorders. Equally, there is a strong implication that the cytoskeleton has a pivotal role in the pathogenesis of these conditions. However, the relative role of structural determinants versus concurrent biochemical derangements, as the crucial pathological event, remains to be defined. In this discussion the main findings from this series of investigations will be considered with emphasis on the relative role of structural and biochemical alterations. For ease of description the cytoskeletal proteins will be discussed separately. It should be noted that the cytoskeleton is a functional unit and the biochemical derangements operant in traumatic brain injury may effect all it's components.

The principal aim of this investigation was to characterise the effects of acute subdural haematoma (ASDH) on the microtubule component of the neuronal cytoskeleton. The study was designed to identify morphological alterations in the cytoskeleton at three time points in the acute phase of injury. In doing so, we hoped to gain an insight into the sequence of events occuring immediately after an ASDH. The findings demonstrate that alterations in the microtubule component of the neuronal cytoskeleton occur following ASDH in the rat. In addition, and perhaps more importantly, these disturbances were detected within 30 minutes of

injury and involved both dendrites and axons. This has important implications in the design of therapeutic strategies which ultimately aim to arrest or reverse the pathological processes involved in this event. The data presented is the first systematic study of the cytoskeleton following ASDH and provides a basis for future investigations.

4.1.1 The rat model of acute subdural haematoma results in multiple insults to the brain

Animal models allow specific aspects of human disease to be investigated. They do not mimic the human condition and such an assumption is flawed [106,174]. The rat model of ASDH results in several features analagous to the situation in humans presenting with this lesion [9,43]. These features allow investigation of the pathophysiology of ASDH. The main neuropathological abnormality following an ASDH, in either the rat model or in humans, is an ischaemic lesion in the ipsilateral cortex beneath the haematoma. In addition, the associated mechanical displacement of the hemisphere, elevated intra-cranial pressure and resultant brain swelling produce a series of insults peculiar to this paradigm. Furthermore, the constituents of the blood itself may play an important role in the evolution of ischaemic cell damage [43,51,52,57]. Therefore, this model is not a model of pure ischaemia. It is a multi-insult model of an important complication of traumatic head injury. The multiplicity of injurious processes associated with ASDH suggest that several biochemical may co-exist and contribute to the pathogenesis of this condition.

4.1.2 MAP 2 is decreased in the ipsilateral cortex following acute subdural haematoma in the rat

Acute subdural haematoma (ASDH) results in a decrease in the protein level of MAP 2 in the adult rat cortex underlying the haematoma. Quantitative Western blot analysis demonstrated that 30 minutes after induction of an ASDH there is a 53% reduction in the protein level of MAP 2 in the ipsilateral cortex. This progresses to a 76% decrease in MAP 2 levels after 4 hours. In animals examined at 2 or 4 hours after production of a subdural haematoma there was an associated reduction in dendritic density as assessed by immunohistochemistry. Ultrastructural examination revealed an abnormal spatial configuration of cortical dendrites beneath the haematoma consistent with disruption of the dendritic cytoskeleton and in agreement with the altered MAP 2 immunostaining.

Within the last five years the role of MAP 2 in traumatic brain injury (TBI) has been examined and several laboratories have reported findings which are in broad agreement with the present study. Although differences in severity, between animal models, cause some discrepencies in the literature, the overall implication is that the somato-dendritic cytoskeleton is sensitive to TBI and that injurious processes initiate degradation of MAP 2. The first publication to examine the effect of TBI on MAP 2 demonstrated that moderate levels of a midline (central) fluid percussion injury in the rat resulted in a significant decrease in hippocampal MAP 2 levels but did not effect cortical levels of MAP 2

[105]. This study suggested that alterations in the distribution of MAP 2 can occur in the absence of neuronal cell damage. Other studies have not supported this basic premise. Rather it appears that a pronounced loss of MAP 2 is associated with degeneration and cell death in cortical neurones. MAP 2 immunoreactivity was profoundly diminished in the cortex and hippocampus ipsilateral to the site of injury following lateral (parasagittal) fluid percussion injury of moderate severity in the rat [118]. This alteration was detected within 10 minutes of injury and persisted up to 7 days after injury. In this study MAP 2 immunolabelling was virtually abolished in the entire injury area which is similar to the appearance of MAP 2 following ASDH. Severe cortical impact injury, which produces contusions similar to those found in humans after traumatic head injury, also caused a loss of MAP 2 protein in the rat cortex within 3 hours of injury [151]. Consequently, there appears to be a range of MAP 2 loss from pronounced loss preceding cell death in zones of severe injury to moderate losses in sublethally injured areas probably not destined for cell death. Therefore MAP 2 may have a useful role in the evaluation of the efficacy of various treatments on attenuating the neuropathological sequelae of traumatic brain injury.

It remains to be determined if this general decrease in MAP 2 is reversible. A recent report suggested that this was the case in mild TBI. In the rat model of mild focal cortical trauma diminished MAP 2 immunostaining was detected at the perimeter of the lesion up to 3 days post injury. However, no loss in immunostaining was detected 21 days after injury [119]. Hence, severe TBI may result in cytoskeletal

abnormalities which cannot be overcome by the cell and may culminate in neuronal cell death. Less severe cytoskeletal alterations could be repaired although functional changes in the neurone may persist.

The decrease in MAP 2 immunoreactivity could be attributable to several factors including the inability to detect the target epitope due to potential post-translational changes following traumatic brain injury [175]. These changes may involve proteolysis since the monoclonal anti-MAP 2 antibody used in this study (Sigma AP-20) does not detect MAP 2 breakdown products following traumatic brain injury [105,176]. Thus the loss of MAP 2 in dendritic processes most probably represents significant disruption of dendritic morphology and presumably alterations in dendritic function. Alternatively, loss of MAP 2 immunolabelling could be associated with mechanically broken dendrites, a possibility that requires investigation with other morphological techniques including Golgi or silver staining.

4.1.3 Laminar arrangement of MAP 2 immunopositive neurones within the ipsilateral cortex following acute subdural haematoma. Is there selective vulnerability of cortical neurones to acute subdural haematoma ?

Following induction of an acute subdural haematoma (ASDH) MAP 2 immunostaining showed a laminar distribution in the ipsilateral cortex. Despite pronounced MAP 2 loss in the cortex beneath the haematoma, a sub-population of pyramidal neurones in layers 3 and 5 were MAP 2 immunopositive. This feature was present 2 hours post ASDH when there

was a clearly defined bi-laminar arrangement of preserved immunolabelling. In animals examined 4 hours post ASDH a different pattern was displayed. Although a proportion of pyramidal neurones within layer 3 remained MAP 2 immunopositive, these were decreased in number and the definition of the cortical layers was more blurred indicating a more pronounced loss of MAP 2 immunoreactivity in the cortex.

The reason for this pattern of immunostaining is uncertain. Neuropathological examination of the brain following ASDH indicated that neurones within the centre of the ischaemic lesion demonstrated minimal changes although the variation in H&E staining suggested that early ischaemic changes were taking place. It is possible that these neurones are only sub-lethally injured and able to resist the decrease in MAP 2 occurring in other neurones which are more severely damaged by the insult such as those in the periphery of the histologically defined lesion. In this proposal subsequent cell death and degeneration would result in loss of MAP 2 immunoreactivity. This is in accord with numerous investigations that suggest loss of MAP 2 is a marker of impending neuronal cell death [119,132,151,177,178]. Unfortunately, the histological stain used in this study, is relatively poor in the recognition of neuronal death at short survival times within the cortex. Silver impregnation of degenerating neurones [179] and immunohistochemical labelling of reactive astrocytes with glial fibrillary acidic protein (GFAP) [180,181] are more useful and could provide definite evidence of the

corelation between MAP 2 loss and neuronal cell death which this study suggests.

Further support that MAP 2 loss is a feature of neuronal cell death following TBI was provided in a recent publication [151]. More importantly, and of clinical interest, their results were the reciprocal of the present study indicating the correctness of our assumption that the neurones in cortical layers 3 and 5 are only sub-lethally injured up to 4 hours after an ASDH. Severe cortical impact injury in the rat, resulted in a marked decrease in MAP 2 immunostaining in apical dendrites of pyramidal neurones within layers 3 and 5 of the injured cortex [151]. These changes in MAP 2 immunolabelling were associated with histological evidence of impending cell death. Taken together, MAP 2 immunostaining is a useful marker of both cell death and possible neuronal viability. It also confirms that strict parallel histological studies are essential for the interpretation of immunohistochemical data.

An alternative, and intriguing, explanation could be that there is a differential vulnerability of cortical neurones to ASDH. This would have clear therapeutic implications designed to arrest or limit neuronal injury in this clinically relevant condition. Investigations in animal models of cerebral ischaemia suggested the concept of "selective vulnerability" [131,182-186]. Simply stated, this proposes that certain neurones are more susceptible to a given insult than others. The physiological basis of this remains uncertain but could be related to anatomical factors, cell membrane receptor characteristics, electrophysiological or biochemical

properties. In animal models of ischaemia, selective vulnerability of neurones in the hippocampus have been described. Similarly, moderate TBI resulted in a decrease in the level of MAP 2 in the hippocampus in the absence of alterations in the cortex suggesting that there is a selective vulnerability of the hippocampus to traumatic injury [105]. Limited information is available concerning the selective vulnerability of cortical neurones. Transient ischaemia in the gerbil model demonstrated that the pyramidal cells in layers 3 and upper layer 6 of the somatosensory cortex were the most sensitive to ischaemia, whereas the neurones in layers 2, 4 and 5 were more resistant to ischaemia [187]. This bi-laminar pattern of neuronal death developed after even short periods of ischaemia (3-10 minutes) and was identifiable at post ischaemic survival times of 6 hours to one month. Why the layer 3 pyramidal neurones and, to a lesser extent the pyramidal neurones in upper layer 6, should be so much more sensitive to ischaemia than other cortical neurones is unknown. In the adult rat, many pyramidal cells in layer 3 are either corticocortical projection cells [188] or callosal projection cells [189] and some neurones at the lower border of layer 5 and the upper part of layer 6 are also callosal projection cells [190]. Therefore it seems that the MAP 2 immunopositive cells identified in the present study, belong to these two classes of projecting neurones. However, the current investigation did not demonstrate a decrease in MAP 2 immunostaining in layers 3 and 5 indicating that, in this model, these neurones are either sub-lethally injured or able to resist injury. As noted above H&E histology did not confirm ischaemic neuronal death and other techniques are required to clarify this issue. With regard to possible increased resistance to injury, it

may be that the blood supply to this region of the cortex offers some protection. Layer 4 of the adult rat somatosensory cortex is characterised by a richer blood supply than other cortical layers [191]. Perhaps a blood borne factor accounts for the decrease in MAP 2 observed in this layer whilst the lower blood supply to layers 3 and 5 account for some degree of protection. Laminar differences in NMDA receptor density could also explain the observed pattern of MAP 2 loss. Cortical NMDA receptors are most numerous in the outer layers (1 and 2) in the adult rat cortex [192,193]. Moreover, glutamate levels are increased in the cortex beneath the haematoma to seven times above basal levels [44]. Therefore, glutamate mediated neuronal death would predominantly effect the outer layers with resultant MAP 2 loss.

4.1.4 Tau-1 is altered in axons, neuronal perikarya and glial cells following acute subdural haematoma in the rat.

Following acute subdural haematoma (ASDH) disturbances in the level and localisation of the microtubule associated protein tau were detected in axons, neuronal cell bodies and glial cells. The temporal profile of these changes indicate that tau is modified 30 minutes after induction of ASDH and these disruptions persisted for up to 4 hours. There was a significant decrease in the level of tau protein in the cortex beneath the haematoma as detected by relative optical density. This was demonstrated at both 30 minutes and 4 hours after induction of the ASDH. Irregular accumulations of immunostained profiles were detected in axons subjacent to the haematoma at all time points examined after injury. At least three possible mechanisms may contribute to the observed alterations in tau immunostaining induced by ASDH and detected by Tau-1 immunostaining. First, tau may be dephosphorylated at one or more sites. Second, tau may be degraded and thirdly, there could be redistribution of existing tau or synthesis of new protein following ASDH. It is likely that a combination of these factors are involved to varying degrees.

The combined use of Tau-1 immunohistochemistry and Western blot analysis suggests that tau is dephosphorylated in response to ASDH. Specifically, the increased Tau-1 neuropil immunostaining observed in the histologically defined lesion is indicative of tau dephosphorylation since the epitope recognised by this antibody is masked in it's

phosphorylated state [167,194]. In addition, the altered electrophoretic profile of tau, with the appearance of additional bands, is also consistent with a change in the phosphorylation state of the protein [195]. Although the significance of this alteration remains unclear it is likely that disruption of the cytoskeleton will result in dysfunction.

Cerebral ischaemia results in an acute alteration in the phosphorylation of many brain proteins by modifying the activity of specific enzymes [196]. Since phosphorylation of cellular proteins is an important regulating mechanism controlling polymerisation of cytoskeletal proteins, changes in the phosphorylation status of proteins may contribute to the pathogenesis of brain damage [197]. Furthermore, since microtubules are inherently unstable structures, the stabilising effect of tau is likely to be important for the integrity of axons that depend on stable microtubules for rapid axonal transport. Ischaemia induced alterations in the phosphorylation of tau could cause modifications in the configuration of this cytoskeletal protein and therefore structural abnormalities in the cell. For example, tau is a component of the cross bridges between microtubules in axons. In the normal state the spacing between adjacent microtubules in the presence of tau is about 20nm. However, alterations in the phosphorylation of tau reduces the stoichiometry and affinity of binding to microtubules in vitro, possibly contributing to disruption of axonal function [91].

Acute subdural haematoma in the rat results in a sevenfold increase in extracellular glutamate levels in the cortex underlying the haematoma.[44]

Increased Tau-1 immunostainining occurred in response to glutamate receptor activation in neuronal cell cultures suggesting that in vivo, dephosphorylation of tau similarly may occur following ASDH in this model.[198] In ischaemia there is disruption of calcium homeostasis and other second messenger systems [199, 200]. Within the ischaemic lesion, the increased Tau-1 immunostaining may result from activation of glutamate receptors and subsequent increases in intracellular calcium levels which could activate calcium dependent enzymes. In vitro, tau is a substrate for many kinases including calcium/calmodulin-dependent kinase II(CaM kinase II), protein kinase C, cyclic AMP-dependent kinase, casein kinase II and proline directed kinases such as MAP kinase [201-203]. These enzymes can alter the phosphorylation status of tau suggesting that the balance of their activities may play an important role in the pathogenesis of ASDH. In the rabbit spinal cord ischaemia model there was a rapid loss of CaM kinase II activity within 30 minutes of ischaemic insult. This enzyme can phosphorylate tau in vitro, causing a reduction in it's electrophoretic mobility [204]. A recent study demonstrated that loss of CaM kinase II activity and dephosphorylation of tau occurred only in the ischaemic segment of the rabbit spinal cord [205]. These workers concluded that loss of CaM kinase II activity and dephosphorylation of tau were early markers of ischaemic damage. Of interest tau was rephosphorylated following reperfusion of the ischaemic segment suggesting that these alterations are not irreversible. Alterations in phosphatase 2B immunoreactivity have been reported following ischaemia although what these antigenic changes mean in terms of phosphatase activity is unclear [206-209]. However, given that phosphatase

2 activities are calcium dependent it is possible that enzyme activity is increase in ischaemic tissue. This could provide a mechanism for the dephosphorylation of tau.

Recent studies support the hypothesis that phosphorylation status of tau is altered following acute brain injury. Six hours of focal cerebral ischaemia in the cat induced increased tau immunostaining in a population of neurones within the ischaemic lesion as reflected by the appearance of Alz-50 immunoreactivity [129]. An early, variable and transient increase in perikaryal tau, as detected by the Tau-1 antibody, was reported following transient global ischaemia in the rat [128]. In the rat model of focal cerebral ischaemia alterations in tau were detected in axons, neuronal cell bodies and glial cells. These changes were revealed within 2 hours of permanent occlusion of the middle cerebral artery and were still present after 6 hours. This investigation concluded that dephosphorylation and/or degradation of tau may account for the observed alterations in the protein [130]. Alterations in tau consistent with dephosphorylation were observed following intra-hippocampal injection of the microtubule disrupting agent colchine[210]. In other paradigms of stress, alterations in the phosphorylation status of tau have been reported. Heat shock for 1 hour followed by a return to normal temperatures for 6 hours leads to an increased phosphorylation of tau in the rat brain [211]. In contrast, heat shock of the human neuroblastoma cell line LAN induces dephosphorylation of tau within 30-60 minutes [212]. Uncoupling of oxidative phosphorylation, as ocurs in ischaemia, in the rat brain cortical slices causes dephosphorylation of tau [213,214]. Overall, these studies

suggest that alterations in tau immunoreactivity is a sensitive indicator of stress. Furthermore, the phosphorylation status of this protein is effected by the balance of numerous enzymes and this may account for the alterations in tau detected following ASDH.

Degradation of tau is likely to be a feature of breakdown of the cytoskeleton following ASDH. Quantitative Wetern blot analysis indicated a significant decrease in the level of tau 30 minutes after induction of an ASDH. Cytoskeletal proteins are susceptible to enzymic proteolysis which may account for this finding. In particular calcium dependent proteases such as calpains have been shown to decrease levels of cytoskeletal proteins following cerebral ischaemia. This possible mechanism will be discussed in another section.

Although the issue of redistribution or synthesis of tau was not specifically addressed in the present study, increased Tau-1 immunoreactivity in axons and neuronal cell bodies following ASDH could be interpreted to indicate redistribution of existing tau or synthesis of new protein. One can speculate that tau is dephosphorylated, rephosphorylated to some extent and accumulates in some cell soma in a degraded form. Although ASDH results in a profound reduction in cerebral blood flow there is not complete ischaemia. In addition, histological examination indicated that certain neurones were only sub-lethally injured, as at the edge of the ischaemic territory. Taken together this suggests that under these conditions there may be some rephosphorylation of tau. In this regard, MAP kinase (ERK-2) has been

shown to be reactivated on reperfusion after transient cerebral ischaemia [215,216]. This kinase can phosphorylate tau on multiple sites. Support for the concept of redistribution of tau following ischaemia, is limited to a few recent studies. Following transient global ischaemia, immunoreactivity with the Tau-1 antibody decreased in the neuropil and increased in the perikarya in all regions of the rat hippocampus [128]. However, in the rat model of focal cerebral ischaemia no increase in immunostaining of either axons or neuronal perikarya was observed when another tau antibody, TP70, was used. This is inconsistent with accumulation or synthesis of tau protein [130].

For brevity it is suffice to note that increased Tau-1 immunostaining was detected in oligodendrocytes within the histologically defined ischaemic territoty. Typically these small cells were most numerous in the white matter although they could be identified scattered throughout the ipsilateral cortex. Increased glial staining for tau was detected following focal cerebral ischaemia in the rat [130] and also in human head injured tissue. Whether this represents an acute response to injury or an increased synthesis of tau is uncertain. However, it does raise the possibility that increased tau immunostaining is related in some way to the resistance of oligodendrocytes to injury. This topic was not central to the hypothesis of this investigation and will not be discussed further.

In conclusion there are numerous factors which may be involved in determining the observed alterations in tau following ASDH. Disturbance of the normal neurochemical environment of the brain effects various

enzymes and second messenger systems. In this context, it appears that the balance of phosphorylation is an important feature which may be central to the involvement of the cytoskeleton in traumatically induced brain injury.

4.1.5 Alterations in β–tubulin following acute subdural haematoma in the rat

This investigation demonstrated increased β-tubulin immunostaining in a defined population of cortical neuronal perikarya underlying the haematoma. These immunopositive cells were detected within 30 minutes of induction of an ASDH and became more prominent up to 4 hours after production of an ASDH. Typically these pyramidal neurones were localised to cortical layer 3 and with increasing survival more cell bodies were strongly β-tubulin immunostained. Cortical layer 3 is usually the least resistant to ischaemic insult suggesting once again that in the rat model of ASDH there exist a group of neurones in the centre of the lesion which are only sub-lethally injured. However, compared to sham operated animals and the contralateral cortex of injured animals there was increased immunostaining of these pyramidal neurones. In addition, axons in the ipsilateral corpus callosum demonstrated altered β-tubulin immunoreactivity with irregular, granular profiles detected at all time points examined. However, quantitative Western blot analysis indicated that there was a significant decrease in the protein level of both bands (high and low molecular weight) at 4 hours after induction of ASDH but not after 30 minutes. Overall, the results suggest that β-tubulin is modified and/or degraded in the rat model of ASDH. The main mechanisms involved are discussed elsewhere in this section and the activation of calpains may be responsible for protein degradation. As noted previously protein phosphorylation is altered in various forms of stress stimuli including ischaemia. This may play a role in the observed alterations in β–tubulin although this aspect of the cytoskeleton has not

attracted much attention in the literature. Severe forebrain ischaemia results in decreased phosphorylation of α-tubulin in the rat cortex [217]. This may result in abnormalities in the polymerisation of this cytoskeletal protein. In broad agreement with this, 5 minutes of ischaemia in the gerbil results in decreased α-tubulin immunostaining in the hippocampus [178]. The monoclonal antibody employed in the present study was not affected by the phosphorylation status of β-tubulin and detects all forms of β-tubulin. Further studies could use alkaline phosphatase pretreatment to determine the of phosphorylation in the response of β-tubulin in this paradigm.

An increased synthesis of β-tubulin following induction of an ASDH would seem unlikely, especially at the acute time points the present study examined. However, in the light of recent studies, this interesting possibility deserves discussion. It may be that there is an initial increase in β–tubulin expression followed by enzymic degradation. There is considerable evidence that mammalian β–tubulin is phosphorylated [218,219]. It is likely that this modification is of great significance in modulating the assembly or properties of microtubules. A recent report demonstrated that of the seven beta isotypes, the phosphorylated one is beta(III), the isotype found almost entirely in neurones [220]. Moreover, the phosphate on the beta(III) isotype was resistant to a wide variety of phosphatases but removal of the phosphate inhibited microtubule assembly in vitro. However, such an inhibition was not evident when microtubule assembly was induced in the absence of microtubule associated proteins. Therefore, dephosphorylation of β-tubulin in the

presence of decreased microtubule associated proteins may result in an increased production of microtubules. Although the mechanism involved is not known, this could account for the increased β-tubulin immunolabelling detected in the ischaemic cortex beneath the haematoma.

There is a precedent in the literature indicating that such an increase in tubulin synthesis occurs following injury to central nervous system. When axons of a peripheral nerve are transected and allowed to regrow, tubulin protein levels rise substantially and there is an enhanced expression of tubulin mRNAs [221-225]. To determine if this change occurred in the central nervous system environment the retinal ganglion cells were examined after cutting the optic nerve [226]. An early increase in tubulin mRNA was detected in axotomised adult rat retinal ganglion cells. This increase, as detected by Northern blot analysis, only lasted for 1 day before a later decrease. It is suggested that the transient increase in tubulin mRNAs may reflect an early regenerative response. It could be that following ASDH there is an increased expression of β-tubulin in an attempt to increase synthesis of microtubules. The stimulus for this process could be a decrease in the levels of micritubule associated proteins such as MAP 2 which is known to stimulate tubulin polymerisation [227]. This requires further investigation but is an intriguing possibility in the modulation of the cytoskeleton.

4.1.6 Ultrastructural alterations in the neuronal cytoskeleton following acute subdural haematoma in the rat.

Transmission electron microscopy demonstrated alterations in the dendritic and axonal cytoskeleton following acute subdural haematoma (ASDH) in the rat. The ultrastructural evidence obtained correlated with the alterations in the neuronal cytoskeleton detected with immunohistochemistry and Western blots. The overall implication is that there is marked disruption of both the dendritic and axonal cytoskeleton in this model of traumatic brain injury. The ultrastructural evidence supports and substantiates the immunohistochemical and immunoblotting data. Although ultrastructural analysis was only performed in animals allowed to survive 4 hours after production of an ASDH, the early alterations detected with immunohistochemical methods suggest that they represent morphological disorganisation of the cytoskeleton.

In the cortex underlying the haematoma there was an abnormal misalignment of dendritic microtubules. This is consistent with the loss of MAP 2 demonstrated in the present investigation. MAP 2 is a component of the cross bridges between microtubules and is necessary for the three dimensional spatial configuration of the dendrite [64,70,91]. Therefore, loss of MAP 2 could result in an abnormal arrangement of the microtubules relative to the longitudinal axis of the dendrite. Perhaps the loss of MAP 2 allowed the microtubules to collapse on each other. It is likely that this leads to functional abnormalities by impairing fast axoplasmic transport. This investigation has demonstrated that ASDH

results in a significant decrease in protein levels of MAP 2. Furthermore, this loss of MAP 2 correlated with the detection of calpain mediated spectrin breakdown products in the cortex beneath the haematoma. Conceivably, the MAP 2 cross bridges could be cleaved by calpain causing the microtubules to collapse and become disorganised.

There was loss of an organised axonal cytoskeleton following ASDH. Typically, there were reduced numbers of microtubules within the axoplasm which consisted of a diffuse, flocculent precipitate. In addition, the neurofilaments appeared compacted, that is, there was a reduced inter-filament spacing, following ASDH. Previous studies in other animal models of traumatic brain injury are in broad agreement with these findings. Quantitative analysis demonstrated a significant loss of microtubules in myelinated axons following optic nerve traction in the guinea pig. In the same material, there was increased spacing of neurofilaments [116,228]. Povlishock and colleagues described neurofilament compaction and loss of microtubules in traumatically injured axons [111]. Moreover, they suggested that loss of the neurofilament sidearms allowed the neurofilaments to collapse on one another with resultant increased packing. However, this alteration was only detected in the more severe forms of injury. Specifically, mild traumatic brain injury was associated with misalignment of the neurofilaments and not microtubule loss and compaction of neurofilaments[111].These morphological abnormalities are not species [113] or model specific [112] indicating that they are operant in most forms of traumatic brain injury.

Dissolution of the axoplasm is thought to result from the proteolysis of cytoskeletal proteins. Calpain, a calcium activated neutral protease, may be involved in this process [170]. Our results demonstrated calpain mediated spectrin breakdown products in white matter tracts of the brain following ASDH but only in animals examined at 2 hours or 4 hours after injury. In addition, the immunolabelling was faint. However, it should be recognised that the Ab38 antibody detects calpain I (μM-calpain) mediated spectrin breakdown products and is only present in low levels in axons [141-143]. A recent report demonstrated calpain I mediated spectrin breakdown products within axons following experimental traumatic brain injury [150]. It should be noted that immunolabelled axons were only identified in the region of a direct tear of the corpus callosum suggesting local disruptive events may be involved. It is also possible that proteases other than calpain cause direct cytoskeletal dissolution. The microtubule associated protein, tau is predominantly localised to axons [94]. Following ASDH, Tau-1 immunostaining was altered in white matter. Since tau is a component of the cross bridges between axonal microtubules [91], modification of this protein could affect the spatial arrangement of the microtubules.

The ultrastructural examination confirmed disruption of both the dendritic and axonal cytoskeleton following ASDH in the rat. The precise mechanisms involved are uncertain at this stage. That calpain or other protease enzymes plays a role seems likely although the exact site of proteolysis remains unclear. It is recognised that the projection domains of microtubules are essential in the maintainence of normal neuronal

calibre [64,70,91]. This function is served by the microtubule associated proteins, MAP 2 and tau, in dendrites and axons respectively. It is conceivable that enzymic degradation of these microtubule associated proteins is responsible for the disorganisation of the cytoskeleton revealed by electron microscopy.

Consideration of mechanisms involved in the alterations in microtubules and neurofilaments after traumatic brain injury can only be speculative. Nevertheless recent evidence has provided a concensus for initial compaction of neurofilaments and loss of microtubules following axonal injury [38,111,112,114,116]. The central hypothesis suggests that activation of calpain I and/or calpain II may account for the different response of microtubules and neurofilaments to traumatic injury. In this proposal calpain I (μM-calpain) is activated by micromolar increases in intracellular free calcium levels following the traumatic insult [147]. This results in cleavage of the neurofilament sidearms causing the neurofilaments to collapse and become compacted [111,112,114]. That is, there is a reduced interfilament distance but retention of their filamentous structure for up to six hours after injury [111,114]. Activation of calpain I (μM calpain) results in post-translational modification of the 150kDa neurofilament subunit (NF-M) and may be involved in shaping the neurofilament network rather than it's degradation [229,230]. This is then followed over a period of hours rather than minutes, by neurofilament misalignment and disassembly [111,114]. Dissolution of neurofilaments does not occur until calcium levels are increased to millimolar levels capable of activating calpain II [147]. Complete loss of neurofilaments

through activation of calpain II (mM calpain) requires calcium concentrations of at least 0.2mM [231]

In addition, calpain I (μM-calpain) activation causes rapid dissolution of microtubules as evidenced by the loss of microtubules in the axoplasm detected in ultrastructural studies. It is assumed that degradation of the microtubule associated proteins also occurs by this mechanism. Axonal microtubules exist as two subtypes, either so-called cold-labile and cold-stable or drug-labile and drug-stable [232-235]. Calcium alone is capable of precipitous disassembly of cold-labile microtubules [236-239]. Cold-stable microtubules are resistant to calcium but are depolymerised when calcium interacts with calmodulin at physiological concentrations such as occur in the brain, where half maximal disassembly of cold-stable microtubules occurs in the presence of 1.0 mM calmodulin and 100 mM calcium [235].

4.1.7 The role of the calcium dependent protease calpain in the mediation of cytoskeletal injury following acute subdural haematoma in the rat.

The present investigation demonstrates that calpain mediated proteolysis of spectrin occurs as early as 30 minutes following induction of an acute subdural haematoma (ASDH) in the rat and continues for up to 4 hours post injury. In addition, immunohistochemical analysis revealed that calpain activity predominated in the cortex underlying the haematoma. Although spectrin is an integral constituent of the submembrane cytoskeleton, this study suggests that calpain may play a role in the degradation of the microtubule component of the neuronal cytoskeleton. Immunohistochemical observations also provided evidence for the cellular localisation of calpain activity after injury. At all survival times examined, spectrin breakdown products (BDPs) were detected in dendrites and neuronal cell bodies but not in axons. At 2 hours and 4 hours post induction of ASDH, calpain degraded spectrin was localised in dystrophic and fragmented dendritic processes. This suggests that dendrites may be particularly vulnerable to calpain mediated proteolysis. The only previous study to examine calpain mediated proteolysis of the cytoskeleton following traumatic brain injury reported similar findings [150].

This investigation has reported early changes in the breakdown of spectrin by calpain following experimental brain injury in the rat. Several points should be considered when using this data to interpret the time course of calpain activation in this model. Calpain activation depends on

the elevation of intracellular calcium levels above a certain threshold [147]. Previous investigations have reported rapid changes in extracellular calcium levels following traumatic brain injury [137,240]. However, whether these translate to a rapid increase in intracellular levels of calcium has not been determined. A recent qualitative study demonstrated accumulation of calcium in the axoplasm of the node of Ranvier following traction of the optic nerve in the guinea pig [116]. As noted in that report, further quantitative studies are required to provide evidence for changes in the concentration of calcium following traumatic brain injury. Therefore, it is possible that increased intracellular free calcium and subsequent calpain activation are delayed following trauma, perhaps due to calcium buffering. An alternative explanation is that acute calpain activation may result in a limited spectrin proteolysis below the detection threshold for the immunohistochemical protocol.

The cerebral cortex underlying the haematoma displayed the most pronounced spectrin breakdown. Histological examination revealed that this area also demonstrates the most marked ischaemic cell change. This close correspondance suggests that cell populations undergoing calpain mediated spectrin breakdown may eventually die. Since we report calpain mediated proteolysis as early as 30 minutes after injury it is likely that spectrin breakdown occurs prior to, rather than as a consequence of neuronal cell death. Studies in experimental models of excitotoxicity and cerebral ischaemia support this hypothesis and raise the possibility that aspects of calpain mediated pathphysiology are reversible [170, 241-243]. Indeed within the last few years several investigations have demonstrated

that calpain inhibitors may have neuroprotective effects in animal models of cerebral ischaemia [243-245]. Moreover, pretreatment with a synthetic calpain inhibitor suppressed the degree of depletion of MAP 2 following permanent occlusion of the middle cerebral artery in the rat [244]. The discovery and refinement of selective inhibitors of calpain remains an important goal [246].

The vulnerability of dendrites following traumatic brain injury has been suggested by decreased levels of cytoskeletal proteins such as neurofilaments and microtubule associated protein 2 (MAP 2) [105,115,151]. In the present investigation, the region of decreased MAP 2 immunostaining in the cortex corresponds closely with the area of pronounced calpain mediated spectrin BDPs immunoreactivity. This suggests that calpain activation may be responsible, in part, for MAP 2 proteolysis. However, this relationship was not detected in the 30 minute survival animals. In this group there was no observed alterations in MAP 2 immunostaining whereas calpain mediated proteolysis was demonstrated. Despite the absence of detected immunohistochemical alterations in the distribution of MAP 2, quantitative Western blot analysis revealed a significant decrease in the protein levels of MAP 2 in the ipsilateral cortex following ASDH. It is unclear why the immunohistochemical protocol failed to detect alerations in MAP 2 immunolabelling but this may reflect the fact that Western blot analysis was the more sensitive technique at this early time point. It is recognised that MAP 2 is more susceptible to calpain mediated proteolysis than spectrin [146,247] supporting the correctness of this assumption. Overall

this study supports the hypothesis that calpain activation occurs within 30 minutes of ASDH and is involved in the degradation of MAP 2. Whether other proteases are involved in the proteolysis of MAP 2 requires elucidation. As noted in previous sections, a decrease in the protein levels of tau and β-tubulin occurred following ASDH in the rat. The demonstration of calpain activation in the cortex is consistent with the central hypothesis that calpain is involved in the degradation of cytoskeletal proteins following traumatic brain injury.

In this study calpain mediated spectrin proteolysis was detected in axons but only in those animals examined at 2 hours and 4 hours after induction of an acute subdural haematoma. Overall, the intensity of axonal calpain mediated spectrin BDPs immunoreactivity was low and restricted to the white matter tracts subjacent to the injured cortex. This indicates that the observed alterations in Tau-1 and β-tubulin within white matter were not entirely due to calpain I activity. This observation is not surprising. Axons are believed to contain primarily calpain II rather than calpain I necessitaing extremely elevated calcium levels to initiate proteolysis [142,143,147]. Such non-physiological concentrations of axonal intracellular calcium may not occur in this experimental model. Alternatively they may not occur within 4 hours of induction of an ASDH. Neither of these points were investigated in the present study. Furthermore, the antibody used in this study, Ab38, is specific for the breakdown products of calpain I activity [170]. Future studies will utilise antibodies directed at the calpain II BDPs to address this issue. It is suffice to conclude that activation of calpain may play a role in the

mediation of traumatically induced axonal injury following acute subdural haematoma.

4.1.8 The mechanistic basis of cytoskeletal damage following traumatic brain injury.

Although this investigation was not designed to provide definitive characterisation of the mechanism(s) involved in cytoskeletal injury after traumatic brain injury, several mechanistically relevant observations merit discussion.

Recent investigations suggest that NMDA receptor activation has a role in mediating calpain induced damage to the cytoskeleton [242,248-250]. In particular it is proposed that NMDA receptor activation interacts with calpain leading to a common final pathway of neuronal injury. There is no doubt that the presence of an acute subdural haematoma alters the neurochemical environment of the brain. Traumatic brain injury induces widespread membrane depolarisation and excessive release of neurotransmitters, including acetylcholine and glutamate [251]. In the rat model of acute subdural haematoma there is an increase in extracellular glutamate to seven times above basal levels in the cortex underlying the haematoma [44]. The ionophore associated with the N-methyl-D-aspartate (NMDA) receptor is permeable to calcium and there is considerable evidence that this property contributes to the role of the receptor in pathological conditions [138]. Calpain has been implicated in a variety of NMDA receptor dependent effects and recent investigations have

suggested that rapid activation of calpain occurs following stimulation of NMDA receptors [248]. Furthermore, translational suppression of calpain reduced NMDA-induced spectrin proteolysis in hippocampal slices [249]. Activation of the NMDA receptor seems to be involved in the observed decrease in MAP 2 immunostaining following cerebral ischaemia in the gerbil, since pretreatment with the NMDA antagonist, MK801 ameliorates this response [252]. Administration of kynurenate, a competitive antagonist at non-NMDA receptors and a non-competitive antagonist at the NMDA receptor, attenuated MAP 2 loss following experimental traumatic brain injury in the rat [118]. However, a recent study did not support the concept of NMDA receptor mediated calcium influx as an important mechanism underlying the loss of MAP 2 immunolabelling following mild traumatic brain injury in the rat [119]

It is also possible that other effects elicited by intense stimulation of NMDA receptors interact with calpain activation. Perhaps the most obvious example of this would be the generation of free radicals, an effect observed to occur following application of NMDA [253, 254]. Proteins damaged by free radicals are reported to be more susceptible to cleavage by calpain thereby extending the range of aberrant effects that follow stimulation of the protease [255].

As discussed earlier the phosphorylation status of tau, MAP 2 and tubulin effects their structural properties with the inference that this translates into functional disturbances. Protein phosphorylation also influences the interaction between calpain and cytoskeletal proteins [140, 256]. The basis

for this complex process is that calcium infux through the NMDA receptor affects second messenger systems controlling protein phosphorylation. Protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase II (CaMKII), are also substrates for calpain [257-261]. Therefore, calpain mediated proteolysis of these regulatory enzymes has the potential to alter the degree of phosphorylation of the cytoskeletal proteins. Ample biochemical evidence indicates that MAP 2 phosphorylation influences it's affinity for microtubules and it's efficacy in promoting tubulin polymerisation [262,263]. Phosphorylation has been hypothesised to effect the conformation of the MAP 2 molecule [264] and seems to determine the susceptibility of the molecule to degradation by calpain [147]. Recent studies have indicated that protection of MAP 2 against calpain is dependent on the degree of phosphorylation of MAP 2 [256,265]. In contrast, NMDA receptor activation induces rapid dephosphorylation of MAP 2 making it more vulnerable to calpainolytic degradation [175]. Phosphorylation is generally considered to protect neurofilament proteins from degradation whereas dephosphorylation increases vulnerability of the neurofilaments to calpain mediated proteolytic degradation [266]. It has been suggested that the high degree of phosphorylation common to the purified NF-H subunit may be responsible for it's relative resistance to calpain mediated proteolysis [267].

The context in which the above interactions would be operative is still unclear. Nevertheless the physiological relevance of these interactions and their interplay are likely to underlie the mechanistic basis of cytoskeletal disruption. It is worthy of speculation that calpain

degradation of phosphatases may render the cytoskeleton more susceptible to calpain mediated proteolysis. The current investigation supports the hypothesis that the phosphorylation status of cytoskeletal proteins is altered following traumatic brain injury and that calpain is responsible, at least in part, for proteolytic degradation of the cytoskeleton.

4.2 ALTERATIONS IN THE NEURONAL CYTOSKELETON FOLLOWING ACUTE SUBDURAL HAEMATOMA IN HUMANS.

The results of this study confirmed that alterations in the neuronal cytoskeleton can be detected in human post mortem brain tissue following traumatic brain injury. In patients sustaining acute subdural haematomas there was a loss of MAP 2 immunoreactivity in the cerebral cortex underlying the haematoma compared to the control cases. In addition, this study demonstrated an increase in Tau-1 immunostaining in the somatodendritic compartment and an altered distribution of β–tubulin immunolabelling in the parietal cortex beneath the haematoma compared to the control cases. Comparison of the results in human postmortem material with those in the rat model of acute subdural haematoma indicate that the alterations in the microtubule component of the neuronal cytoskeleton were similar in both studies. Although the postmortem changes were not as extensively characterised in the human as compared to the rat brain, the results demonstrate that alterations in the neuronal cytoskeleton are a feature of head injured human beings sustaining an acute subdural haematoma. This is important in that it indicates that the rat model of acute subdural haematoma could be a useful tool in the investigation of the pathobiology of traumatic brain injury.

The use of human postmortem tissue presents certain difficulties. There is no doubt that it is difficult to correct for every premorbid variable. For example, the mechanism of injury can vary within a group of patients sustaining an acute subdural haematoma. In addition, factors such as

associated systemic injuries, coexistent diseases, delays in treatment and the nature of treatment can vary greatly. However, since the aim of this particular study was to identify alterations in the neuronal cytoskeleton in humans and, to compare these with the results obtained in the well controlled experimental model, it is reasonable to accept these limitations as being inherent to the use of postmortem tissue. Obvious in any consideration of a study of this nature is the potential for artifactual change. As noted in the Materials and Methods section, considerable differences exist in the intervals between death and fixation of brain tissue. Although such a variable can contribute to potential erroneous evaluations, it is reasonable to accept this because this study was essentially a neuropathological comparison between experimental studies, in which delays in fixation are not a factor, and human postmortem tissue.

A recent investigation examined the stability of the neuronal cytoskeleton during the postmortem interval [268]. These workers demonstrated that similar cytoskeletal changes occurred in human postmortem tissue and rat brain analysed at various postmortem intervals. The main suggestion from this study was that alterations in the cytoskeleton detected in postmortem tissue were of questionable significance. The reason for this appeared to be that the microtubules and microtubule associated proteins (MAPs) are not biologically stable after death. Therefore caution is required in the interpretation of cytoskeletal alterations in studies using human postmortem tissue.

Overall the results of the present investigations indicate that similar alterations in the cytoskeleton can be detected in humans and rats following acute subdural haematoma. Despite the difficulties in the use of postmortem tissue, this work supports the hypothesis that alterations in the neuronal cytoskeleton play a role in the mediation of traumatic brain injury.

4.3 ALTERATIONS IN AXOLEMMAL PERMEABILITY FOLLOWING TRAUMATIC BRAIN INJURY.

The present study confirmed the results of previous investigations by demonstrating that alterations in the axolemma are a feature of traumatically induced axonal injury [111]. Comparison between two extracellular tracers of different molecular size indicated that there was no evidence of an increased detection of flooded axons when employing a tracer of smaller molecular size. This suggests that in this injury paradigm, trans-axolemmal influx of the extracellular tracer is an" all or none" phenomenon. That is, there is no evidence of a differential permeability change following traumatic brain injury wherein the axolemma would first preferentially leak to tracers of smaller molecular weights. From this study it appears that when the axolemma fails it does so in a non-specific manner.

Ultrastructural tracers serve the important purpose of defining tissue or cellular compartments and their interconnecting channels. The ideal tracer substance should be (a) nontoxic and physiologically inert, (b) composed of uniform particles of known size, (c) immobilised in its in vivo position by fixation, (d) capable of being accurately localised when viewed with the electron microscope and , (e) demonstrable at low concentration. In addition a small molecular size is essential for many applications. For example,membrane channels as narrow as 20nm have been demonstrated with colloidal lanthanum [269], a tracer smaller than almost all others in use. Because of its toxicity, colloidal lanthanum can only be applied to

fixed rather than living tissue specimens. This complicates and limits the interpretations made with this tracer. Clearly there is a need for an extracellular tracer of smaller molecuar size which is non-toxic at physiological concentrations. Microperoxidase (MP) is such a tracer (molecular weight 1900 Daltons, molecular diameter 2nm).

Since the horseradish peroxidase (HRP) method was first applied to the peripheral nervous system [270] and subsequently to the central nervous system [271] it has become one of the most common experimental methods for demonstrating neuronal connectivity in the nervous system. HRP has a molecular weight of 40, 000 Daltons and a molecular diameter of 5nm. In contrast, microperoxidase (MP) is an ultrastructural tracer of low molecular weight. The initial investigation which proposed the use of MP demonstrated that MP penetrated an extracellular compartment from which HRP was excluded [272]. This finding was consistent with the smaller molecular size of MP compared to HRP. On this basis the present study was designed.

The investigation was designed to examine a particular aspect of axolemmal permeability. Previous studies employed (HRP) as an extracellular tracer [111,112,114]. The use of a macromolecular tracer with a molecular weight of 40,000 units has inherent restrictions as oulined above. It could be argued that such a molecule may not enter an injured axon because it is too large to "pass through "the axolemma. The axolemma of an injured axon may be disrupted to allow the passage of smaller molecular weight species such as calcium ions as outlined in the

previous sections. If there was a differential change in axolemmal permeability following traumatic injury then an extracellular tracer of smaller molecular weight should detect an increased number of reactive axons. This study did not support the concept that such a process was operant. Clearly this conclusion can only be made on the assumption that HRP and MP have similar properties as related to diffusion and binding to intracellular protein. The fact that the extracellular tracer of smaller molecular weight (MP) detected significantly less reactive axons than HRP suggests that this is not the case. To my knowledge there is no relevant literature available on this issue. It is possible that the smaller MP molecule does enter an increaed number of reactive axons but does not bind to the intracellular proteins and refluxes out of the axon rendering it undetectable to our experimental procedure. Therefore, it is suffice to note that this study supports the concept that disruption of the axolemma following traumatic brain injury is an all or none phenomenon.

The mechanistic basis by which normally excluded molecules gain intra-axonal access following traumatic brain injury remain to be elucidated. It is unlikely that organic macromolecules are gaining access through ionic channels. Furthermore, both tracers examined appeared free in the axoplasm as opposed to being bound to the membrane. This free appearance indicates that it is unlikely that endocytosis is the underlying mechanism of influx. General axolemmal failure, as proposed in Wallerian degeneration and ischaemia [273-276] could be excluded by the focal appearance of intra-axonal peroxidase activity and the lack of cytoskeletal dissolution. Recent investigations suggest that the

mechanism of axolemmal permeability is a complex dynamic process and may involve attempts by the axon to reseal the disrupted membrane.

Following axotomy extracellular tracers can be detected intra-axonally. Several investigations have demonstrated tracer influx after slice axotomy [277-279]. In these studies, the axolemmal permeability is transient and usually lasts for up to 30 minutes after axotomy. This "sealing" of the axolemma occurs by constriction of the severed tip of the fibre and involves a calcium dependent process. An ongoing study by Blight and colleagues suggests that the tracer may not enter the axon via the cut end of the fibre [280]. Using the in vitro model of excised spinal white matter these workers applied extracelluar HRP following axotomy. When the axons were sliced with a scalpel they were found to reseal and exclude further influx of HRP within 30 minutes. In contrast, axons subjected to crush injury, where the axon is not severed, maintained their altered axolemmal permeability for up to 3 hours. This suggests that the mechanics of the axonal injury may effect the nature of axolemmal disruption. It is possible that some of the axons are subjected to shear or strain forces in the axolemma following a crush injury [281] producing alterations in axolemmal permeability similar to those observed in the animal model of traumatic brain injury examined in the present study. The in vitro model allowed this group to modify the extracellular environment and examine the process of axolemmal sealing after an injury. Following slice axotomy an extracellular media containing no calcium ions impaired resealing of the axolemma such that HRP could influx for up to 3 hours after axotomy [280]. A similar disturbance occurs

after traumatic or crush axotomy indicating that the type of injury has the potential to inhibit repair mechanisms within the axon. Blight interprets this to suggest that traumatic injury, in this case crush injury, has caused the uptake of calcium ions by glia and neurones, resulting in a decrease in the concentration of calcium in the extracellular space necessary to stimulate axolemma resealing (Blight, personal communication).

The focus of this study was the axolemma and not the associated detailed cytoskeletal changes which are documented elsewhere [111,112,114]. Concerning the relationship between these reactive axonal changes and alterations in the axolemma, the key question is what factor(s) trigger the neurofilament compaction, loss of microtubules and mitochondrial dilation following traumatic brain injury. The rapid passage of macromolecules and/or smaller molecular species, such as ions, through the compomised axolemma could contribute to the ensuing axonal damage. The possible role of calcium has attracted a great deal of research attention in this regard. In the present study, swollen dilated mitochondria were detected in reactive axons within 5 minutes of traumatic injury. This may reflect a compensatory reaction to calcium influx [282]. Mitochondria are known to absorb calcium ions when cytoplasmic concentrations are elevated. The increased mitochondrial concentration of calcium stimulates dilation or swelling of the organelle [282-284]. That mitochondrial dilation is consistently linked with altered axolemmal permeability in the current study, suggests that the mitochondria are absorbing excess intra-axonal calcium that has entered the axoplasm through the disrupted membrane. In this hypothesis the

influx of calcium through the axolemma would be capable of altering the intra-axonal environment. The associated cytoskeletal changes may involve the calcium activated neutral proteases, calpain, which has been discussed in a previous section.

Current studies in our laboratory support the hypothesis that calcium is involved in the observed cytoskeletal changes (Povlishock, personal communication). In a preliminary study, calcium chelators were infused intra-thecally in conjunction with HRP. Following experimental traumatic brain injury the brains were prepared for ultrastructural analysis. There was decreased neurofilament compaction and no mitochondrial dilation in the chelator treated animals compared to the non-treated group. Although calcium chelators did not provide complete protection, they altered the morphology of the HRP permeable axons suggesting that calcium influx has an important role in the pathobiology of traumatically induced axonal injury.

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