SUBSTANCE P IN TRAUMATIC BRAIN INJURY

ANDREW CHRISTIAN ZACEST

DEPARTMENT OF PATHOLOGY

THE UNIVERSITY OF ADELAIDE

DEPARTMENT OF NEUROPATHOLOGY

INSTITUTE OF MEDICAL AND VETERINARY SCIENCE

THESIS SUBMITTED FOR THE DEGREE OF MASTERS OF SURGERY

MARCH 2004

page

Table of Contents 3

CHAPTER 1. LITERATURE REVIEW.

Statement of access

I give consent to this copy of my thesis, when deposited in the University Libraries, being available for photocopying and loan.

Andrew Christian Zacest

Declaration

I certify that this thesis does not contain any material that has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge it does not contain any material which has been previously been written or published by another person, except where due reference is made.

Andrew Christian Zacest

March 2004.

Acknowledgements

This thesis would not have been possible without the contributions of the following people who gave so freely of their time and to whom this work is dedicated. It was an honour to work with a group of researchers who can each be rightly regarded as leaders in their fields.

Bob Vink, who took me on as a naïve researcher and gave guidance as well as independence throughout the year

Peter Blumbergs, whose expertise with neuropathology underpinned the interpretation of all the results

Jim Manavis, whose technical expertise made possible the results

Mounir Ghabriel, my prior neuroanatomy tutor, whose expertise with the electron microscope and the blood brain barrier opened a new realm of research opportunity

Gafar Sarvestani, whose expertise with the confocal microscope produces those images that captured a thousand words

Dale Caville, whose expertise printed the manuscript

Cathy, Penny, Bernice, Cai, Rowena and all the lab staff whose technical expertise made the many hours in the lab fun

My wife Gorana and my daughter Annalisa, with whom I could spend more time this year and who were my support team

My parents, who have proudly encouraged all my academic endeavours and particularly my father who rightly told me on my 21st birthday that " Scholarship is its own reward ".

Abbreviations

Ar	Argon
βAPP	beta amyloid precursor protein
BBB	blood brain barrier
CNS	central nervous system
Cm	centimetre
CGRP	calcitonin gene related protein
CD	compact disc
Cy5	indopentamethine cyanine
DAB	diaminobenzene
ELIZA	enzyme linked immunoassay
EM	electron microscopy
FITC	fluorescein isothiocyanate
GABA	gamma amino butryic acid
GFAP	glial fibrillary acid protein
Gm	gram
He	helium
Hr	hour
ICAM	inter cellular adhesion molecule
IL	interleukin
L/min	litre per minute
KD	Kilo Dalton

M	metre
Ml	mili litre
Mw	mili watt
MCA	middle cerebral artery
Mg	magnesium
MHC	multi histocompatability complex
MRS	magnetic resonance spectroscopy
N	number
NA	numerical aperture
Ne	neon
NK	neurokinin
NO	nitrous oxide
NMDA	N methyl D aspartate
Nm	nano meter
PET	positron emission tomography
PGP	protein gene product
PPT	pre pro tachykinin
PMT	photomultiplier tube
SNC	spinal nucleus caudalis
SP	substance P
STT	spinal trigeminal tract
TNC	trigeminal nucleus caudalis

TNF	tissue necrosis factor
ULAPO	universal positive low APO chromatic
VIP	vasoactive intestinal peptide

List of figures

Figure 8.1 H&E section of rat frontoparietal parasagittal cortex 5hr following diffuse impact. Subarachnoid haemorrhage(⇒) and neuronal dark cell change are evident (←).(x100)

Figure 8.2 H & E section of rat fourth ventricle 5hr following diffuse impact, showing haematoma (H).Cerebellum (C) and medulla (M).(x40)

Figure 9.1 APP positive axonal swellings (←) in rodent corpus callosum 5hr following diffuse impact. (x400)

Figure 9.2 Axonal swellings (←) and increased neuronal APP in rodent parasagittal cortex 5hr following diffuse impact. (x400).
Figure 9.3 APP positive axonal swellings (←) in rodent pyramidal decussation 5hr following diffuse trauma. (x400)

Figure 9.4 Perivascular APP positive granules (→) in rodent parasagittal cortex 5hr following diffuse impact. (x400)

Figure 10.1and 10.2. Gross examination and coronal section of rat brain 5hours following fluid percussion injury. Left parieto occipital subdural haematoma with underlying cortical contusion with mass effect. Left(L) and right(R) indicated.

Figure 11.1 Left parietal haemorrhagic contusion underlying subdural haematoma in a rodent 5 hours following fluid percussion injury (.x100)

Figure 11.2 Adjacent pyramidal neurones (P) exhibit red tinctorial change against a background of microcytic oedema (E) in the neuropil. (x400)

Figure 12.1 Area of haemorrhage (H) with infarct (I) in left parietal rodent cortex 5hr following fluid percussion injury. (HE x200)

Figure 12.2 Right parasagittal subdural haematoma with mild cortical contusion in rodent 5 hours following fluid percussion injury. (HE x 100).

Figure 13.1 APP positive axonal swellings (←) in left corpus callosum of rodent 5hr following fluid percussion injury.(x400)

Figure 13.1 APP positive perivascular granules (→) in left parietal hemipshere of rodent 5hr following fluid percussion injury.(x400)

Figure 14.1 Left parietal cortex pyramidal neurones (P) adjacent to haemorrhagic contusion show intense SP granular morphology 5hr following fluid percussion injury. (x400)

Figure 14.2 Left pyramidal non pyramidal neurones (N) adjacent to area of infarction show positive SP staining in contrast to non staining adjacent neurones.(x200)

Figure 15.1 and 15.2. Left parietal cortical venules adjacent to haemorrhagic contusion show increased perivascular Sp granularity.(x400)

Figure 16.1 Increased SP perivascular granularity in rodent parasagittal cortex 5hr following diffuse injury and also in pyramidal neurones. (P).(x400)

Figure 16.2 Rodent parasagittal cortical vessels 5hr following diffuse injury.(x400)

Figure 17.1 Increased SP immunoreactivity in rat caudal medulla 5hr following diffuse injury.Spinal trigeminal tract (T), nucleus (N).(x100)

Figure 17.2 Rat spinal trigeminal nucleus (N) and tract (T) 5hr following injury. Although background staining is greater,some increased neuronal staining in the nucleus is evident (←).(x200)
Figure 18.1 EM of rodent control parasagittal cortical vessel stained for SP.

Figure 18.2 EM of rodent cortical vessel stained for SP 30 minutes following diffuse impact injury. Notice the increased numbers of immunolabelled unmyelinated axons (→) compared to the control.

Figure 18.3 Cortical rodent vessel 30min following diffuse injury stained for SP. Stained axons are prominent. Intraluminal platelets(→) exhibit membrane staining for SP.

Figure 18.4. High magnification of endothelium of cortical rodent vessel stained for SP 30 minutes following diffuse injury. Small labelled axons are close to endothelium (→) but the endothelium itself is unstained.

Figure 19.1 and 19.2. Confocal images of control rodent cortical vessel double labelled for PGP 9.5 (green) and SP (red) showing SP containing perivascular axons (orange) (⇐)(x600)

Figure 20.1 and 20.2 Confocal images of rodent cortical vessel and neurone 5hr following diffuse injury double labelled for APP (green) and SP (red) showing injured perivascular axons and upregulation of cortical neuronal SP (yellow).(⇐)(x600)

Figure 21.1 Confocal image of rodent astrocytes in deep parasagittal cortex double labelled for GFAP(green) and SP (red) 5 hours following diffuse injury showing no colocalisation.

Figure 21.2 Confocal image of astrocytes in rodent corpus callosum 7days following diffuse injury double labelled for GFAP(green) and SP(red) and showing no colocalisation.

Figure 22.1. Human spinal trigeminal tract and nucleus stained for SP in caudal medulla (control).(x40)

Figure 22.2. SP containing nerve terminals (←) of human spinal trigeminal tract. The neurones (N) of the nucleus do not stain for SP.(x400).

Figure 23.1 Human frontal cortex stained for SP. The pyramidal neurones are prominent (←)(x40)

Figure 23.2. A human cortical venule surrounded by SP labelled nerve terminals (control) (←). Pyramidal neurones (P).(x400)

Figure 24.1 Prominent neuronal red cell change (←) in the frontal cortex of an 86 year old man with a compound depressed skull fracture with venous infarction who survived 4 days. Scattered haemorrhage is also present.(x400)

Figure 24.2 Microcytic oedema of the cortical neuropil (N) in the frontal cortex of a 13 year old girl with diffuse cerebral oedema who survived 2 days.Perineuronal fixation vacuolation (P) is also prominent.(x200)

Figure 25.1 APP positive axons (→) along a cortical venule in a 21 year old who died within hours with an acute subdural haematoma. Increased neuronal APP positivity is also evident in pyramidal neurones (P).(x400)

Figure 25.2 APP positive axons (A) with retraction balls (B) and perivascular axons (P) in an 18 year old patient with bilateral contusions who suvived 3days. (x400)

Figure 26.1 H&E section of frontal cortex underlying acute subdural haematoma (H) in a 41 year old man who survived 1day. Some pyramidal neurones (P) show red cell change.(x200).

Figure 26.2 Same cortex as per 26.1 stained for SP. Notice prominence of granularity in pyramidal neurones (P).(x200)

Figure 27.1 Increased perivascular SP granularity in the frontal cortex of a 36 year old with cerebral oedema who survived 1day.(x400)

Figure 27.2 SP perivascular profile in a 21 year old with an acute subdural haematoma who died within hours.(x400)

Figure 28.1 Prominent SP immunostaining in the cortex of a 23 year old with cerebral contusion who survived 6days.(x40)

Figure 28.2 At higher power the more distinct morphology of the astrocyte is apparent (←).(x400)

Figure 29.1 Confocal image of a cortical venule in 25 year old, double labelled for PGP 9.5 (green) and SP (red), showing intense perivascular colocalisation (yellow).(x600)

Figure 29.1 Confocal image of a cortical venule in 2 year old, double labelled for PGP 9.5 (green) and SP (red), showing perivascular colocalisation (yellow).(x600).

Figure 30.1. Confocal image of cortical vessel of 52 year old with acute subdural haematoma at 7 days, double labelled with PGP 9.5(green) and SP(red). The perivascular end nerve fibres and the SP granules in pyramidal neurones (P) colocalise (yellow).(x600).

Figure 30.2.Confocal image of cortical venule of a 13 year old with diffuse cerebral oedema at 2 days, double labelled with PGP 9.5 (green) and SP(red) showing intense perivascular colocalisation (yellow).(x600).

Figure 31.1. Confocal image of cortical arteriole in 41 year old with acute subdural haematoma at 1 day, double labelled with APP(green) and SP(red), showing APP positive perivascular nerve fibres (yellow).(x600)

Figure 31.2. Confocal image of cortical venule in 13 year old with difuse cerebral oedema acute at 2 days, double labelled with APP(green) and SP(red), showing APP positive perivascular nerve fibres (yellow).(x600)

Figure 32.1. Confocal image of cortical astrocyte double labelled with GFAP(green) and SP(red) in 48 year old man (control).(x600)

Figure 33.1.Confocal image of cortical astrocyte in 41 year old with acute subdural haematoma at 1day, double labelled for GFAP(green) and SP(red). Compared to figure the astrocyte soma (S) is enlarged and perivascular processes (P) prominent.(x600)

Figures 33.2 and 33.3. Confocal images of cortical astrocytes in 52 year old with acute subdural haematoma at 7 days, double labelled with GFAP(green) and SP(red). Some colocalisation is evident (orange) on the soma.(x600)

Figure 33.4. Confocal image of cortex of 23 year old with bilateral cerebral contusions at 6 days, double labelled with GFAP(green) and SP(red) showing intense astrocytosis.(x100)

Figure 33.5.Confocal image of above case showing SP(red) localised to the soma of astrocytes.(x600)

List of Tables **page**

List of appendices **page**

Abstract

The mechanisms of cerebral oedema in traumatic brain injury, the biggest killer of persons under 45 years, remain incompletely understood. Recent evidence from a rodent model suggests that neuropeptides released following traumatic brain injury may play a significant role in the aetiology of cerebral oedema. This study used 2 animal models of traumatic brain injury and post mortem human material, to characterise the distribution of SP in experimental and fatal human traumatic brain injury using immunohistochemistry.

Our results demonstrate increased SP immunoreactivity in perivascular cortical unmyelinated axons and pyramidal neurones early following injury in both rodents and human cases. Increased astrocyte binding of SP was observed in some human cases while increased immunoreactivity in the rodent trigeminal nucleus caudalis was also observed. Colocalisation studies suggest mechanical perivascular axonal injury could be an important mechanism of release of this neuropeptide, in both rodent models and human cases. The reduction in perivascular and neuronal SP immunoreactivity over time in hypoxic and oedematous human tissues suggests that hypoxic-ischaemia may also contribute to its release.

Our findings suggest an important role for substance P in perivascular axons, cortical neurones and astrocytes following injury, support earlier work implicating an important role for neurogenic inflammation in experimental TBI and suggest these processes could occur in human TBI.

CHAPTER 1. LITERATURE REVIEW.
TRAUMATIC BRAIN INJURY and SUBSTANCE P.

1.1 Overview of the Pathology of Traumatic brain injury

1.1.1 Introduction
1.1.2 Primary and secondary injury
1.1.3 Axonal injury
1.1.4 Hypoxic-ischaemic injury
1.1.5 Cerebral swelling and cerebral oedema
1.1.6 Traumatic vascular and microvascular injury
1.1.7 Metabolic and cellular sequelae
1.1.8 Neuroprotection

1.2 Tachykinins in primary afferent neurones

1.2.1 History and overview
1.2.2 Structure and receptors
1.2.3 Synthesis, axonal transport, release and plasticity of expression
1.2.4 Tachykinin distribution in the CNS
1.2.5 Biological actions– general
1.2.6 Biological actions - vascular
1.2.7 Inactivation of tachykinins

1.3 Neurogenic inflammation

1.3.1. Definition
1.3.2 Pathological models
1.3.3 Plasticity of neuronal expression during neurogenic inflammation

1.4. The Sensory Innervation of cerebral blood vessels.

1.4.1 Introduction
1.4.2 Neuroanatomy
1.4.3 Neurophysiology
1.4.4 Neuropathology and neurogenic inflammation

1.5 Tachykinins in CNS pathology

1.5.1 Cerebral inflammation, blood brain barrier and oedema
1.5.2 General and cerebral ischaemia
1.5.3 Non apoptotic cell death
1.5.4. Traumatic brain injury- animal models and human

1.6 Substance P antagonists in CNS disorders.

1.6.1. Introduction
1.6.2. Human CNS pathology

1.7 Conclusions.

1.8 Aims and Hypotheses

1.1.Traumatic Brain Injury.

1.1.1. Introduction

Traumatic brain injury is a significant public health issue with an estimated incidence of greater than 200 per 100 000 per year [1] and is the most common cause of death in patients younger than 44 [2]. Head injuries are also the most common cause of trauma death in those patients who survive to reach hospital [3]. Unfortunately in spite of improvements in mortality from motor vehicle accidents in the last 30 years [4, 5] approximately one third of patients with a severe head injury, defined as having a Glasgow Coma Score of ≤ 8, will die in spite of maximal treatment in neurosurgical centres [6]. For those who survive there are often long term neurological sequelae resulting in disability [7]. The cost to society for the treatment of these patients, which includes medical, rehabilitation and residential care, legal fees and loss of income, is enormous [8, 9].

1.1.2. Primary and secondary injury.

Traumatic brain injury has been previously divided into primary and secondary types. Primary injury is considered to occur as a result of mechanical forces occurring at the point of impact and includes laceration of vessels and axonal shear. Secondary injury occurs as a complication of

the primary insult and includes haematoma formation, hypoxic ischaemia and raised intracranial pressure. The distinction between the two has been appreciated for over a quarter of a century [10] and although somewhat artificial has persisted as this concept has obvious pathological, clinical and therapeutic utility.

An important assumption based on this is the idea that primary injury is largely irreversible and the possibility that secondary injury may be attenuated by appropriate and early therapeutic interventions including haemodynamic support, surgery and neuroprotective. For example the major improvements in mortality from traumatic brain injury to date have been as a result of preventive strategies e.g. seatbelts, reduction of speed and alcohol in reducing the primary injury burden and in the prevention and treatment of secondary injury, notably hypotension, hypoxia and raised intracranial pressure [11].

More recently however a greater understanding of the delayed biochemical and physiological processes following primary injury has lead to the development of pharmacological therapies to attempt to halt the evolution of these processes [12]. While current therapies continue to focus on the prevention of primary injury and treatment of secondary injury, it is likely that future therapeutic advances in the treatment of traumatic brain injury will arise mainly from a greater understanding of these factors involved in the evolution of primary injury [13].

1.1.3. Axonal injury and APP.

The term axonal injury refers to the impairment of axoplasmic transport secondary to a number of processes including trauma and hypoxia which result in a failure of interneuronal communication. Following traumatic brain injury the axonal responses to injury include axonal swelling and disconnection and these are sensitive microscopic pathological markers of injury.

The evolution of axonal injury has been characterised experimentally and Povlishock et al [14] have identified focal cytoskeletal misalignment and changes in axonlemmal permeability as intiating factors leading to the impairment of axoplasmic transport, cytoskeletal collapse and eventual axonal disconnection following injury. From these experimental observations the concept of primary axotomy with immediate axonal disconnection and secondary axotomy developing hours after injury has evolved [15]. Therapeutically this concept has obvious appeal, although to date no effective interventions have been developed for clinical use.

The contemporary microscopic diagnosis of axonal injury has relied on the identification of axonal swellings and retraction balls using immunocytochemistry. Although silver stains and neurofilament proteins have been used in the past, _ amyloid precursor protein (APP) has

become the method of choice and in human material can be identified as early as 1.75 hours following injury [16]. APP is a membrane spanning glycoprotein and plays an important role in neuronal growth, plasticity and also undergoes fast axonal transport [17]. Following trauma animal studies have demonstrated a marked neuronal upregulation of APP and APP mRNA in excess of that seen in axons, suggesting a possible protective role [18]. The macroscopic correlate of diffuse axonal injury in humans may include small haemorrhages most frequently located in the corpus callosum, internal capsule and superior cerebellar peduncle. Axonal injury may be focal or diffuse, the latter being defined as the presence of diffuse damage to axons in the cerebral hemispheres, corpus callosum, brain stem and cerebellum [19]. While initially the concept of diffuse axonal injury was descriptive of the most severe cases a spectrum of injury is now appreciated. This may range from brief unconsciousness with or without residual neurological impairment [20], to patients who survive for some time and show an admixture of normal and damaged axons in the same microscopic area [21] up to and including patients with persistent vegetative state [22]. Moreover, axonal injury may be associated with a wide range of injuries including motor vehicle accidents, in which it was originally described and in which deceleration forces are diffuse and longer [23], falls [24] and gunshot wounds [25]. In addition non traumatic axonal injury e.g. around infarcts has been long

described [26]. Finally axonal injury can be produced experimentally in animal models of traumatic brain injury [27, 28].

1.1.4. Hypoxic-ischaemic injury

The fundamental importance of hypoxic-ischaemia as a secondary injury factor following traumatic brain injury cannot be overstated. Ischaemic brain damage is found in up to 90% of patients who survive for several hours after traumatic brain injury [29] and may arise from either reduced oxygen content (hypoxia) or reduced global cerebral perfusion (hypotension, cardiac arrest) or reduced focal perfusion. Factors that may contribute to hypoxic-ischaemic injury following traumatic brain injury include loss of cerebral autoregulation, perilesional hypoperfusion, raised intracranial pressure and an increased susceptibility of the injured brain to hypoxia. Histologically ischaemic injury to neurones may manifest itself by loss of cytoplasmic basophilia, so called "red cell" change. Axons may develop APP positivity, reflecting impairment of axoplasmic flow. Hypoxic-ischaemic injury may result in infarction (pannecrosis of tissue) initiating a vicious cycle leading to break down of the blood brain barrier, brain swelling and, if uncontrolled, death from raised intracranial pressure [12].

1.1.5. Cerebral swelling and cerebral oedema.

Cerebral swelling following traumatic brain injury can be considered to be secondary to increased vascular engorgement, increased brain water

content (cerebral oedema) or both. Cerebral oedema may be further classified into vasogenic, cytotoxic, hydrostatic and osmotic [30]. Vasogenic oedema refers to the impairment of the blood brain barrier leading to accumulation of protein rich fluid in the extracellular space and is commonly seen in relation to contusions and intracerebral haematomas. Cytotoxic oedema refers to the accumulation of intracellular water following hypoxic ischaemic injury affecting cellular membranes and ionic haemostasis. In human traumatic brain injury both forms often coexist [31] and recent work now suggests that cytotoxic oedema is the predominant type. For example, using magnetic resonance techniques, focal human lesions have been shown to have a predominant cytotoxic component [32], a finding confirmed in a comparable rodent cortical impact model [33].

The underlying pathogenesis of cerebral oedema following trauma is poorly understood in humans but has been studied in detail in animal models. Diffusion weighted magnetic resonance imaging, a technique which is able to measure the distance excited water molecules can move over time has been useful in this regard [34].

The pathogenesis has been shown to be dependent on the nature of injury using animal models. For example using a rodent fluid percussion model, in which there is a prominent hypertensive surge, Hanstock et al [35] demonstrated that early water accumulation was largely vasogenic for at

least 4 hours, and Holmin et al [36] described a biphasic course of oedema in another rat cerebral contusion model with oedema maximal at 2 and 6 days, more comparable to human pathology. In contrast, using a diffuse impact injury model, Ito et al [37] showed that the addition of hypoxia or hypotension lead to predominantly cytotoxic oedema, findings similar to those in ischaemic stroke [38]. Further research using this model led Barzo et al [39, 40] to propose a biphasic pathophysiological response of vasogenic and subsequent cytotoxic oedema characterised by a modest increase in brain water within the first hour (due to vasogenic oedema) but a progressive increase up to 24hrs (due to cytotoxic oedema). In summary then evidence from experimental models suggests that vasogenic oedema occurs early following trauma but that cytotoxic oedema is predominant thereafter and is exacerbated by hypotension or hypoxia. Delayed oedema may occur around cerebral contusions with most recent evidence suggesting cytotoxic oedema is predominant. Changes in blood brain barrier (BBB) permeability, the precursor to vasogenic oedema, have also been characterised in these models and correlate well with these findings. In the rodent fluid percusssion model [41], characterised by early surges in blood pressure and variability of injury [42], the BBB remains open diffusely for 4 hours, can be further disturbed by hypoxia and hypotension but oedema typically occurs only on the injured side [43]. In the rodent impact acceleration model [27, 28],

which avoided these hypertensive surges, Barzo et al [44, 45] described a early transient opening of the blood brain barrier (resolving by 30min), which was prolonged by the addition of ischaemia and hypotension (resolving by 60min). Using different models of diffuse impact some investigators have shown maximal BBB permeability at 4-5 hours using Evan's blue [46], while others have shown no BBB opening [47]. In conclusion, blood brain barrier changes appear to occur early following experimental brain injury, are prolonged by hypertension, hypoxia and hypotension and appear to work permissively in the formation of vasogenic oedema.

1.1.6. Traumatic vascular and microvascular injury.

Traumatic brain injury may be associated with a wide spectrum of vascular injury that includes cerebral contusions, intracerebral haematomas and extraparenchymal haematomas including acute subdural and extradural haematomas.

Cerebral contusions result from localised mechanical forces which damage the small blood vesssels and neural parenchyma. Pathologically this damage may initiate a cascade of processes leading to secondary breakdown of the BBB, vasogenic oedema and infarction of adjacent tissue secondary to thrombosis of blood vessels and may evolve over days. Contusions most frequently involve the inferior frontal and inferolateral temporal lobes where the brain comes into contact with the

irregular bony surface of the anterior and middle cranial fossa and directly beneath skull fractures [12].

Intracerebral haematomas probably result from deformation and or rupture of intrinsic blood vessels, are often multiple, vary in their size and location and like contusions are often associated with marked perilesional hypoperfusion and hemispheric ischaemia [48]. They are also dynamic lesions and suggested mechanisms of evolution and expansion include coagulopathy secondary to intravascular coagulation, vessel wall weakening secondary to pH changes or cellular damage and changes in cerebral blood flow e.g. following decompressive surgery [49].

Acute traumatic subdural haematomas may be classified into 2 types. The first are related to or contiguous with contusions and lacerations, which occur in association with severe diffuse primary brain injury. The "burst lobe" refers to this extreme pattern of injury. The second type is caused by rupture of the superficial bridging veins or arteries with comparatively little primary brain injury. These lesions are associated with a high mortality rate (30-70%), which can be improved with earlier operative intervention [50]. One important factor related to this is the extent of ischaemic hypoxic injury observed in the underlying tissues [29], presumed to be as a result of raised intracranial pressure and reduced cerebral perfusion pressure, although other aetiologies e.g. microvascular occlusion and coexistence of diffuse axonal injury have also been

suggested from rat models [51, 52].Cerebral swelling due to reactive hyperaemia and cerebral oedema, both cytotoxic secondary to ischaemic membrane injury and release of excitatory neurotransmitters and vasogenic due to disruption of the blood brain barrier commonly accompany these processes.

Traumatic brain injury also causes significant derangements in the cerebral microvasculature, which have been characterised in animal models and observed in human tissue removed from head injured patients. These include perivascular astrocytic swelling [53], endothelial microvacuolation, diapedesis of red cells and increased leucocyte adherence [54-56], mobilisation of brain macrophages [57], neutrophils [58] and subsequent intracerebral inflammation in areas of contusion and perivascularly [55]. Increased permeability of the BBB has been associated with increased endothelial nitric oxide synthase release in rat models [59] and significant increases in von Willebrand factor and thrombomodulin have been measured in human patients, particularly in those who later went on to develop delayed intracerebral haematomas [60]. In addition a large number of inflammatory mediators including bradykinin, histamine and arachidonic acid, which may be released following TBI, have been shown to increase BBB permeability [61].

1.1.7. Metabolic and reactive cellular sequelae.

The metabolic sequelae of traumatic brain injury are a consequence of both the direct shearing effects on cellular membranes with release of neurotransmitters and the impairment of tissue perfusion, termed flow-metabolism uncoupling, resulting in anaerobic metabolism with the production of lactate. These processes have been studied by a variety of techniques including positron emission tomography (PET), direct tissue microdialysis [62], jugular bulb measurement and MR spectroscopy [63]. Furthermore, an initial reduction in cerebral blood flow following traumatic brain injury may compound these problems [64].

The metabolic sequelae that have been best characterised following traumatic brain injury include massive rises in intracellular calcium [65], intracellular lactate, extracellular glutamate from nerve terminals [63], free radicals following reperfusion [66], lipid degradation and pro inflammatory mediators [67] and most recently declines in intracellular free magnesium [68].

Cellular responses may be adaptive or maladaptive following injury. Reactive astrocytosis, characterised by increased staining of Glial Fibrillary Protein (GFAP) is commonly seen following injury and occurs in response to inflammation and regenerative processes [69]. In contrast, apoptosis or programmed cell death has also been recognised following neural trauma [70].

1.1.8. Neuroprotection

Neuroprotection involves the initiation of treatment given after the onset of trauma with the purpose of attenuating the intensity of the brain injury. Unfortunately, at the time of writing, in spite of numerous studies and initial success in animal models, no effective pharmacological therapies have been developed for clinical use to date. Important reasons cited for this may be the inherent heterogeneity and multifocal nature of traumatic brain injury, limitations of animal models and the inability of single therapeutic agents to target multiple pathological mechanism [71, 72]. Currently novel neuroprotectant drugs including magnesium, cyclosporine and neuropeptide antagonists as well as non pharmacologic therapies e.g. hypothermia and hypertonic saline are being investigated [73]. From past experience, it appears likely that future successful therapies or combination of therapies will have to target multiple pathological mechanisms involved in traumatic brain injury. Prerequisites for this will be the comprehensive characterisation of these mechanisms in animal models and corroboration with human studies.

1.2. Substance P.

1.2.1. History and overview.

In 1931, a 24 year old Swedish pharmacologist, Ulaf von Euler, was in the process of characterising the tissue distribution of the recently discovered neurotransmitter acetylcholine. Using tissue extracts, particularly from equine brain and intestine, he observed that muscle contraction following their application to rabbit jejunum could not be abolished by atropine, the antagonist to acetylcholine and proposed the effect must be due to another substance. This unidentified substance, which also lowered blood pressure upon intravenous injection, was designated P, for the powder made after it was evaporated [74]. In 1953 his student Pernow [75] and separately Lembeck (cited in [76]) identified high concentrations of the substance in the dorsal horns of the spinal cord and proposed a role as a neuronal sensory transmitter associated with pain transmission. The 12 amino acid sequence was finally identified by Chang et al in 1971 [77]. This step allowed the development of antibodies and radioimmunoassay techniques [78] and lead to the first descriptions of SP immunoreactivity in the central and peripheral nervous systems of the rat by Hokfelt [79] in 1975. This knowledge and the demonstration of

its release from primary afferent neurones in the spinal cord [80] led to extensive neuroanatomical and neurophysiological studies. The development of SP anatagonists in the 1980s then allowed pharmacological studies to broaden the understanding of the potential role of tachykinins in pathological processes [81]. As a direct consequence current research into this neuropeptide is being directed toward potential clinical roles in conditions like depression [82], emesis [83], nociception [84]and most recently neuroinflammatory pathologies including stroke [85] and traumatic brain injury [86].

1.2.2. Tachykinin structure and receptors.

Tachykinins are a family of peptides which are defined by a common C terminal amino acid sequence and along with substance P include neurokinin A and B [87, 88]. Substance P contains 12 amino acids, and derives from the preprotachykinin (PPT) gene, which codes for other tachykinins. These exert their actions via NK1,NK2 and NK3 receptors, with substance P having the highest affinity for NK1 [89]. These receptors are transmembranous and intracellularly coupled to G proteins [76]. While identification of these receptors and SP mRNA have utilised in situ hybridisation techniques in the past to gain functional information, identification of NK receptors in the human brain may be performed by functional PET studies in future clinical applications [90].

1.2.3. Synthesis, axonal transport, release and plasticity.

Following gene transcription, substance P is synthesised in the ribosomes of the neuronal perikaryon, packed into storage vesicles and transported along axons to both central and peripheral branches of primary sensory neurones for further enzymatic packaging and release [76, 91]. Hamar et al [92] estimated from an in vitro L5 dorsal root ganglion preparation that only 30% of the SP underwent rapid axonal transport and moved with a velocity of 4.9mm/hr, results similar to those calculated in sciatic and vagal nerves [93]. Furthermore he determined that 4 times as much SP was transported peripherally as was transported centrally and that the ganglion turnover time was 3.6 hr. Axonal transport may also be reduced by cooling or completely blocked by topical application of capsaicin [94]. Substance P and an increasing number of other colocalised neuropeptides e.g. CGRP are released from the end terminals of sensory nerves. Release may be stimulated by a number of noxious stimuli and compounds including heat, protons, bradykinin, tryptase, acetylcholine and capsaicin and also inhibited by opiates [95], galanin and neuropeptide Y [96]. Capsaicin, a ligand for the recently cloned vanilloid receptor and also the pungent agent in hot chilli peppers, is the best characterised of these. It has a highly selective effect of releasing substance P from unmyelinated C and poorly myelinated A mammalian primary sensory neurones by activating and sensitising them to further stimuli. Indeed this specificity of action has been used to experimentally define substance P containing

neurones as capsaicin-sensitive neurones. Repeated capsaicin administration can lead to depletion of SP as well as down regulation of genetic transcription of tachykinins [97]. This property has been exploited experimentally particularly in neonatal rats [98, 99].

Another important property of sensory neurones containing substance P is the capacity for plasticity of neuropeptide expression following axotomy [100, 101], partial ligation [102] or inflammation [103, 104], which results from induction of the neuropeptide gene [105] and NK1 receptor [106] expression as early as 3 hours following induction of experimental inflammation. This response is thought to be integral to nociception in the spinal cord and brain stem and will be covered later [107].

1.2.4 Distribution of tachykinins.

1.2.4.1. Introduction. Substance P has been demonstrated in many tissues including skin, adipose, cardiac, respiratory, gut and widely throughout the central and peripheral nervous system [89, 108]. Of particular importance to this review, SP containing nerves are found in perivascular plexuses around both arteries and veins in animals, particularly in adventitia and with greater density around arteries than veins [109]. Finally, although SP immunoreactivity has been demonstrated on a number of non neuronal cells e.g. vascular endothelium [110], monocytes and macrophages [111] there is now increasing evidence to suggest non neuronal elements including

macrophages [111] and brain endothelial cells [112] can synthesise and release SP in experimental conditions.

1.2.4.1. CNS distribution. The results of numerous and extremely comprehensive original and review papers in the literature demonstrate that substance P, substance P immunoreactive neurones and NK1 receptors are widespread throughout the CNS in both animals [88, 89, 113-117]and post mortem human tissue [118-123]. Although minor species differences exist common brain areas rich in substance P include the trigeminal ganglion, spinal trigeminal tract, periaqueductal gray, globus pallidus, striatum, interpeduncular nuclei, substantia nigra, medial amygdala and hypothalamus. For the purposes of this review however only the spinal cord, cerebral cortex, spinal trigeminal tract and cerebral arteries will be considered in detail.

1.2.4.3. Spinal Cord. In the spinal cord substance P has been identified in a small subset of Aδ and C fibres in the dorsal horn laminae (1-11 of Rexed), Lissaeur's fasiculus, and in smaller amounts around the central canal and ventral horn [34]. Of the neuronal cell bodies in the dorsal root ganglion between 15-20% may be positive for substance P [116]. Ultrastructural examination has revealed dense core vesicles at the nerve endings in the spinal substantia gelatinosa [124]. As already mentioned immunoreactivity for substance P has also been shown to be diminished in the substantia gelatinosa following dorsal root section [79], 7 days

following direct spinal cord trauma [125] and up regulated following inflammatory stimuli [126]. In addition the findings of accumulation of SP caudal to transected spinal cord in cats [127] and increased NK1 receptor immunoreactivity in adjacent interneurones following inflammatory stimuli [100, 128], gives further credence to a role for SP as a modulator of afferent neuronal activity and suggests its rostral and second order transport by axoplasmic flow.This role is further supported by evidence of the colocalisation of NK1 and receptors for glutamate, an excitatory neurotransmitter implicated in neuropathic pain [129].

1.2.4.4. Cerebral cortex. Substance P containing cell bodies and axons have been identified in the cerebral cortex of several mammalian species [89, 115, 117] and humans [118, 120, 121, 123, 130]. In the rat a bilaminar distribution of substance P positive neurones and fibres i.e. layers II and IV can be demonstrated in most cortical areas, but not in the molecular layer. This was in contrast to the monkey which showed immunoreactive fibres in all cortical layers [115]. In human post mortem tissue Mai et al [123] observed SP immunoreactivity in most cortical areas studied. Similar to the monkey a more diffuse cortical distribution of neurones and fibres has been noted in human cortical layers II,IV [121], varicosities in layer III [123] with NK1 receptor immunoreactivity most prominent in layers 1-III [118]. In the human hippocampal cortex a high density of SP positive neurones and fibres has been demonstrated

[120], suggesting an important role in memory. Taken together and accepting species and cortical area variability, these findings suggest the presence of an extensive cortical SP network, more prominent in humans. The origins, connections and functions of SP cortical neurones and fibres are incompletely understood but an intrinsic as well as an extrinsic circuit from the nucleus laterodorsalis tegmenti has been demonstrated in rodents [114, 121].

Further insights into a functional role for SP in the cortex can be gained from antagonist studies. For example SP injection induced status epilepticus in rats [131] and NK anatagonists have improved seizure control in kainic acid induced seizures in rat striatal and hippocampal neurones[132]. These findings suggest a role for substance P in modulating cortical neuronal excitability.

1.2.4.5. Spinal trigeminal tract and nucleus. Substance P localisation within tracts and nuclei in the brain stem has been extensively reported in mammals, particularly the rat [113, 114, 133] and humans [134-136].

In the rodent the trigeminal nucleus caudalis, best defined caudal to the closure of the fourth ventricle, shows dense SP immunoreactivity of primary afferent nerve terminals, particularly concentrated in laminae I and outer II (substantia gelatinosa), analgous to the spinal cord. These afferent fibres arise from the central branches in the trigeminal and sensory ganglia. Interestingly, although densely supplied with terminals

the substantia gelatinosa was devoid of SP receptor immunoreactive cells, which were confined largely to lamina I in a subset of cells (5%), but also lamina III [133]. Regional quantitation studies performed on rats post mortem using radioimmunoassay have confirmed very high levels of SP in the spinal nucleus, second only to the substantia nigra [137]. In addition the solitary tract nucleus, receiving afferent fibres from the ganglia of VII,IX,X, the rostral nucleus ambiguus, dorsal vagal nucleus and medullary raphe nucleus have been shown to exhibit variable amounts of immunopositivity [114, 117].

In the human, the above arrangements are largely comparable [134]. The spinal trigeminal tract, commencing from the trigeminal root in the pons, contains fibres from the ophthalmic (ventral), maxillary (intermediate) and mandibular (dorsal) divisions as well as sensory fibres from the facial, glossopharyngeal and vagus nerves, which occupy the dorsomedial regions as it traverses the brain stem until synapsing upon the medial nucleus. The sharp segregation of terminal fibres within the spinal nucleus means the nucleus caudalis, an oral prolongation of the apical grey substance of the dorsal horn of the spinal cord, receives input predominanly from receptive fields over the forehead, cheek and jaw, including dura and cerebral blood vessels [138, 139]. Within the nucleus caudalis, the subnucleus gelatinosus is the largest and forms a horseshoe area densely immunoreactive for SP at levels caudal to the obex. Above

the level of the obex, SP immunoreactivity appears in patchy bundles. Comparable to the rat the dorsal motor nucleus of vagus, nucleus of the tractus solitarius and medial medullary reticular formation showed modest positive staining.

Ontological differences in SP immunoreactivity, which are again comparable to the rodent have been studied in humans. Del Fiacco et al noted a more densely packed immunofluoresence in the spinal gelatinous subnucleus in human newborns compared to adults [135], while the opposite was true for the substantia nigra. Substance P cortical immunoreactivity has also been shown to be reduced in patients with Alzheimer's disease [140] and Parkinson's disease [141].

1.2.4.6. Cerebral blood vessels.

Nerve fibres containing substance P have been demonstrated in the walls of cerebral arteries in several mammalian species including rat, cat, guinea pig and man [142-149]. Most SP fibres have been found in the adventitia and adventitia-media border of arteries as small as pial vessels and in greater density on vessels of the rostral circle of Willis.

Convincing anatomical evidence from lesioning and retrograde injection experiments in animals suggests that these substance P fibres to the rostral circle of Willis cerebral vessels derive from the ophthalmic division of the trigeminal nerve [147, 148, 150], while those from the caudal basilar artery may arise from cervical dorsal root ganglia.

Humans studies on the innervation and possible function of neuropeptides in the cerebral vasculature have, however, been limited. Nevertheless SP containing nerves at the intima-media border of cerebral arteries have been demonstrated and quantitation of vessel SP performed, with a steady decline noted with age in humans [144]. In vitro administration of SP has resulted in endothelial dependent relaxation in cerebral arteries [151]. SP immunoreactivity in intimal cells has been demonstrated in post mortem human middle cerebral artery using electron microscopy [149].

1.2.4.7. Other non neuronal cell types.

A number of other cell types, resident in the CNS, have been shown to either express the tachykinin receptor, produce tachykinins or both. Astrocytes have been shown to possess NK1 receptors in culture in mice [152], following injury in rabbits [153] and gerbils [154], in the forebrain of normal human infants [155] and in post mortem tissue of patients with multiple sclerosis [156]. Cultures of rodent astrocytes exposed to SP produced IL1 via a calcium dependent mechanism [157], similar to IL2 production in macrophages stimulated by SP [158]. Astrocytes in vitro have the ability to synthesise SP [159]

Human microglia, the resident macrophages of the CNS, have also been identified as possessing NK1 receptors as well as the ability to produce tachykinins [160].One in vitro microglial culture study was unable to demonstrate activation of microglia with SP alone, unlike astrocytes,

although other cytokines were effective [161]. These findings may indicate an earlier activation of astrocytes with later involvement of microglia and also the potential capacity for autocrine signalling. Together with data from antagonist studies [162, 163], accumulating evidence suggests a potentially important role for SP in inflammatory cells of the CNS.

1.2.5 Biological actions of tachykinins – general.

The biological actions of tachykinins, mediated through their receptors, include neuronal stimulation, particularly in the CNS, endothelium dependent vasodilatation, smooth muscle contraction, plasma protein extravasation, mast cell degranulation, stimulation of inflammatory cells and stimulation of secretions [81]. The resultant effects produced by tachykinins released from sensory nerve endings including increased permeability of blood vessels, secondary production of inflammatory mediators, interaction with adhesion molecules, extracellular matrix and mediation of leucocyte migration by interaction with endothelial, mast and immune cells have been termed neurogenic inflammation [164].

1.2.6. Biological effects of tachykinins – vascular.

1.2.6.1. Blood flow. The mechanism of tachykinin vasodilatation via activation of endothelial NK1 receptors is known to be at least partially dependent on endothelial nitrous oxide (NO) generation [165]. Similar responses have been demonstrated in vivo in the cerebral circulation of

animals following administration of tachykinin agonists [166]. As a result a possible role for the regulation of cerebral blood flow by tachykinins has been proposed [167]. One important question raised, and still not answered from these studies is the precise mechanism by which endothelial tachykinin receptors are activated by tachykinins.

1.2.6.2. Plasma protein extravasation. The mechanism of plasma protein extravasation through post capillary venules is also incompletely understood. The simplest explanation is that tachykinins released from perivascular nerves act directly on venular endothelium. Alternatively, given the dense SP innervation of arterioles, authors have suggested that SP released at this level may reach venules through the blood stream. The thickness of arteriolar walls, which would increase the diffusion distance has been raised as a potential impediment to this. Alternatively, tachykinins may be derived from intravascular sources. A combination of mechanisms may occur [81].

Abundant evidence exists to suggest that SP causes plasma protein extravasation via its interaction with target cells that produce vasoactive mediators. SP activates neutrophils to aggregate, release enzymes [168] and bind to endothelium [169] and platelets are activated [170]. Polymorphonuclear leucocytes increase production of oxygen radicals when stimulated by SP [169] Macrophages and monocytes, cells important to inflammation, possess NK1 receptors [111] and produce

interleukins when exposed to substance P in vitro [158]. SP can induce degranulation of mast cells with release of histamine, a potent permeability factor [171] and antihistamines can reduce tachykinin induced oedema [172]. SP induces the expression of intercellular adhesion molecules on endothelial cells [173] by intracellular calcium mobilisation [174]. Capsaicin induced dermal oedema was not attenuated when an NK1 antagonist was coinjected, suggesting activation of other vasoactive mediators [175]. Finally SP can induce mobility of endothelial capillary cells [176].

1.2.6.3.Tachykinin induced changes in vascular ultrastructure.

A few studies have specifically examined substance P induced changes in vascular ultrastructure associated with oedema. Dimitriadou et al [177] reported changes in the microvessels of rat dura and tongue as a result of electrical stimulation of the trigeminal ganglion and noted an increase in luminal and abluminal pinocytic vesicles, occasional endothelial blebs and microvilli and intact tight junctions. These changes were confined largely to the post capillary venules in the dura but also included arterioles in the tongue. Opening of intercellular gap junctions was also noted in rat dural microvessels by Ghabriel et al [178] following intravenous injection of substance P. Interestingly, although Markowitz et al [179] were able to demonstrate neurogenically mediated edema in dural, eyelid and gingival tissues this could not be shown in brain, under

normal physiological conditions with an intact blood brain barrier. Whether tachykinins could have a role in cerebral oedema when the blood brain barrier may be open e.g. trauma or ischaemia is still unknown.

1.2.7. Tachykinin inactivation.

Excluding desensitisation, tissue diffusion and enzymatic degradation are the main mechanisms of tachykinin inactivation in peripheral tissues and the CNS. Of several enzymes discussed in the literature, endopeptidase 24.11 has been characterised as an important peptidase at the peripheral and central ends of capsaicin sensitive nerve endings in the CNS. For example in a rat model of neurogenic inflammation intravenously injected endopeptidase 24.11 attentuated plasma extravasation. Tachykinin inactivation also appears to occur rapidly once released from the protected environment of nerve terminals. Gazelius et al determined that following antidromic stimulation of dental pulp the SP level of stimulated whole pulp was 45% that of control after 30minutes incubation and significantly less if the pulp was homogenised [180].

1.3 Neurogenic inflammation and substance P.

1.3.1. Definition.

Although vasodilatation of lower limb vessels from stimulation of divided posterior lumbar roots was reported by Stricker in 1876 and from stimulation of sciatic nerve earlier by other investigators, it was Bayliss who comprehensively characterised the origin of these fibres as being primary afferent sensory neurones with their cells in the dorsal root ganglion and used the term "antidromic" to refer to the process by which conduction of nerve impulses in a direction contrary to usual produces effects in the organ of origin of these fibres [181]. Since this report, the concept of neurogenic inflammation has evolved to describe the processes of vasodilatation, plasma extravasation and neuronal hypersensitivity caused by the release of neuropeptides, including substance P and calcitonin gene related peptide (CGRP), from sensory neurons [182]. These biological effects of tachykinins, which have been detailed in the previous chapter, result from either the direct or indirect actions through the secondary production of inflammatory mediators. While our understanding of the principles of neurogenic inflammation through animal models, antagonist studies and other technological aids has come a long way, it is sobering to acknowledge that over one hundred

years later we are just starting to appreciate the role of neurogenic inflammation in human and CNS pathology in particular and the potential role of neurokinin anatogonists in treating them [164].

1.3.2. Pathological models.

Neurogenic inflammation has been induced in several experimental pathological models and this has been useful in studying the pathological effects of tachykinin release, sensory neurone plasticity and how these may potentially relate to human pathologies. Alves et al [183] demonstrated that neurokinin antagonists reduced plasma extravasation and oedema formation in a rat paw inflammation model. Substance P antagonists as well as somatostatin and morphine inhibited the vasodilatation and plasma extravasation induced by saphenous nerve stimulation in another rodent model [184]. In a mouse pleurisy model neurogenic inflammation characterised by a mononuclear cellular infiltrate was induced by substance P and was markedly reduced by pre-treatment with substance P antagonists [185]. In guinea pig airways neurogenic plasma exudation, induced by using vagal stimulation and potentiated by lipopolysaccharide, was significantly attenuated by NK1 antagonists [186]. Substance P antagonists have also attenuated neurogenic inflammation in a cyclophosphamide-induced model of bladder inflammation [81]. In animal models of arthritis substance P caused a significant increase in plasma extravasation and vasodilatation

when injected into the synovial cavity of the rat in addition to inducing mast cell degranulation [104].

1.3.3.Neuronal plasticity in neurogenic inflammation.

Tachykinin containing primary afferent neurones may undergo profound phenotypic changes in response to various subacute or chronic pathological stimuli, which may include nerve injury or inflammation. For example Ahmed et al demonstrated increased tissue levels of SP and CGRP in both the synovium and the dorsal root ganglia of rats in response to adjuvant arthritis induced by inoculation with mycobacteria. This corresponded to an increase in the number and intensity of SP immunoreactive dorsal root ganglia cells 11 days after innoculation and correlated with quantative radioimmunoassay. Further studies using this model have also demonstrated a reciprocal reduction in sympathetic responsiveness in these models, which may be of importance to impaired autoregulation in other pathologies. Similar observations of tachykinin release or genomic upregulation have been made in cat athritic models using antibody microprobes [187] and rodent hindpaw inflammation models using preprotachykinin mRNA in situ hybridisation [105] techniques.

Similar changes in the trigeminal ganglion, the functional homologue of the dorsal root ganglion, have also been demonstrated. Neubert et al [103] used in vivo microdialysis and radioimmunoassay techniques to

demonstrate intraganglionic release of SP in response to turpentine induced unilateral orofacial inflammation.

Finally significant morphological changes have been characterised in primary afferent neurones in response to noxious stimuli. Mantyh et al [188] elegantly described loss of cell surface NK1 receptor immunoreactivity in the soma and dendrites of spinal neurones in lamina 1 of rat spinal cord 5 minutes following intradermal injection of capsaicin and replacement by a coarse intracellular pattern characterised by swollen varicosities connected by thin fibres. These changes were interpreted as being secondary to the endocytosis of NK1 receptors in response to and in proportion to the amount of released SP and returned to normal within 1 hour following injection. Intrathecal injection of N-methyl-D-aspartate (NMDA) into rats has also produced similar morphological changes as a result of the presynaptic facilitation of SP release [189]. Internalisation of SP via NK1 receptors has also been observed following intravenous administration of SP within post capillary venules in rat trachea with the maximal number of immunoreactive endosomes at 3 minutes and gradual return to baseline by 120 minutes [190]. As indicated by the authors of this paper, NK1 receptor endocytosis is probably an important means of cellular desensitisation to tachykinins and of limiting excessive oedema.

Another method of characterising neurogenic inflammation in target tissues is quantitation of the depletion of tissue SP. Brodin et al [191]

showed that in dental pulp, a tissue richly innervated by trigeminal nerve endings, SP concentration, as determined by radioimmunoassay, was significantly depleted following brief antidromic stimulation of the inferior alveolar nerve compared to controls and that this was blocked by somatostatin P. Alternately tissues with chronic neurogenic inflammation may be charactersied by higher levels of neuropeptides as demonstrated recently by Awawdeh et al [192]. Non radioactive assays are at present commercially available to perform quantitation.

1.4 The Sensory innervation of cerebral blood vessels - the trigeminovascular system.

1.4.1. Introduction.

Cerebral blood vessels are innervated by a combination of sympathetic, parasympathetic and trigeminal sensory nerve fibres all of which are believed to be important in cerebrovascular regulation [151]. It is well established that the sympathetic nerves arise from the superior cervical ganglion [150, 193], the parasympathetic nerves arise chiefly from the sphenopalatine ganglion via from the greater petrosal nerve of the facial nerve, with lesser contributions from the otic and carotid ganglia [193-195] and sensory nerves arise from the trigeminal ganglion [150, 196, 197]. In addition the physiological responses following stimulation of these nerves under normal experimental conditions have been characterised in animals i.e. vasodilatation with trigeminal stimulation [198, 199], vasodilatation with sphenopalatine stimulation[193, 194] and vasoconstriction with sympathetic stimulation [193]. Finally it has been demonstrated that these perivascular nerves contain numerous peptides including noradrenaline and neuropeptide Y in sympathetic fibres, acetylcholine, vasoactive intestinal peptide (VIP) and nitrous oxide (NO) in parasympathetic nerves and tachykinins and CGRP in sensory nerves. Consequently a role for these neuropeptides in cerebrovascular regulation in health and disease has been hypothesised [167]. Evidence to support

this hypothesis has come from in vitro studies using human pial, middle and anterior cerebral arteries which have demonstrated relaxation of precontracted arteries with substance P which was endothelium dependent [200] and studies showing a reduction of CGRP in human [201]and animal cerebral vessels [202, 203] following subarachnoid haemorrhage.

Although cerebral dysautoregulation underpins a number of important human pathologies including traumatic brain injury, ischaemic stroke and migraine, caution is required in extrapolating this and other data to be discussed to human pathology and the relevance of findings from animal or human studies which examined large calibre vessels is uncertain.

1.4.2. Neuroanatomy of substance P containing perivascular neurones.

Numerous studies have described the occurrence and rich distribution of substance P and other vasoactive peptides in sensory nerve fibres surrounding the adventitia of mammalian blood vessels and of the cerebral blood vessels in particular [143, 145, 148, 167].

The origin and central connections of substance P and CGRP containing perivascular fibres, the so called "trigeminovascular system", have been studied extensively in animals using a variety of techniques including nerve and ganglion section with immunohistochemistry and retrograde transport [150, 196, 197, 204-207]. These have shown the origin of these

fibres are in neurones in the trigeminal ganglion with peripheral branches running mainly via the ophthalmic (nasociliary branch) and maxillary divisions of the trigeminal nerve to supply the proximal circle of Willis vessels including the proximal basilar artery. The distal basilar and vertebral arteries have been shown to be supplied from fibres with cell bodies localised to the 1st and 2nd cervical spinal ganglia. Importantly the smallest vessels thus studied were pial arteries. Also identified in these studies was a significant convergence of afferents from vascular and non vascular structures e.g. skin in the ganglion, explaining referred pain. The central endings of trigeminal neurones then synapse in the brain stem trigeminal nuclear complex depending on their function. Second order neurones concerned with cortical craniovascular nociception, located primarily in the principal sensory and rostral spinal trigeminal nucleus, project via the thalamus to the primary somatosensory cortex[139, 194]. Second order neurones mediating trigeminovascular and nociceptive reflexes are located predominately in the spinal trigeminal nucleus pars caudalis [208-210]. As well as predominant ipsilateral connections some crossover has been demonstrated [211] These may project to the parabrachial nucleus or rostral ventral lateral medulla, the blockade of the latter, which projects to spinal sympathetic preganglionic neurones, completely abolishing reflexive pressor responses. Other second order neurones have been described which project to the facial nucleus and then

to post ganglionic para sympathetic fibres and have been implicated in a trigemino-parasympathetic reflex to increase cerebral blood flow. Although a recent study has convincingly demonstrated SP and CGRP immunoreactive fibres in the human trigeminal nucleus caudalis extending to C2 [212], only limited confirmatory human evidence exists [213] for the anatomy of these central connections and as such extrapolation from animal data must be considered inferential.

1.4.3. Neurophysiology of substance P containing perivascular neurones.

The above neuroanatomical pathways suggest a potentially important functional role of the trigeminovascular system in vascular nociception and cerebral blood flow and physiological studies in animals and humans have given further support to this. Most studies, but not all [193], have demonstrated an increase in cerebral blood flow in cortical vessels following direct ganglionic stimulation [199] and indirectly via stimulation of the superior sagittal sinus in cats [198]. This has also been elegantly and reversibly demonstrated in a rodent model of cerebral vasospasm, in which, following corneal stimulation, there was an increase in cerebral blood flow associated with an increase in systemic arterial blood pressure and decrease in cerebrovascular resistance, indicating activation of the trigeminovascular system. In addition, destruction of the sphenopalatine ganglion abolished the cerebral blood flow response,

further supporting evidence of a trigeminoparasympathetic reflex [214]. Further support for independence of this reflex comes from studies involving sympathectomy in which increased cerebral blood flow can be demonstrated following application of a noxious skin stimulus [215]. In summary these studies suggest cooperation between the trigeminal system and the parasympathetic system as regulators of cerebral blood flow in experimental models. It is unclear whether such reflexive responses are important in pathological situations, although this possibility has been suggested by one author [194].

As would be expected from the studies above, trigeminal ganglionectomy has been shown to markedly attenuate post occlusive cortical hyperaemia in 2 studies in cats [216, 217] and modify pial arteriolar responses (by allowing unchecked sympathetic drive) [218]. SP antagonists have also been shown to attenuate similar cerebrovascular responses [219]. Although there are no comparable human physiological studies, profound vasopressor responses (hypertension with reflex bradycardia) are well known during neurosurgical operations particularly with manipulation of the trigeminal ganglion.

A role for the trigeminovascular system in nociception has also been well demonstrated in physiological models and human patients. The subnucleus caudalis of the spinal trigeminal tract has been confirmed as being important following stimulation of dural arteries [220], trigeminal

ganglion [211] and dura [221-224] using microelectrodes and antibody probe techniques. Further evidence of this comes from pharmacological studies using NK1 antagonists [225, 226] and serotonin agonists [222], which as well as blocking neurogenic dural inflammation reduced the release of substance P and c-fos mRNA in the nucleus caudalis.

1.4.4 Pathology involving the trigeminovascular system.

The extensive evidence of a role of the trigeminovascular system in cerebral blood flow and vascular nociception has led to hypotheses of an important role in a number of human pathologies including subarachnoid haemorrhage[201-203, 227], trigeminal neuralgia [228] and vascular head pain including migraine headache [229-231]. Indeed, in experimental models of these pathologies, either activation of afferent nerve endings and /or their second order neurones centrally in the nucleus caudalis, can be demonstrated.

The best characterised of these experimentally is migraine headache. Recent work has shown colocalisation of serotonin receptors with SP in the human spinal trigeminal tract, a significant finding in light of the observation that antimigraine drugs, including serotonin agonists, modulate neuronal firing in subcaudalis and C2 dorsal horn neurones following neurogenic inflammation or administration of nitrates intravenously [232, 233]. Furthermore neuropeptide levels have been measured during migraine attacks in humans and elevated blood levels of

CGRP but not SP have been identified [231], a finding consistent with the current view that CGRP, a more potent vasodilator and more abundant than SP, is more likely to be important in vascular pathologies involving vasodilatation [234] However, the most recent human randomised placebo controlled trial involving an inhibitor of neurogenic inflammation administered intravenously however showed no difference to placebo [235].

1.5. Tachykinins in CNS pathology.

1.5.1. Post traumatic inflammation

Cerebral inflammation is a well characterised and important secondary injury process involved in a number of CNS pathologies including ischaemia, trauma, autoimmune diseases and infection. Given the extensive evidence of the effects of tachykinins on inflammatory and immune cells (reviewed in [236]), an important role for tachykinins in inflammatory processes of the CNS has recently been considered. Cerebral inflammation has been well characterised following traumatic brain injury and animal contusional models have shown accumulation of polymorphonuclear leucocytes [54] similar to findings in human contusions in which massive increases in macrophages, reactive microglia, polymorphs and T lymphocytes have been shown [237]. As important as inflammatory cells are cytokines, including interleukins and tissue necrosis factor (TNF), which are released from injured vascular cells. Release of these cytokines has been shown in vivo in rats to contribute to intracerebral inflammation, cell death by apoptosis and increased blood brain barrier permeability leading to oedema [238], while cytokine antagonists have been shown to significantly reduce neuronal damage in rats following traumatic brain injury [239, 240]. Recently SP has been shown to be released from cytokine activated brain endothelium [112].

As mentioned previously SP has been shown to modulate the permeability of dural vessels resulting in oedema and significant morphological changes following both intravenous injection of SP and antidromic stimulation [177, 178]. Catalan et al [241] postulated that SP may have a role at the BBB by noting that SP caused a shift of protein kinase activity from the cytosolic to membrane fraction in bovine microvessels. Most recently it has been shown that in a rat brain endothelium model, SP antagonists attenuated the increased blood brain permeability, upregulation of MHC class 2 molecules, increased expression of ICAM-1 and associated cell morphological changes induced by cytokines. These preliminary findings in animal models suggest a potentially important and as yet unrecognised pathogenic role for substance P in cytokine induced damage of the BBB.

Beyond its neuroimmunomodulatory function at the blood brain barrier, SP has also been implicated in inflammatory processes within the neuropil, including astrocytic and microglial activation. Mantyh et al [153] identified a high density of substance P binding on rabbit transected optic nerves, similar to the findings of Lin et al, who reported reactive astrocytes with SP immunoreactivity in gerbils following traumatic brain injury [154]. SP immunoreactive astrocytes have also been noted around multiple sclerosis plaques in humans, and in astrocytosis induced in rodent models of African trypanosiasis [162, 163]

1.5.2. Post ischaemic release of tachykinins.

1.5.2.1. General.

Previous studies demonstrate at least 2 potential sources of SP release following ischaemia. A number of studies have demonstrated that sensory nerves release SP following ischaemia, low pH or following the release of bradykinins, prostaglandins and leukotrienes [81, 242]. SP has also been demonstrated to be directly released from the endothelium of coronary vessels during hypoxia [243] and from the endothelium of peripheral vasculature during increased flow [244]. In addition, as described before, SP can be directly released from inflammatory cells.

Recently Souza et al [242] described a marked suppression of plasma extravasation, neutrophil activation and haemorrhage following mild ischaemia and reperfusion injury in rats treated with capsaicin neonatally and therefore inferred that sensory nerves were the main source of neuropeptides released following ischaemia/reperfusion injury. In addition SP antagonists, given after ischaemia but before reperfusion, reduced elevations in TNF(tissue necrosis factor) and IL10 in gut and lung. Although it was not possible from this study to infer whether neuropeptide release preceded or followed the release of other proinflammatory mediators, Cao et al [245] showed NK1 antagonists played a pivotal role in inflamed microvasculature, suggesting that

neuropeptides may have an important role in amplifying inflammatory responses.

Substance P release has also been demonstrated following ischaemic myocardial injury as evidenced by a reduction in myocardial injury following administration of SP antagonists in rats with dietary Mg deficiency, but not normal rats [246]. Indeed prior to ischaemic injury and during dietary magnesium deficiency the serum SP level was abnormally elevated and preceded the elevation of other inflammatory mediators, suggesting SP mediated and then potentiated the tissue inflammatory response. This finding suggests that neuropeptides may potentiate rather than initiate inflammatory responses following ischaemia. This finding is also of particular relevance to traumatic brain injury as traumatic brain injury produces sustained declines in intracellular magnesium [68]. Given the early success of magnesium therapy as a neuroprotectant in traumatic brain injury substance P antagonists might hold similar promise.

1.5.2.2. Cerebral ischaemia.

Similar findings have been made in studies examining cerebral ischaemia. Using a rodent focal cerebral ischaemic rat stroke model, Yu et al [85], demonstrated that intracerebroventricular administration of a neurokinin receptor antagonist reduced infarct volume 24hrs following focal ischaemia. Using a similar model a detailed study by Stumm et al [247]

characterised SP and tachykinin receptor expression following cerebral ischaemia. They observed an ischaemia induced increase in SP and NK1 cortical neuronal expression, maximal at 2 days post infarct with strong SP and NK1 perikaryonal staining in lamina V cortical pyramidal and nonpyramidal neurones. In addition a transient induction of NK1 was observed in venular endothelium, perivascular granularity was increased but this was absent from activated astroglia or microglia. These observations further suggest a possible role for tachykinins in blood brain barrier permeability and cortical neuronal activation in ischaemia.

The evidence of a role for tachykinins in human cerebral ischaemia is limited at this time and direct clinical evidence is limited only to observations of increased serum SP taken from patients with symptoms of transient ischaemic attack or completed stroke [248]. However, SP immunoreactivity was reported by Yamanouchi et al [249] to be increased in trigeminal tract fibres in the brainstem of victims of sudden infant death syndrome which correlated with the degree of astrogliosis and was postulated to be related to ischaemia.

1.5.2.3. Programmed cell death.

Delayed cell death, as distinct from necrosis, has been increasingly recognised as an important secondary mechanism following neuronal injury. While apoptosis is the best characterised of these with a distinctive morphology and specific immunohistochemical profile, it has been

increasingly recognised that alternative patterns exist. A SP-NK1 mediated non apoptotic form of programmed cell death derived from cultures of hippocampal, striatal and cortical rodent neurones has recently been described [250]. The significance of this finding is yet to be determined in animal or human studies.

1.5.2.4. Traumatic brain injury.

Inspite of the extensive characterisation of multiple secondary injury processes following traumatic brain injury in animal models, neurogenic inflammation has not, until recently been described. Vink et al [86] recently described improved motor and cognitive outcome and reduced blood brain barrier permeability following traumatic brain injury, using an impact acceleration model, after administration of a NK1 antagonist 30 minutes following injury. These results were not significantly different to those obtained when capsaicin pretreated rodents were used. These results were consistent with release of SP from perivascular fibres post injury and suggested a potentially important role for neurogenic inflammation in cerebral oedema.

The only human study to attempt to address the question of release of neuropeptides following traumatic brain injury examined levels of CSF SP within 24 hours of injury and found them to significantly lower than those of controls, in contrast to all other measured proinflammatory markers [251].

1.6. Substance P Antagonists in Human CNS disorders.

1.6.1. Introduction.

One of the most promising aspects of neuropeptide research has been the development of tachykinin antagonists that have evolved at the time of writing to treat a number of human central nervous system pathologies including pain, emesis, depression and schizophrenia. In addition to the work from animal antagonist studies, particularly with respect to ischaemia, trauma and apoptosis, the functional insights and therapeutic applications derived from this work may yield important secondary benefits for patients with traumatic brain injury in the future.

1.6.2. Tachykinin antagonists and human CNS disorders.

Despite a well characterised role in nociception, substance P antagonists have been shown to reduce acute post operative pain compared to placebo in only one study [84] with the remainder showing no difference [252]. There may be more promise in the treatment of chronic deafferentation pain syndromes if encouraging animal studies suggesting a benefit [253, 254] can be replicated in humans. Indeed human studies have characterised depletion of substance P containing axons in the medulla and spinal substantia gelatinosa of patients with hereditary diminished pain sensitivity [255].

Cisplatin induced emesis was reduced by a selective neurokinin receptor antagonist in a clinical human placebo controlled trial [83] and in animals

[256]. The presumed loci of pharmacological blockade were the nuclei of the tractus solitarius and area postrema.

NK antagonists have also proven clinical antidepressant and anxiolytic activity in humans [82, 257], following similar observations in animals. Putative sites of action include projections from the amygdala to the hypothalamus and periaqueductal grey, which show dense immunoreactivity for substance P [258] and the prefrontal regions[259]. Other investigators have noted increased firing of dorsal raphe nucleus neurones following administration of substance P antagonists via a habenulo-raphe projection [260, 261].

Finally NK antagonists have been trialled in schizophrenic patients without clinical efficacy despite initial observations that antipsychotic drugs reduced SP content and synthesis in mesolimbic-dopaminergic regions [262].

1.7 Conclusions.

Substance P is a neuropeptide which is released from capsaicin sensitive sensory nerves and has an important and long established role in nociception and neuroinflammation in the peripheral and central nervous system, where it is widely distributed. Further important roles for this neuropeptide have emerged recently in the pathology of conditions such as depression, epilepsy, schizophrenia, emesis and learning. However, its potentially most important role may yet be characterised as an important secondary injury factor in ischaemic stroke and traumatic brain injury with a role in cerebrovascular regulation, oedema formation, axonal transport, apoptosis and the glial response to injury. With its dense localisation in sensory nerve fibres around cerebral blood vessels, its involvement in neurogenic inflammation and the encouraging early results of antagonist drugs in animal models of traumatic brain injury, the role of substance P is worthy of further investigation in these disorders.

1.8 Hypotheses

1. SP is anatomically localised in the peripheral and central ends of the trigeminovascular system in rodents and humans. These will correspond to nerve fibres around cerebral vessels of the microvasculature of the cerebral cortex and white matter and the spinal trigeminal tract of the medulla respectively.

2. SP is released from its peripheral and central ends following traumatic brain injury in rodent models and humans.

3. SP contributes to cerebral oedema following traumatic brain injury in experimental and human TBI.

4. SP is involved in the glial response to TBI in animal models and humans.

Aims

1. To characterise the SP immunoreactivity of the central and peripheral ends of the trigeminovascular system as defined above in the normal rodent and human.

2. To characterise the changes in SP immunoreactivity at these sites following experimental TBI in 2 rodent models and human TBI over time, with respect to perivascular nerve fibres, neurones and astrocytes.

3. To correlate any SP immunoreactive changes with neuropathological features of experimental and human TBI including axonal injury, ischaemic-hypoxia and cerebral oedema.

CHAPTER 2. METHODOLOGY

2.1 RODENT TRAUMATIC BRAIN INJURY

2.1.1 Animals used and ethics approval.

2.1.2 Impact acceleration brain injury model

2.1.3 Fluid percussion brain injury model

2.1.4 Brain perfusion fixation

2.1.5 Tissue processing

2.1.6 HE and immunohistochemistry.

2.1.7 Tissue selection and analysis.

2.1.8 Transmission Electron Microscopy

2.1.9 Laser scanning Confocal microscopy

2.2 HUMAN TRAUMATIC BRAIN INJURY

2.2.1 Case selection and exclusions.

2.2.2 HE and immunohistochemistry

2.2.3 Tissue analysis

2.2.4 Laser scanning confocal microscopy.

2.1. RODENT TRAUMATIC BRAIN INURY

2.1.1. Animals used and ethics approval.

All animal experiments were performed within the guidelines of the National Health and Research Council and were approved by the animal ethics committees of the Institute of Medical and Veterinary Science (diffuse injury 19/03, fluid percussion injury 39/03) and the University of Adelaide (diffuse injury M-40-2003, fluid percussion M-38-2003). Adult male Sprague-Dawley rats 350 – 450gm were used. Animals were housed in a conventional rodent room on a 12hr day-night cycle and were provided with a standard diet of rodent pellets and water ad libitum. A summary of the experimental animals used is provided in table 1.

2.1.2. Impact acceleration brain injury model

Animals were placed in an induction box and anaesthetised using halothane 3% with oxygen 1.5l/min. Once anaesthetised and there was no response to painful stimuli (paw) the animal was removed from the box, placed prone and halothane 2%/oxygen 1.5l/min anaesthesia maintained via a nose cone. The scalp was widely shaved and 0.5ml plain lignocaine 1% was infiltrated into the scalp for local anaesthesia. A midline incision was made and scalp edges were retracted to identify the bregma and lambdoid sutures. A stainless steel protective disc was secured between these with polyacrylamide adhesive and allowed to dry for 5 minutes.

Once dry the anaesthetised animals were placed prone on a 10cm foam cushion, with the head centred underneath the injury device and secured with tape (figure 1.1). Using the injury method described by Marmarou et al [27, 28], a 450 gm brass weight was dropped from a height of 2m through a cylindrical tube to impact upon the stainless steel disc (figure 1.2). Following injury the animal was quickly removed to avoid rebound impact.

Following impact the animals were moved to a 37°C heating pad to assess heart rate and ventilation. As apnoea was observed in all injured animals, animals were manually ventilated using an oral airway until spontaneous ventilation resumed or the animal died. The protective disc was then removed and the scalp closed with either suture or surgical clips (9mm Autoclip wound clips, Becton Dickinson). Animals were then observed for recovery of consciousness, heart rate and respirations until stable. All animals also received a 5ml injection of saline subcutaneously following trauma to prevent dehydration. All sham injured animals had only the scalp incision and wound closure, prior to sacrifice.

2.1.3. Fluid percussion brain injury model

The fluid percussion injury device (Biomedical Engineering, University of Virginia, USA), which consisted of a pendulum, a horizontal fluid column and distal outflow nozzle was assembled, filled with normal saline to exclude air bubbles and tested manually prior to animal usage

(figure 2.1). Animals were placed in an induction box and anaesthetised using 3% halothane and 1.5l/min 02. An endotracheal tube (Norton, USA) was then placed using direct laryngoscopy and correct i.e. endotracheal position was confirmed by equal bilateral chest expansion. The tube was secured by suturing it to the cheek to prevent dislodgement during surgery or injury, to ensure airway patency and facilitate ventilation as required. Under general anaesthesia (2% halothane, 1.5L/min02), endotracheal intubation and mechanical ventilation (70 breaths per minute, tidal volume 2.5ml, Harvard ventilator), the animals were placed prone on a 37°C heat pad and the head immobilised by a rigid fixation device inserted firmly into the ears. The scalp was shaved and 0.5ml of lignocaine infiltrated. A midline incision was made and the scalp edges retracted to expose the sagittal, coronal and lambdoid sutures. Next a left 5mm parasagittal burrhole was fashioned using a hand held trephine and the dura exposed, but not breached. A plastic Leur-loc connector with external diameter enough to fit snugly into the burrhole was then secured using quick drying dental cement and then filled with normal saline in order to ensure no air once connected to the injury device (figure 2.2). The animal was then temporarily disconnected from the ventilator and connected to the fluid percussion device.

Injury was performed according to the method described by McIntosh et al [41] by means of releasing the pendulum, which had been preset to

deliver an impact force of approximately 2 atmospheres. The impact could be manually felt by the assistant holding the animal as a pressure pulse and it could also be confirmed that there was a good seal around the connector by absence of fluid breaching the connector seal. One animal received 1 hit and the second 2 hits.

Following impact the plastic connector device was removed from the burrhole and dura inspected. Underlying haemorrhage could be observed and also cortical swelling in one animal. The scalp wounds were closed with surgical clips. The animals were returned to the heating pad and assessed for spontaneous ventilatory effort and heart rate. Both animals required mechanical ventilation until adequate spontaneous ventilation and gag reflexes permitted safe removal of the endotracheal tube.

1 sham animal was also anaesthetised and underwent craniotomy prior to sacrifice at 5 hours.

2.1.4. Brain perfusion fixation.

Animals were sacrificed at a variety of time points to allow comparative tissue analysis and these included 30minutes, 5 hr, 1 day, 2 day, 3 day and 7 day following injury. Animals were group housed in a conventional rodent room on a 12 hour day-night cycle and provided with rodent pellets and water ad libitum.

At the time of sacrifice animals were anaesthetised with 3% Halothane and 1.5L/02 and maintained at 2% halothane and 1.5L/02 .The animals

were placed supine and the abdominal and chest cavities were opened to expose the heart. A blunt 19gauge needle was inserted via the left ventricle into the ascending aorta and 5000U of Heparin was administered as an anticoagulant for all cases except those used exclusively for examination by electron microscopy to minimise any possible blood brain barrier disturbance due to the negative charge of heparin. Following perfusion with by 4% paraformaldehyde (ph 7.4), the fixed intact brain was removed and stored in 4% paraformaldehyde solution.

2.1.5. Tissue processing

The whole fixed rat brain was placed hemisphere down into the concavity of a rat brain blocker (PA 001 450-600gm type, David Kopf Instruments, Tujunga, CA) with the depression between cortex and cerebellum fitting the corresponding ridge of the blocker (figure 3.1,3.2). Rostral to caudal coronal sections at 2mm intervals were then taken with a razor blade and these were separately impregnated with wax overnight for cutting the following morning. This procedure allowed a means of standardisation of the rodent brain for comparison in tissue analysis which could be correlated with a standard stereotactic rat brain atlas [263]. It also allowed creation of a rat "brain bank", from which comparable sections could be taken in future projects.

2.1.6. HE staining and immunohistochemistry.

5_m paraffin sections were cut from all blocks by tissue microtome for analysis. Tissue sections were routinely stained for haematoxylin and eosin (HE), Weil's haematoxylin for myelin and immunolabelling with amyloid precursor protein (APP) and substance P antibodies. For double immunolabelling sections were also stained for Glial Fibrillary Acidic protein (GFAP), a well established marker for astrocytes [264], PGP (protein gene product) 9.5 primarily as a marker for nerve fibres and APP as a marker for injured axons. The details of routine HE, Weil and immunostaining, their primary antibodies, their dilutions and retrieval solutions are summarised in appendices 1, 2 and 3.

For all immunolabelling of material for light microscopy the protocol in appendix 3 was followed with the following exceptions. Sections with prominent formalin pigment staining were treated with picric acid solution for 15 minutes, prior to immersion in methanol. For confocal microscopy 10_m thick sections were used. Following addition of the primary antibody and washing in PBS (Phosphate Buffer Solution), fluorescent staining was performed using fluoroscein isothiocyanate (FITC) and Alexa 546 to conjugate with their respective antibodies. After washing in PBS, slides were mounted using an aqueous mounting solution with antifade and sealed using varnish. Positive and negative controls were routinely used for all antibodies.

2.1.7. Tissue selection and analysis.

We used a combination of standard histological, immunohistochemical, double immunolabelling and electron microscopic techniques to characterise the pattern of traumatic brain injury (primary and secondary features) and distribution of substance P in this study. Regions of particular interest in the rodent models were the central (spinal trigeminal tract in the medulla) and peripheral (perivascular) ends of the trigeminovascular system and the region of parasagittal cortex underneath the impact site that has been shown in other studies using MR imaging (MRI) to develop vasogenic oedema maximally at approximately 5hr following injury [39]. Specifically HE staining allowed the identification of haemorrhage, oedema, necrosis and neuronal dark cell change and neuronal red cell change. APP immunostaining was used to characterise axonal injury by the presence of axonal swellings and retraction balls, particularly in perivascular nerve terminals. Neuronal APP staining was also as a marker of APP upregulation. Weil staining was used to identify loss of myelin staining and myeloarchitectonic microanatomy.

SP immunostaining was used to observe patterns of SP staining and also in conjuction with double immunolabelling for PGP 9.5, APP and GFAP to positively identify the anatomical distribution of SP in perivascular nerve terminals, injured axons and astrocytes in normal and post traumatic tissue.

Electron microscopy was used to assess SP immunopositive structures, particularly those not clearly seen at LM level including the endothelium and small perivascular nerve fibres at different time points. 30 minutes was selected as this corresponds to the time that substance P antagonists are administered in rodent outcome studies. 5hr was selected as this represents a time of maximal post traumatic vasogenic oedema (viewed by MRI) and 7 days was selected to examine for reactive astrocytosis. To control for variability of immunostaining including background staining which might confound comparative tissue analysis, immunostaining of all final data sets was performed in one sitting. Positive and negative immunostaining controls were used.

2.1.8. Transmission Electron Microscopy

Parasagittal 60_m sections of paraformaldehyde fixed rat brain including cortex, cerebellum and brain stem were cut using a Lancer series 100 Vibratome. Immunolabelling for SP with negative controls was performed using the modified protocol described in appendix 3. Antigen retrieval was not used in these specimens as an initial experiment showed no microscopic difference between sections with and without antigen retrieval. 1x1mm cubes of parasagittal frontoparietal cortex from under the impact site were removed for processing for transmission electron microscopy (appendix 4).Tissue was not counterstained to allow better visualisation of SP. Ultrathin (70-80nm) sections were cut on a Reichert-

Yung microtome and placed on copper grids. Sections were analysed using a PhilipsCM100 transmission electron microscope with SIS Megaview II CCD camera and AnalySIS software (Munster Germany) at Adelaide Microscopy, University of Adelaide.

Cortical blood vessels were identified by their characteristic morphology at low power and the perivascular SP labelled profiles were examined and identified by their morphology at higher power. Perivascular nerves (myelinated and unmyelinated), neurones, astrocytes, pericytes, endothelial cells and intravascular cells could be thus identified and their immunopositivity or negativity recorded. This procedure was performed for all time points studied with their respective negative controls and a range of vessels 4 – 10 sampled in each specimen. This data was stored electronically and CD copies made for further analysis.

2.1.9. Laser scanning confocal microscopy.

Confocal images were produced using the BioRad Radiance 2100 confocal microscope (BioRadMicroscience Ltd, UK) equipped with three lasers, Argon ion 488nm (14mw); Green HeNe 543nm (1.5mw); Red Diode 637nm (5mw) outputs and Olympus IX70 inverted microscope. The objective used was a 10- 60x UPLAPO with NA=1.4 oil. The dual labelled cells were imaged with two separate channels in a sequential setting. The FITC was excited with an Ar 488nm laser and the emission was viewed through a HQ515/30nm narrow band filter in PMT1. The

Cy5 was excited with Green HeNe 543nm laser light and the emission was viewed through a long pass barrier (E600LP) to allow only far red light wavelengths longer than 600nm to pass through PMT2. Automatically all signals from PMTs 1 and 2 were merged. The imaged data were stored on a CD for further analysis using a confocal assistant software program for Microsoft Windows (Todd Clark Brelje, USA).

2.2 HUMAN TRAUMATIC BRAIN INJURY.

2.2.1 Case selection and exclusions.

Archival post mortem material was selected from patients who had sustained head injuries, had died and had undergone post mortem and detailed neuropathological examination (n=29) and also a second cohort of patients with a known past medical history of epilepsy who had died but subsequent post mortem and neuropathological examination had found no abnormality (n=10). This second group served as case controls for the purpose of this study.

The head injury group were further subdivided into patients with mild head injury (GCS 14-15), who had sustained a concussion but died from an unrelated injury or cause, severe head injury (GCS <8) or paediatric (age < 14) groups. These subdivisions were formed to allow examination of the potential role of injury severity and age on neuropeptide release following traumatic brain injury.

In addition the head injury group was divided into 2 groups to allow specific comparison of the medullary spinal trigeminal tract and cerebral cortex with age matched controls. This would permit us to characterise neuropeptide release from the central and peripheral ends respectively of the trigeminovascular system following TBI in humans.

Criteria for selection for each of the patient cohorts were as follows. To permit direct anatomical comparison of the trigeminal spinal tract only those cases with sections available between the inferior olivary nucleus and commencement of the cervical cord were included. Cases were age

matched but could not be sex matched always. Two control cases, one with Down's syndrome and one with cerebellar atrophy were also excluded as there appeared significant depletion of SP in the spinal tracts in numerous sections. The cases used for cortical examination were selected on the basis of having common post traumatic neuropathological lesions including cerebral contusions, subdural haematoma and oedema. All cases, exhibited at least one microscopic feature of secondary injury including oedema, red cell neuronal change and inflammatory infiltrates. A wide distribution of survival times (from instant death to 1 week survival) was selected. These time points were specifically selected to allow observation and inference about patterns of neuropeptide expression immediately following trauma and in response to secondary injury particularly hypoxic ischaemia and oedema. To permit direct anatomical comparison with the control material only comparable cortical regions were examined for each case e.g. frontal with frontal cortex. The neuropathological features of the individual cases are summarised in appendix.

2.2.2 HE staining and immunohistochemistry.

Wax blocks containing sections of medulla and cortex were selected from the human archival material as above for sectioning. Transverse sections of 5_m thickness were then cut by tissue microtome for analysis. Tissue

sections were routinely stained for haematoxylin and eosin (HE), Weil, amyloid precursor protein (APP) and substance P. Double immunolabelling was performed using combinations of APP, GFAP and PGP 9.5. Routine HE and immunohistochemistry for light and confocal microscpy was performed as previously described for in the rat.

2.2.3. Tissue analysis

Tissue analysis was essentially similar to the rat with the following important differences. HE analysis was important in characterising secondary injury specifically allowing the identification of haemorrhage, oedema, necrosis and ischaemic hypoxic injury. To control for variability of immunostaining including background staining which might confound comparative tissue analysis, immunostaining of all final data sets was performed in one sitting.

2.2.4. Laser scanning confocal microscopy.

The preparation of tissue for confocal examination was identical to the rodent.

CHAPTER 3 – RESULTS.

3.1 RODENT TRAUMATIC BRAIN INJURY

3.1.1 Model mortality and morbidity

3.1.2 SP distribution in normal control rodent

3.1.3 Neuropathology of impact acceleration model – HE and APP profile

3.1.4 Neuropathology of fluid percussion model – HE and APP profile

3.1.5 Changes in SP distribution following traumatic brain injury – LM

3.1.6 Changes in SP distribution following traumatic brain injury – EM

3.1.7 Confocal microscopy – SP and PGP 9.5

3.1.8 Confocal microscopy – SP and APP

3.1.9 Confocal microscopy - SP and GFAP

3.2 HUMAN TRAUMATIC BRAIN INJURY

3.2.1 SP perivascular profiles in normal control cerebral cortex and subcortical white matter

3.2.2 SP distribution in normal control cerebral cortex and subcortical white matter

3.2.3 SP distribution in normal control spinal trigeminal tract (STT) by level

3.2.4 Neuropathology of trauma cases

3.2.5 Changes in SP distribution in cerebral cortex, subcortical white matter and perivascular profiles following traumatic brain injury

3.2.6 Changes in SP distribution in STT following traumatic brain injury

3.2.7 Confocal microscopy – SP and PGP 9.5

3.2.8 Confocal microscopy – SP and APP

3.2.9 Confocal microscopy – SP and GFAP

3.3 SUMMARY OF RESULTS.

3.1 RODENT TRAUMATIC BRAIN INJURY

3.1.1 Model mortality and morbidity.

The overall mortality for the diffuse impact acceleration model was approximately 15% with apnoea, usually lasting a few minutes, being the major morbidity and also cause of death. Animals were excluded from analysis if skull fracture was evident. With the fluid percussion model there was no mortality observed, however the 2 animals used were both intubated prior to injury and mechanically ventilated. Apnoea following injury usually lasted a minimum of 20 minutes before the animal could be safely weaned from the ventilator. Additionally both animals injured using the fluid precussion model had a right hemiparesis following recovery of consciousness, although this was transient in one animal.

3.1.2 SP distribution in normal control rodent

Three key regions of interest were examined in the normal rodent to characterise the distribution and morphology of SP immunoreactivity. These were the parasagittal cortex and corpus callosum under the site of impact and the spinal trigeminal tract and nucleus caudalis in the medulla below the closure of the fourth ventricle.

The cortical perivascular spaces were a region of prominent SP immunoreactivity and a number of characteristic morphologies could be distinguished and later confirmed by confocal analysis. Astrocytes, characterised by round to oval nuclei, pale chromatin and prominent

enveloping perivascular processes exhibited a linear to finely granular immunoreactive pattern (figure 4.2). In contrast larger, discrete granules, some in groups of 3-6 and resembling an "endplate" morphology and others in linear paravascular formation could be identified at high power (x400) on the majority of cortical vessels. There was a greater preponderance of these on venules compared to arterioles and they were difficult to identify on capillaries (figure 4.1). These profiles were interpreted and later confirmed by confocal examination to be nerve terminals. In addition larger discrete granules were observed around vessels in the corpus callosum but appeared fewer and there was scant astrocyte immunopositivity, largely confined to the soma (figure 5).

The parasagittal frontal cortex with corpus callosum sectioned coronally between +1 0 and –0.4mm from the bregma was chosen for reference comparison [263]. Within the cortex a weak staining of cortical neurones, particularly non pyramidal was present. The molecular layer of the cortex was generally void of immunolabelling. Ependymal cells of the lateral ventricles, and particularly choroid plexus, if present, were immunopositive. A prominent fibrillar and granular SP immunoreactivity, morphologically similar to nerve end terminals seen in the medulla, was observed in the overlying dura which continued interhemispherically and

involved the walls of the dural veins and arteries and their cortical penetrating branches.

The caudal or closed medulla was a useful reference landmark to observe the central endings of the trigeminal neurones and the spinal trigeminal tract could be reliably defined immunohistochemically starting from the closure of the fourth ventricle to the commencement of the cervical cord (a distance 13.68 – 14.6mm caudal from the bregma [263]). Intense varicose SP staining could be identified in the crescent shaped tracts without adjacent staining of the STN in the normal control animal (figure 6.1,6.2). The constancy of the anatomical features of this region made it a useful positive and negative immunolabelling control.

3.1.3 Neuropathology of the impact acceleration model.

A number of distinct neuropathological changes could be observed following the impact acceleration model of injury, although variability was also prominent in the cases reviewed. These are summarised in table 2.

On gross examination, frank basal subarachnoid haemorrhage was evident in one of the 5hr survivors but not over the cerebral hemispheres (figure 7.2). On light microscopy variable amounts of subarachnoid haemorrhage was seen circumferentially around the entire cortex and also variable amounts of intraventricular haemorrahge (figures 8.1,8.2). Variable neuronal dark cell change, particularly in the parasagittal

cortical neurones was apparent, compared to the control animals in all cases (figure 8.1). No microscopic evidence of oedema nor ischaemic-hypoxia (neuronal red cell change) was evident in the trauma cases at any time point.

Neuronal somatic APP expression was markedly upregulated in all neurones following injury in all cases compared to normal and sham controls (table 3, figure 9.2). APP positive axons could also be variably demonstrated in the corpus callosum, rarely in the cerebral hemisphere just superior to the corpus callosum, and most reliably, when present in the specimen in the pyramidal decussation of the medulla (figure 9.1, 9.3). Perivascular APP positive granules could also be consistently demonstrated in a large (40%) number of vessels particularly in the cortex under the impact, with lesser amounts around vessels in the white matter tracts, deep nuclei and least in the brain stem (figure 9.4).

3.1.4 Neuropathology of fluid percussion model – HE and APP profile

On gross examination prominent left parietal subarachnoid haemorrhage was evident in both specimens. In one of the cases a left parietal cortical haematoma was also present and extended over 8mm (rostral to caudal) (figures 10.1,10.2).

The neuropathological findings of the cases injued by the fluid percussion model are summarised in table 2, 3 and figures 11.1,11.2,12.1, 12.2. Both

cases demonstrated haemorrhagic contusion of the left parietal lobe extending from the cortex to and involving dissection of the ipsilateral corpus callosum, similar to a burst lobe. This was associated with some focal parasagittal oedema and variable neuronal red cell change. One case also had an overlying acute sudural haematoma. The right hemispheres was relatively normal. APP positive axons could be identified mainly in the left corpus callosum, adjacent to the contusion and a few scattered throughout the left basal ganglia and brain stem (figure 13.1). There were widespread perivascular APP positive granules, but these were no more numerous adjacent to lesions (figure 13.2). One case had 2 discrete areas of cortical infarction.

3.1.5 Changes in SP distribution following traumatic brain injury – LM

Traumatic brain injury induced in both rodent injury models was associated with a marked increase in SP immunoreactivity, compared to the normal and sham controls, which was maximal at 5hrs (table 3). In the cortical sections a return to normal baseline staining in some sections could be appreciated as early as 1 day, whereas in the medulla increased staining was apparent as late as 7 days (impact acceleration).

The most significant changes occurred perivascularly in the cortex. In all 5hr cases, there was a marked increase in the cortical perivascular granularity, which was a combination of increased coarse discrete

granules (nerve fibre) and fine granularity of astrocytic processes (figure 16.1, 16.2). Although variability in immunolabelling intensity existed it was apparent that the animal with the most HE microscopic evidence of trauma i.e.ventricular and subarachnoid haemorrhage exhibited the greatest increase in perivascular granularity. The immunostaining of processes and perikarya of cortical pyramidal neurones was also subtley increased at 5 hours compared to control animals (figure 16.1).

Within the medulla there was a marked increase in background staining following trauma, with some increased staining of neurones of the STN and a less pronounced increase in perivascular staining (figure 17.1,17.2).

In the 2 fluid percussion injured animals the same changes were evident however the increase in neuronal SP granularity was more pronounced, particularly in pyramidal neurones. This was most exaggerated in neurones with red cell change adjacent to the cortical contusion in case 1 and lesser so underneath intact cortex with overlying subarachnoid haemorrhage (figure 14.1). In one of the fluid percussion case neurones in the ischaemic core exhibited increased SP immunoreactivity (figure 14.2). Perivascular granularity seemed greater than controls (figure 15.1,15.2).

3.1.6 Changes in SP distribution following traumatic brain injury – EM

Significant changes in SP immunolabelling around cortical vessels in injured rodent cortex were noted following trauma at the ultrastructural level with electron microscopy. Compared to normal and sham controls the number of perivascular SP immunolabelled profiles (which could be verified by comparison to a time matched negative control), was clearly most numerous at 30 minutes following trauma (figures 18.1,18.2). These immunopositive profiles were largely unmyelinated axons. There was no discernable difference in the immunolabelling profile of intravascular cells, interpreted as being platelets or endothelial cells between trauma, sham or normal controls. It is of particular note that most immunolabelled axons were located within 1-5_m of a vessel and that at higher magnification small immunolabelled axons could be identified within 1 _m of the endothelium (figure 18.3,18.4).

3.1.7 Confocal microscopy – SP and PGP 9.5

Colocalisation of SP and PGP 9.5 was clearly shown around rodent cortical vessels and proved that the source of the discrete perivascular deposits of SP were within nerve terminals. These were best demonstrated in thin walled venules (figures 19.1,19.2).

3.1.8 Confocal microscopy – SP and APP

Colocalisation of SP and APP was also clearly shown around rodent cortical vessels (figure 20.1) and demonstrated that perivascular nerve terminals containing SP were injured following impact acceleration trauma. Again these were best demonstrated in thin walled venules. In addition colocalisation of SP and APP was seen in some neurones (figure 20.2).

3.1.9 Confocal microscopy - SP and GFAP

There was no colocalisation between SP and GFAP around rodent cortical blood vessels in control and traumatised animals, proving that SP is not contained within astrocytes (figure 21.1,21.2) and that this is not changed following trauma.

3.2 HUMAN TRAUMATIC BRAIN INJURY

3.2.1 SP perivascular profiles in normal control cerebral cortex and subcortical white matter .

Considerable variation in perivascular SP morphology could be observed within the control population. The cortical vessels had greater amounts of perivascular SP than those of the white matter. Profiles observed included focal collections of granules unrelated to cells, focal or linear granules associated with glial cells, diffuse spread or combinations of the above (figure 23.2). Rarely vessels were bare. Arterioles and venules were both invested with profiles and characteristically granules were located outside the muscularis layer in arterioles and closer to the endothelium of the thinner walled venules. Capillaries were difficult to conclusively identify but some could be shown to have SP positive perivascular profiles. No endothelial binding of SP could be seen in controls, but occasional immunopositive mononuclear cells could be identified in the vascular lumina.

3.2.2 SP distribution in normal control cerebral cortex and subcortical white matter

In contrast to the caudal medulla SP immunoreactivity was ubiquitous in the human cortex and to a lesser extent the subcortical white matter of the cases chosen as controls. Only the frontal and parietal cerebral cortices were examined in this study. The most striking feature in the cortex was

the coarse perikaryonal granular staining of the pyramidal neurones prominent in all but the molecular layer such that gray and white matter may be distinguished on gross inspection (figure 23.1). A finely granular SP immunoreactivity was seen within the dura. Although these morphologies were constant, variability in the intensity of immunolabelling was a feature within the control population

3.2.3. SP immunostaining of the normal spinal trigeminal tract

The archived nature of the post mortem human material available for this study did not allow as strict a degree of standardisation of sectioned material, particularly in the brainstem compared to the rodent. Accordingly case selection was based on the availability of sections of the lower medulla, a region of the spinal trigeminal tract that was easily recognisable. The morphological variability of the human STT is summarised in appendix 5 and the level chosen for comparison between cases is shown in figure 22.1. Anatomically this corresponded to a region of the medulla caudal to the closure of the fourth ventricle and cephalad from the commencement of the dorsal horns of the cervical spinal cord. At this level the tract assumes a characteristic bilateral crescent morphology as the intensely immunostained end fibres with varicose terminals encircle the spinal nucleus. Neurones of the spinal nucleus caudalis were unstained as were surrounding blood vessels (figure 22.2).

3.2.4 Neuropathology of selected trauma cases

Samples of cerebral cortex and subcortical white matter were selected from 7 cases of acute subdural haematomas, 2 cases of cerebral oedema, 1 contusion, 1 post traumatic cortical venous thrombosis and one case of post traumatic middle cerebral artery (MCA). The summary of their neuropathological autopsy findings and the neuropathological profiles of the cortical samples are summarised in appendix 6 and table 6.

All cases but 2, who died instantly, had unequivocal neuronal red cell change as a marker of ischaemic hypoxic injury (figure 24.1). Prominent oedema was noted in 9 cases and intraparenchymal haemorrhage was also noted in nine (figure 24.2). Of the thirteen cases eleven had evidence of axonal injury in the cortical section examined (figure 25.1,25.2) and eleven had APP positive perivascular profiles. Three of these were selected for later confocal examination and each of these had proven injured perivascular SP containing nerve fibres. Mononuclear inflammatory cells were the most common intravascular and intraparenchymal inflammatory cells seen.

3.2.5 Changes in SP distribution in cerebral cortex, subcortical white matter following traumatic brain injury.

Although heterogeneity was common throughout the pathological material examined, three chief criteria were selected that showed the greatest morphological difference between the control and the trauma

cases. These were an increase in the perivascular SP immunolabelling profiles, pyramidal neuronal granularity, and an increase in glial SP immunolabelling in cortex and white matter following traumatic brain injury. The neuropathological features and SP profile of all the cerebral cortical cases are summarised in table 8.

The most obvious difference evident in perivascular staining was the early increase in the number of SP immunoreactive non cellular perivascular profiles, resembling end terminals, following trauma (figure 27.1,27.2). The perivascular pattern became more heterogeneous with time and secondary injury factors with the inclusion of increasing numbers of inflammatory cells (both intraluminal and perivascular), glial staining and the absence of discrete end plate morphologies. Within areas of microcystic oedema and neuronal red cell change heterogeneity was present but with a tendency toward a reduction of profiles and discrete end terminal changes were rarely seen.

An increase in the SP granular immunolabelling of pyramidal neurones was the most obvious difference observed in the trauma cases (figures 26.1,26.2). This was slightly increased in those cases with the shortest survival and most apparent with increasing survival times. Importantly however, the presence of marked neuronal red cell change and/or microcystic oedema, usually evident after 1-2 days, was associated with a significant reduction of granularity. Indeed this heterogeneity of

granularity could be regularly observed in the most injured tissues (reduced) and their more normal adjacent areas (increased). This contrasted to the normal controls in which a blander homogeneity was the rule (figure 23.1).

An increase in glial SP immunolabelling was a prominent feature of some trauma patients and was most evident several days following injury as a more prominent somal labelling and more distinctive labelling of the processes. This contrasted to the control patients who had a weak homogeneous glial immunolabelling pattern (figure 28.1,28.2).

3.2.6 Changes in SP morphology in STT following traumatic brain injury.

Using the criteria of anatomical comparison discussed above, no significant differences in the morphology of SP immunostaining of the STT or STN were noted between aged matched controls and trauma cases of varying survival and pathology. These cases are summarised in table 5. Morphological changes that were specifically looked for in the nucleus included neuronal perikaryonal and process immunostaining. Variablity in tract morphology and the degree of background staining secondary to artefact were common.

3.2.6 Confocal microscopy – SP and PGP 9.5

Colocalisation of SP and PGP 9.5 could be demonstrated in all cases examined. These included normal controls aged 2, 25, 48, 82 years and trauma patients of varying ages and pathologies (13 year old girl with diffuse oedema, 52 year old man with acute subdural haematoma and an 82 year old man with a post traumatic venous thrmbosis) (figures 29.1,29.2,30.1,30.2). Interestingly colocalisation occurred only at the ends of perivascular nerves and not along their course, in veins more commonly than arteries and much closer to the endothelium in veins than arterioles, where cololocalisation typically occurred outside the muscularis layer. Within the small population sampled above the number of colocalised perivascular profiles appeared greater in the post traumatic group. These findings prove the existence of SP containing perivascular nerve terminals in controls and head injured patients with a variety of ages and pathologies.

3.2.7 Confocal microscopy – SP and APP

Colocalisation of SP and APP could be demonstrated in all 3 cases selected for confocal examination. They included an 82 year old man with a post traumatic cortical venous thrombosis, a 41 year old man with acute subdural haematoma and a 13 year old girl with diffuse oedema. Each case was initially selected on the basis of having APP positive axonal injury on light microscopy in the selected cortical sections.

Interestingly, in all samples the degree of colocalisation was variable so that not all SP containing nerves were injured and similarly not all injured perivascular nerves contained SP. Colocalisation in nerve terminals could be seen in arterioles as well as venules. Some neuronal colocalisation also occurred, but the morphology of this was usually quite distinct from perivascular nerves. These findings prove that SP containing perivascular nerves are injured in the microvasculature of human head injured patients (figures 31.1,31.2).

3.2.8 Confocal microscopy – SP and GFAP

There was evidence of weak colocalisation of SP and GFAP in normal and post traumatic human cases. The trauma cases included a 41 year old man with an acute subdural who had survived 1 day, a 23 year old woman with bifrontal contusions who survived 6 days and a 52 year old man with an acute subdural haematoma who had survived 7 days. The morphology of the astrocytes was significantly different between the control and the trauma cases. The control case exhibited astrocytes with a compact soma and short perivascular processes, whereas the post traumatic cases had enlarged somata and elongated processes (figures 32.1,33.1). Although there was evidence of colocalisation in one case (figure 33.2,33.3) i.e. a colour change was evident (orange), SP generally stained separately from GFAP on the soma of astrocytes. This pattern of

staining was suggested that SP might be bound to the astrocyte menmbrane on a receptor.

3.3 SUMMARY OF RESULTS

Substance P is widely distributed throughout the central nervous system of the human and rat including nerve terminals that invest arterioles and venules of the cerebral cortex and subcortical white matter microvasculature and those that terminate in the spinal trigeminal tract of the medulla. Cortical pyramidal neurones contain substance P in both species, while astrocytes of the cortex and subcortical white matter exhibit weak substance P immunoreactivity. Neurones of the spinal trigeminal nucleus and endothelial cells of rodent brain microvasculature are immunonegative.

Following traumatic brain injury changes in SP immunoreactivity occur predominantly at and around the peripheral endings of SP containing perivascular neurones in the rodent and human CNS.

In two experimental models of TBI there is an early increase in perivascular substance P immunolabelled profiles, which can be observed at early as 30 minutes in a rodent diffuse injury model. The morphology of these profiles at light microscopy, supported by confocal analysis and study of their ultrastructure using electron microscopy show them to be predominantly SP containing unmyelinated axons. Cortical pyramidal neurones demonstrate increased SP granularity in an experimental rodent

cortical contusion model characterised by oedema and neuronal red cell change. Colocalisation of SP and APP in perivascular nerve fibres and some cortical neurones demonstrates that these changes occur in injured nerve fibres and neurones.

The time course and the association of these changes with secondary injury parallel observations made from selected human head injury cases, however heterogeneity is common and prominent SP immunolabelling of astrocytes can be demonstrated in some human cases. In humans confocal examination suggests that SP may be bound to a membrane receptor.

Figure 1.1 An anaesthetised rat with protective skull disc is positioned under brass weight prior to diffuse impact injury.

Figure 1.2 A 450gm brass weight is hoisted 2m up a hollow PVC tube prior to impact

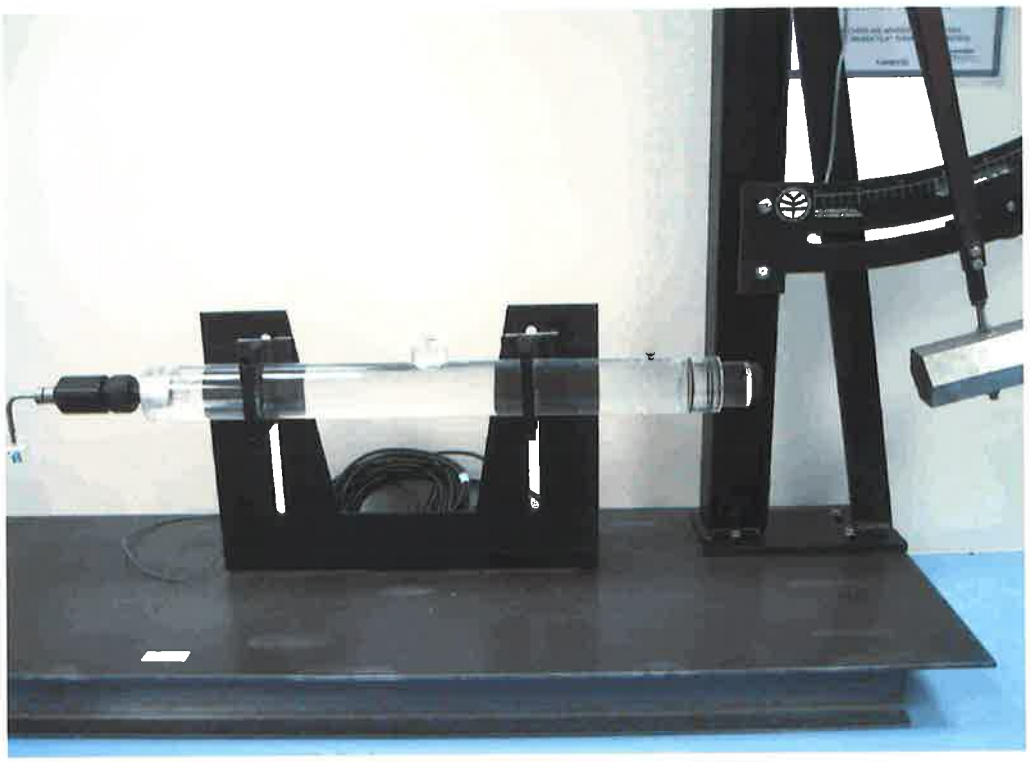


Fig 2.1 The fluid percussion injury device.

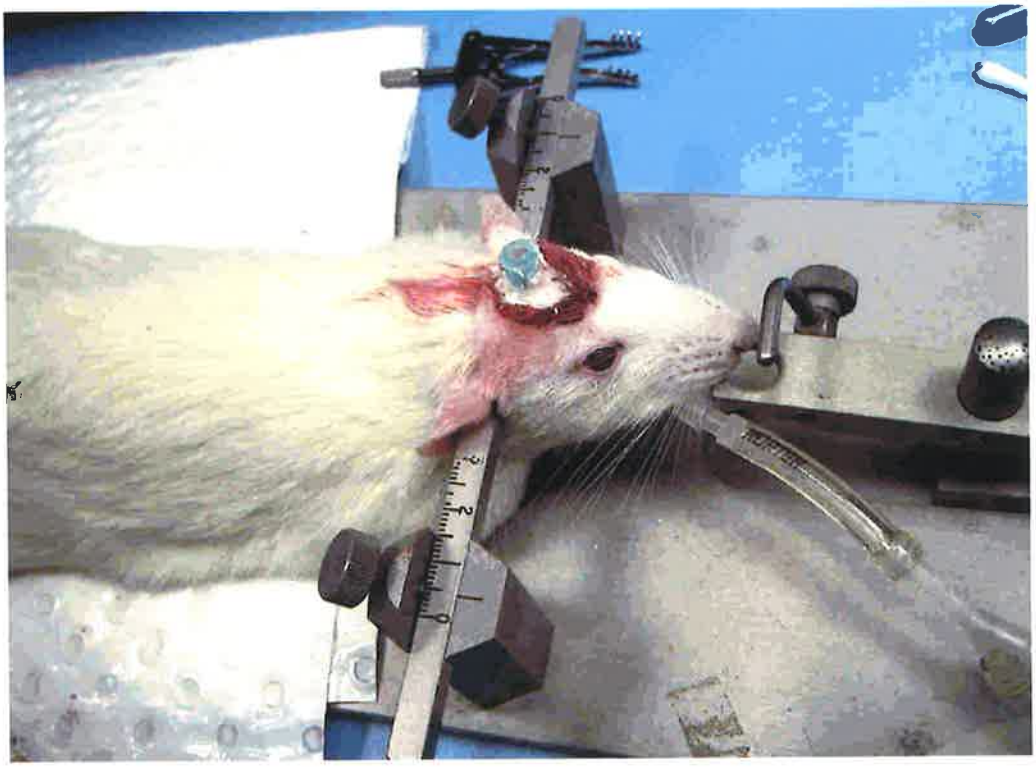


Fig 2.2 The anaesthetised, intubated and ventilated rat has had a left parasagittal burrhole with cemented plastic connector inserted prior to fluid percussion injury.

Figure 3.1The Kopf (450-600gm) rat brain blocker

Figure 3.2 A rat brain is positioned in the blocker and 2mm axial sections are cut with a razor blade.

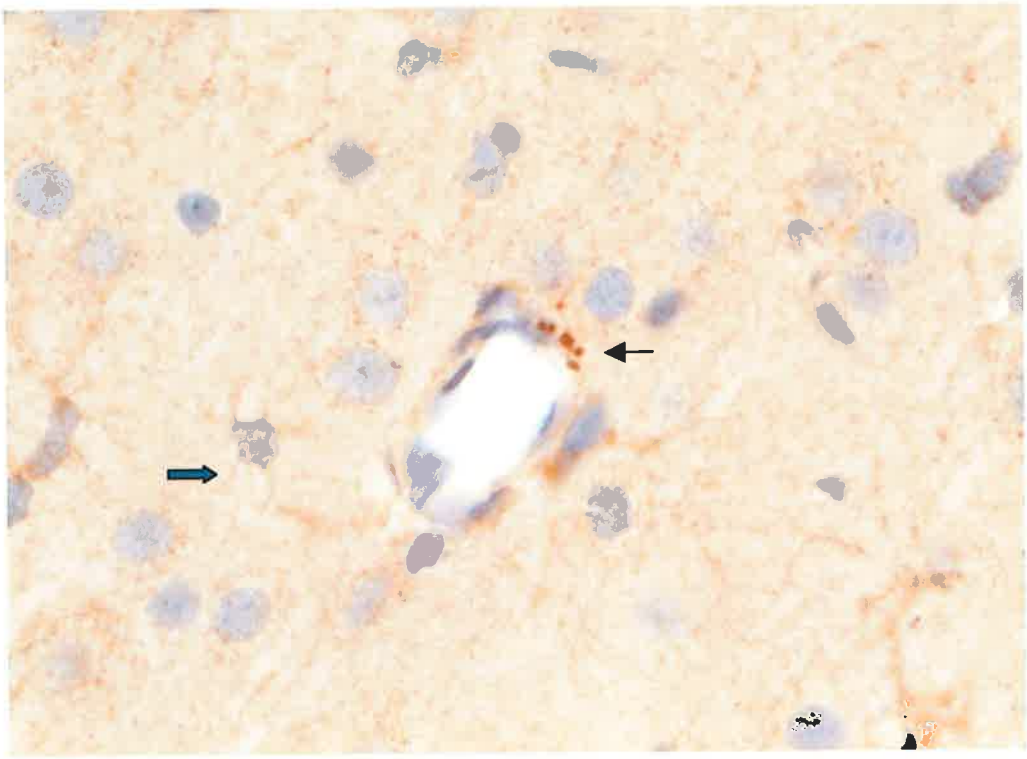


Figure 4.1 Control rat cortical vessel. Notice the discrete SP granules (←) and weak granular perikaryonal staining of pyramidal neurones (⇐) (x400).

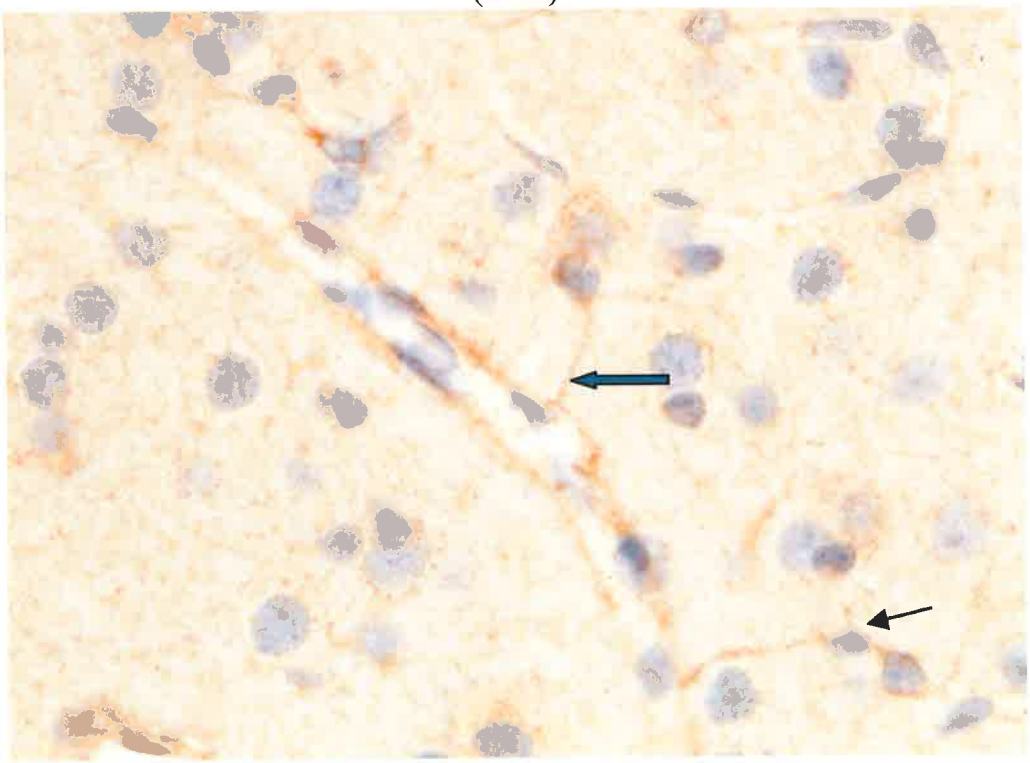


Figure 4.2. Control rat cortical vessel. Notice encircling astrocytic processes (⇐) and soma (←)(x400).

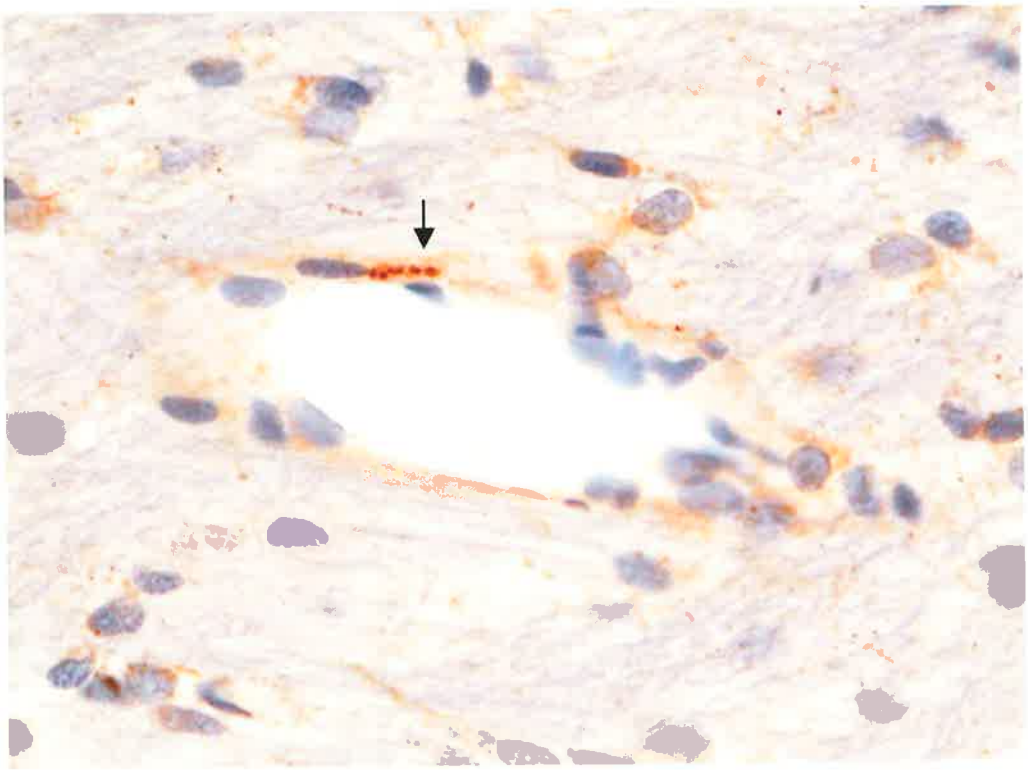


Figure 5. Control rat white matter (corpus callosum) vessel. Note discrete perivascularr SP granules (←) and sparse astrocytic staining (x400).

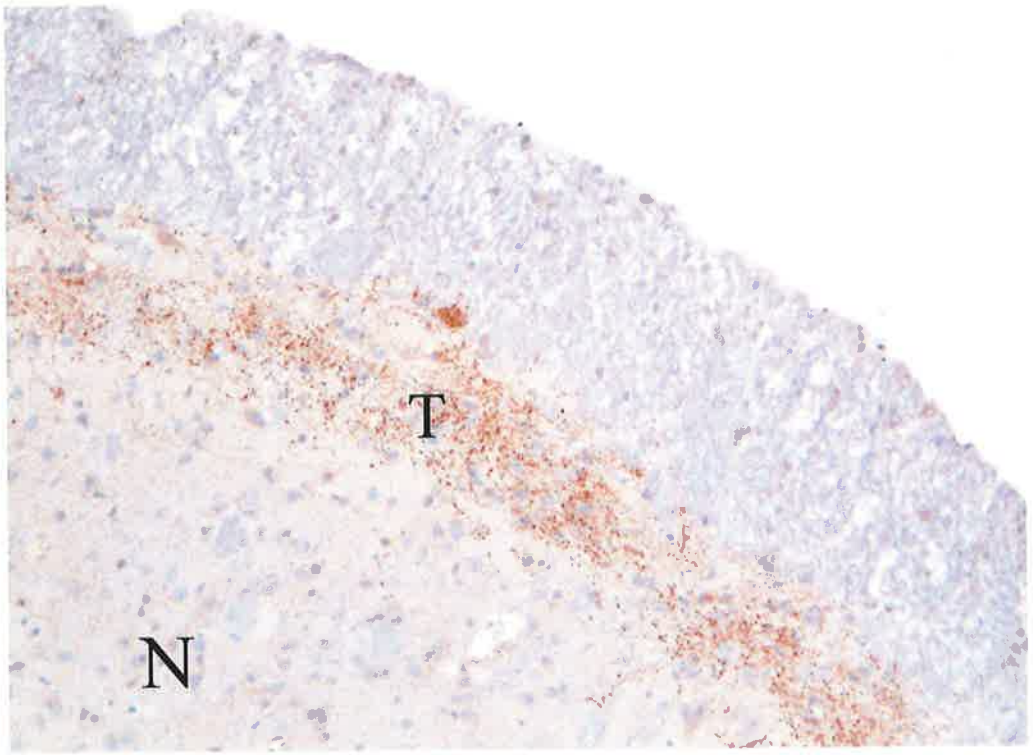


Figure 6.1Control rat. Section of spinal trigeminal tract (T) in caudal medulla with the nucleus medial. (x100)

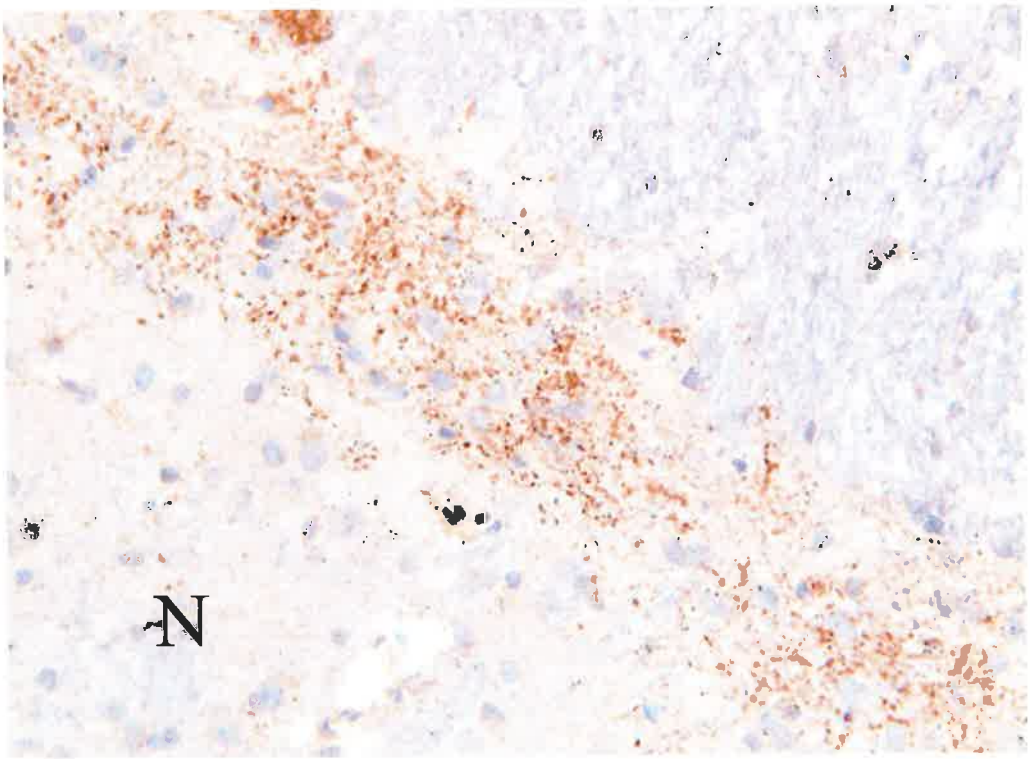


Figure 6.2 Control rat. Spinal trigeminal tract with varicose morphology of central nerve fibres with terminals.The neurones of the nucleus do not stain.(x200).

Figure 7.1 and 7.2. Gross examination of rat brain 5 hours following diffuse impact injury. A small amount of basal brainstem haemorrhage is evident.

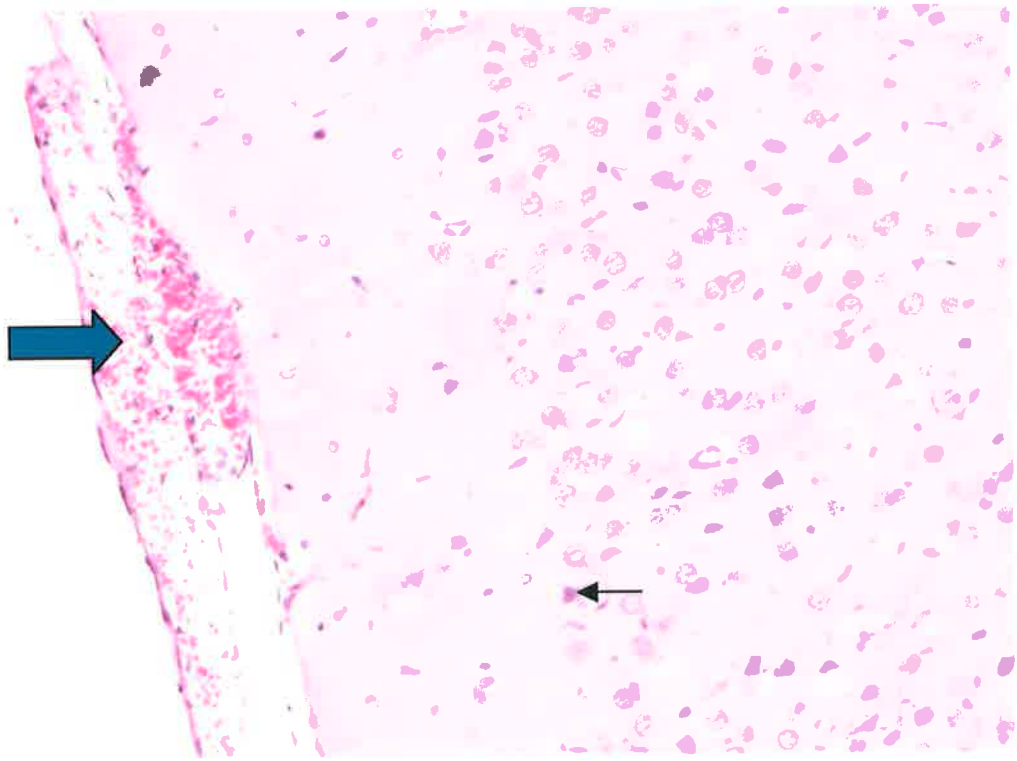


Figure 8.1 H&E section of rat frontoparietal parasagittal cortex 5hr following diffuse impact. Subarachnoid haemorrhage(⇒) and neuronal dark cell change are evident (←).(x100)

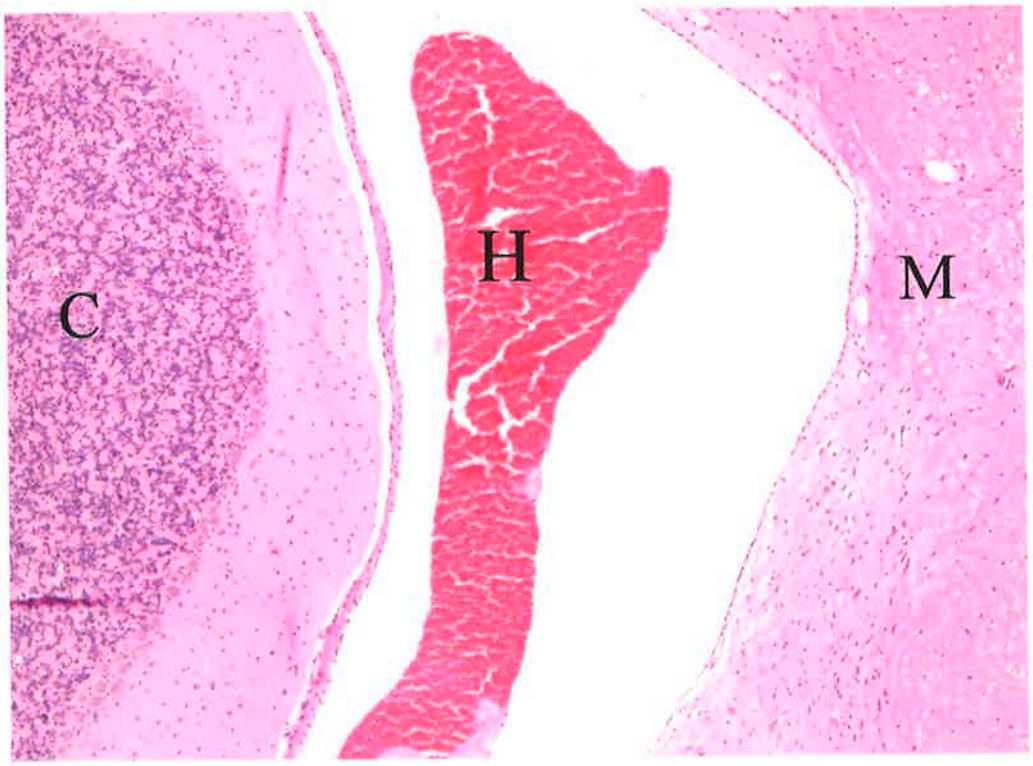


Figure 8.2 H & E section of rat fourth ventricle 5hr following diffuse impact, showing haematoma (H).Cerebellum (C) and medulla (M).(x40)

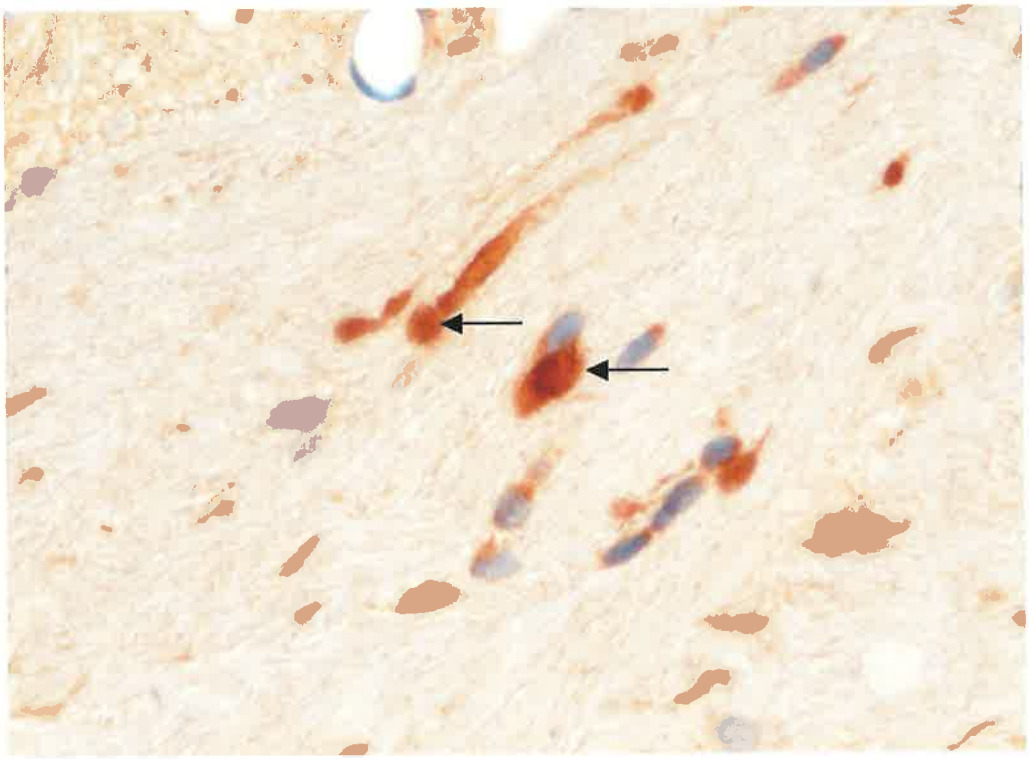


Figure 9.1 APP positive axonal swellings (←) in rodent corpus callosum 5hr following diffuse impact. (x400)

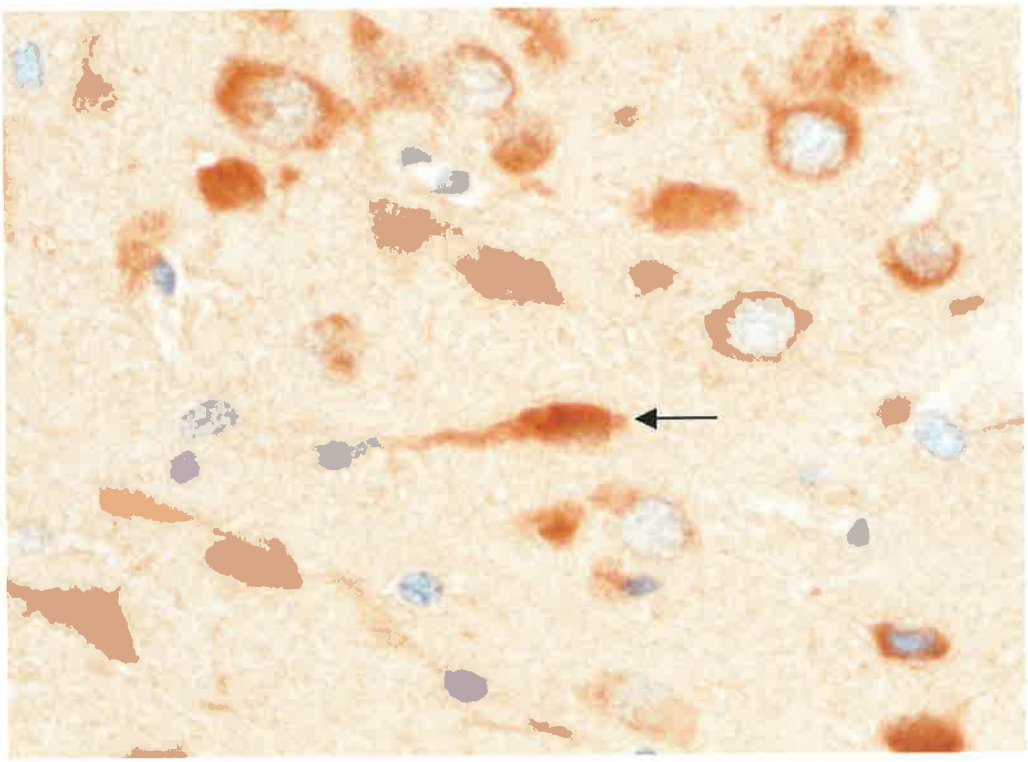


Figure 9.2 Axonal swellings (←)and increased neuronal APP in rodent parasagittal cortex 5hr following diffuse impact. (x400).

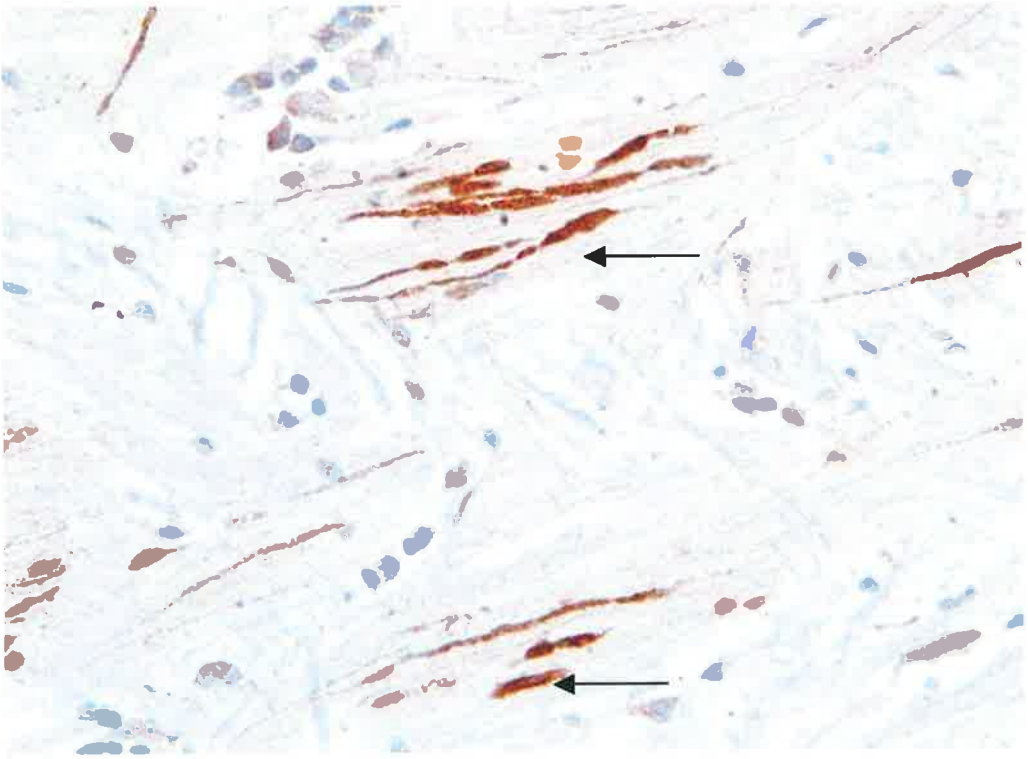


Figure 9.3 APP positive axonal swellings (←) in rodent pyramidal decussation 5hr following diffuse trauma. (x400)

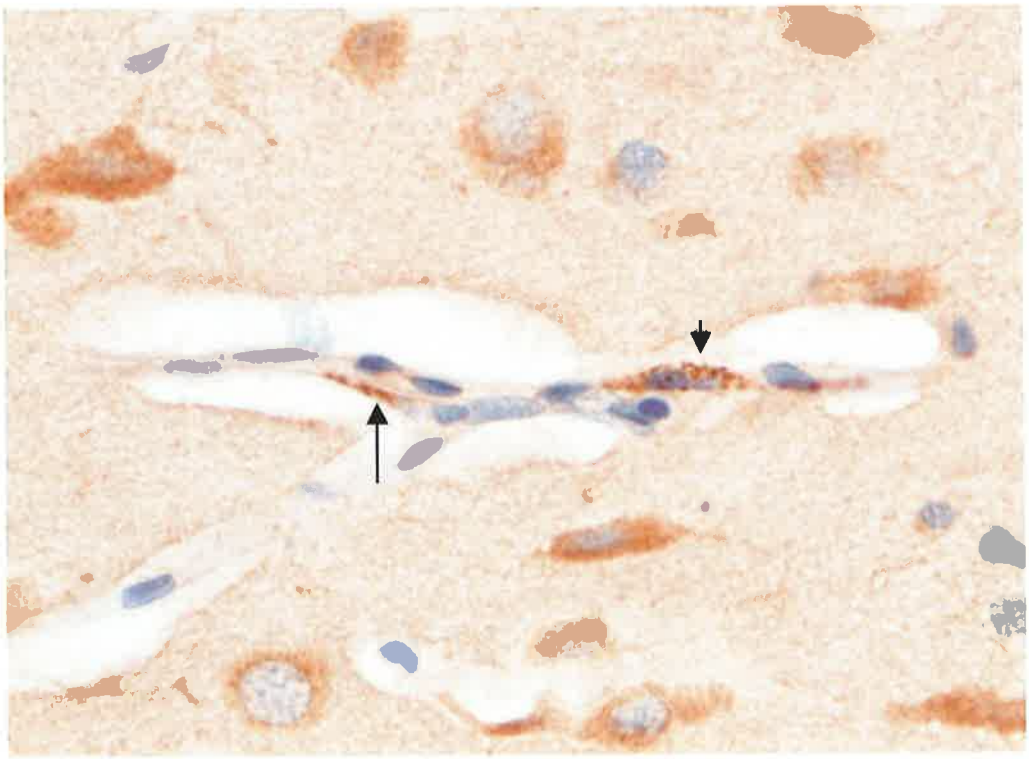
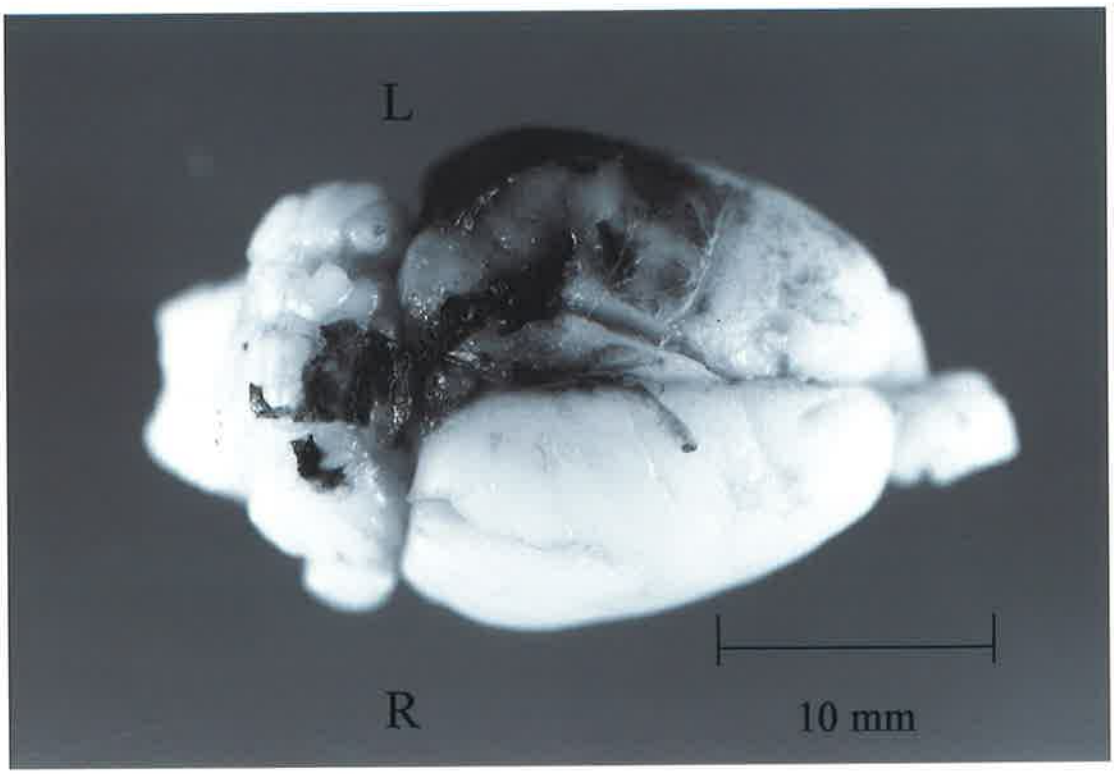
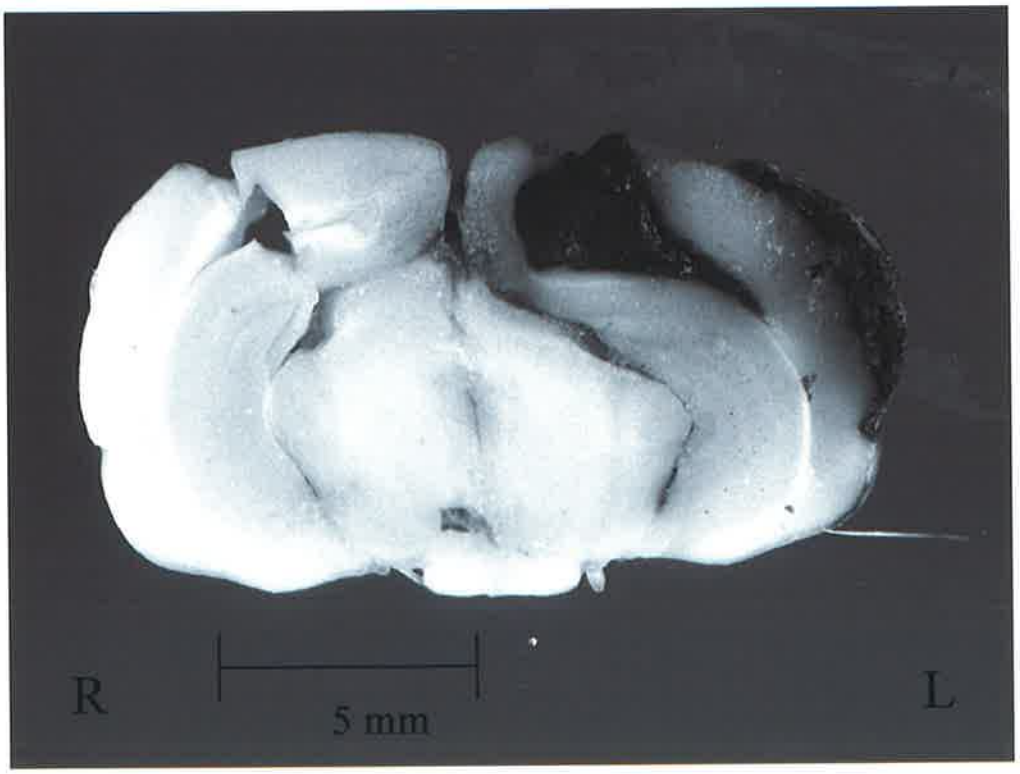


Figure 9.4 Perivascular APP positive granules (→) in rodent parasagittal cortex 5hr following diffuse impact. (x400)



igure 10.1and 10.2. Gross examination and coronal section of rat brain 5hours following fluid percussion ijury. Left parieto occipital subdural haematoma with underlying cortical contusion with mass effect. Left (L) and right (R).



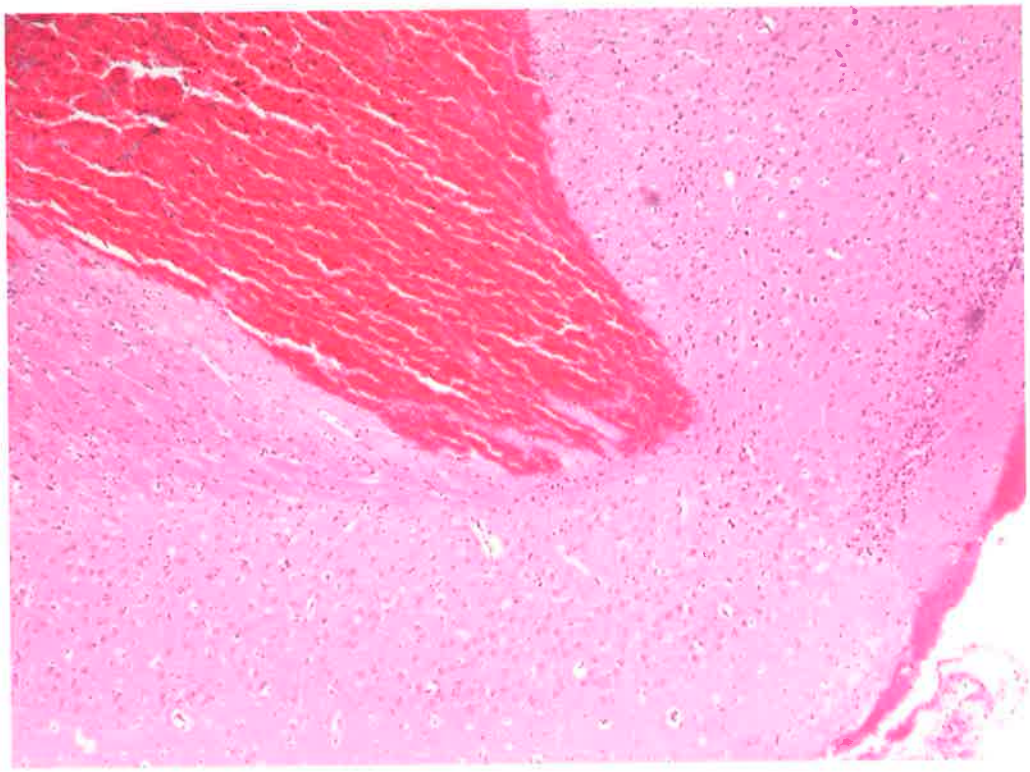


Figure 11.1 Left parietal haemorrhagic contusion underlying subdural haematoma in a rodent 5 hours following fluid percussion injury (.x100)

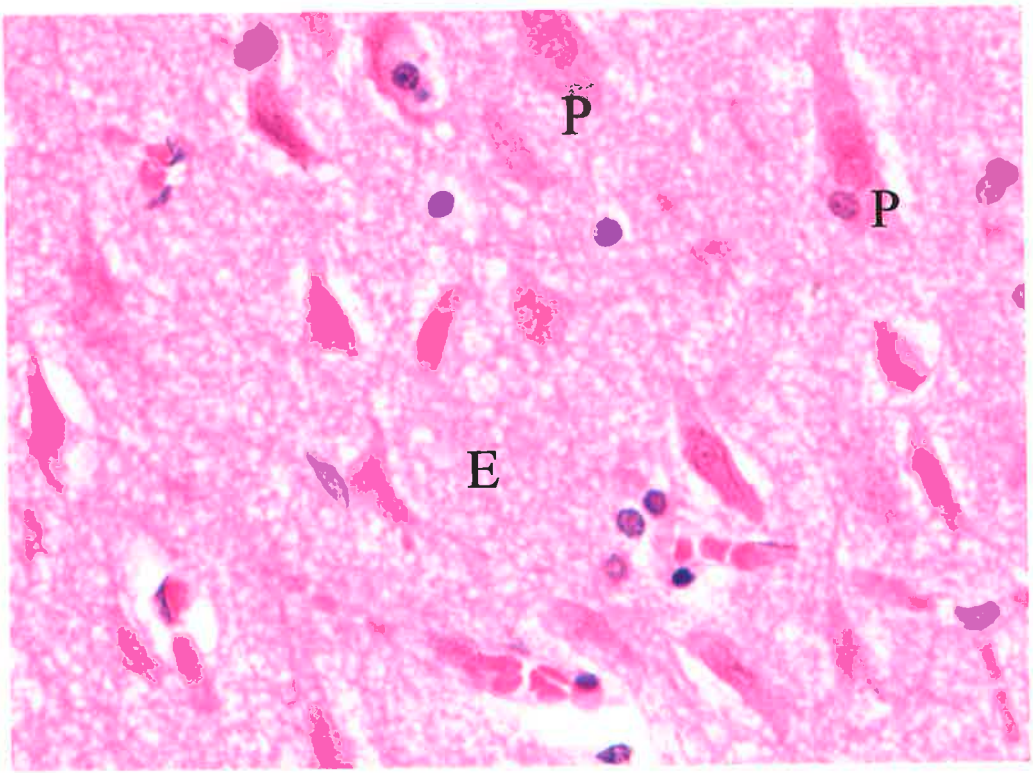


Figure 11.2 Adjacent pyramidal neurones (P) exhibit red tinctorial change against a background of microcytic oedema (E) in the neuropil. (x400)

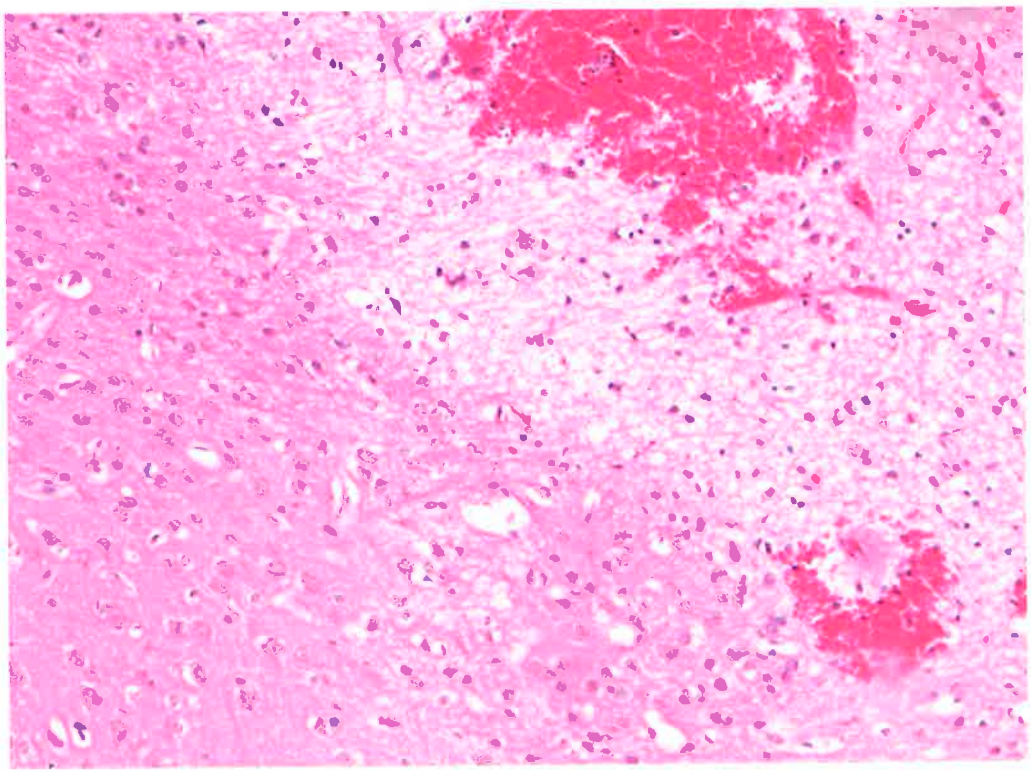


Figure 12.1 Area of haemorrhage (H) with infarct (I) in left parietal rodent cortex 5hr following fluid percussion injury. (HE x200)

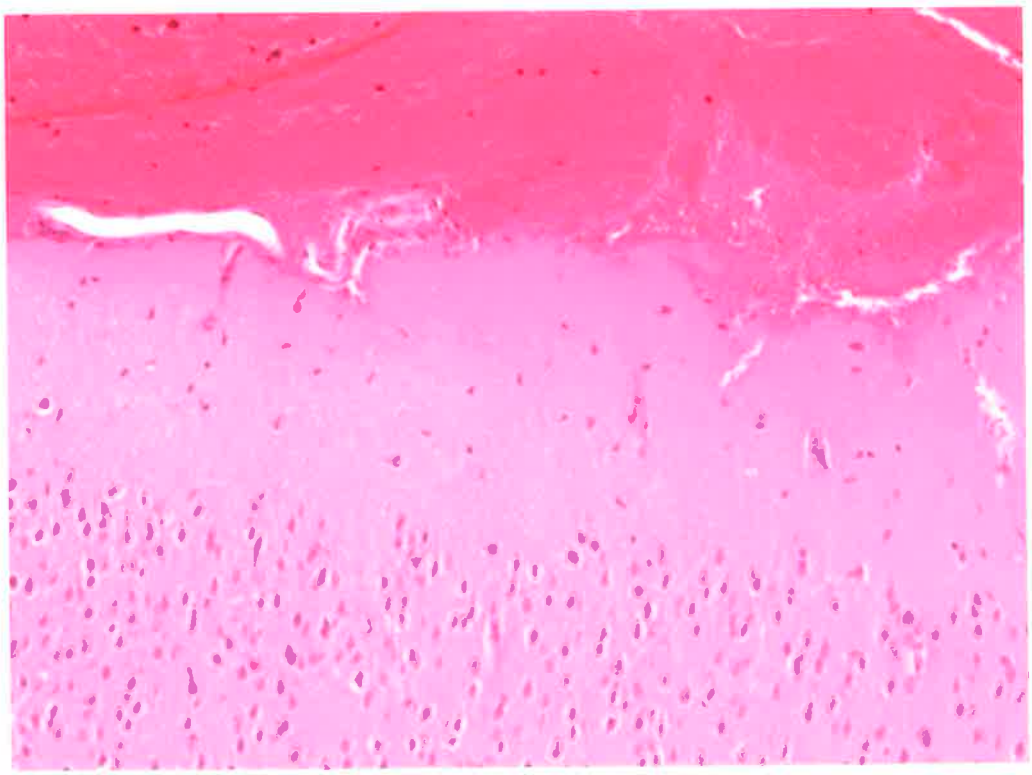


Figure 12.2 Right parasagittal subdural haematoma with mild cortical contusion in rodent 5 hours following fluid percussion injury. (HE x 100).

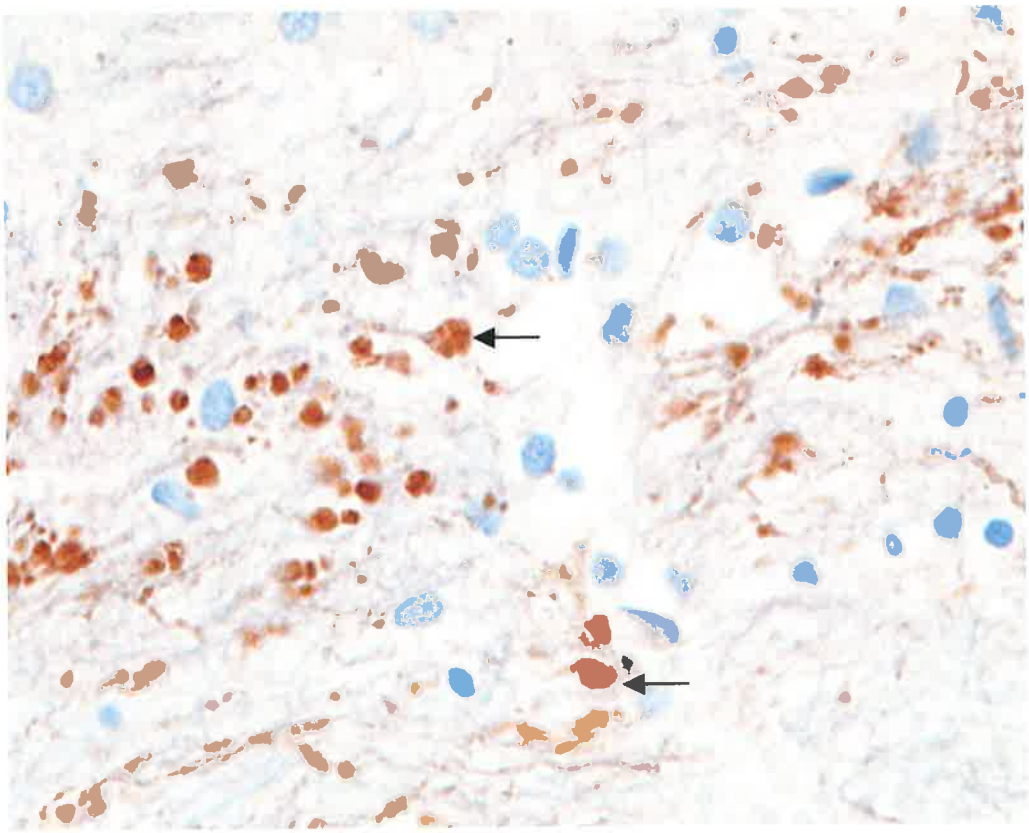


Figure 13.1 APP positive axonal swellings (←) in left corpus callosum of rodent 5hr following fluid percussion injury.(x400)

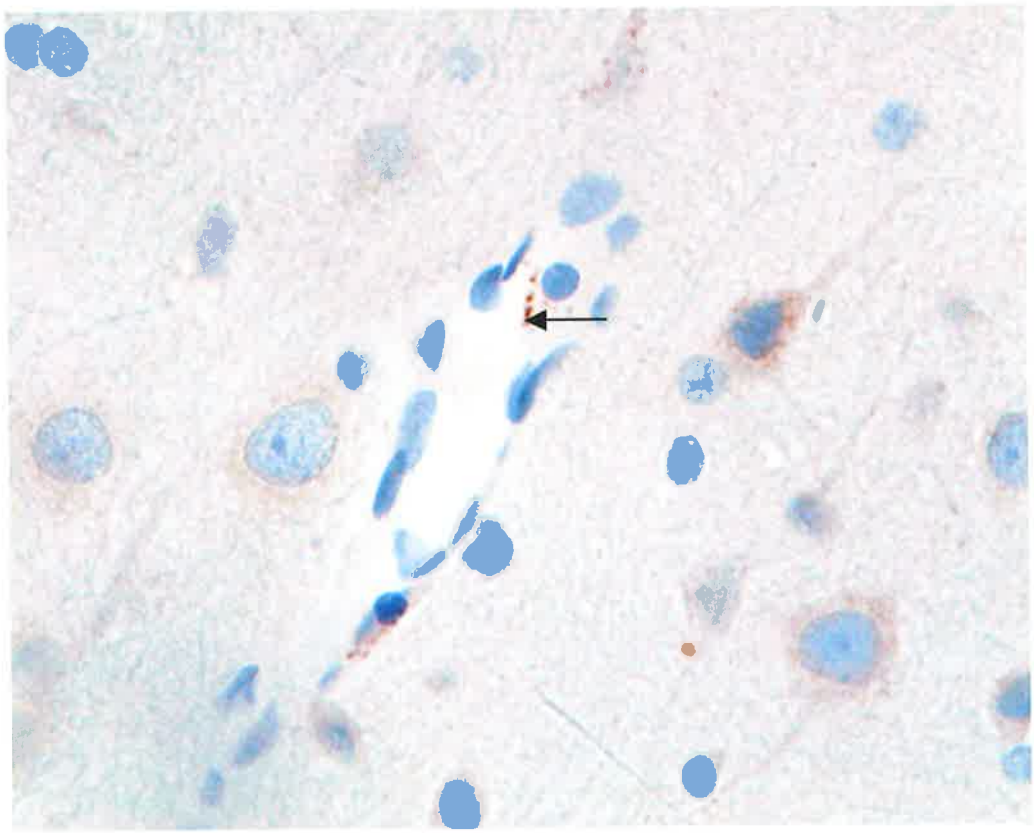


Figure 13.1 APP positive perivascular granules (→) in left parietal hemipshere of rodent 5hr following fluid percussion injury.(x400)

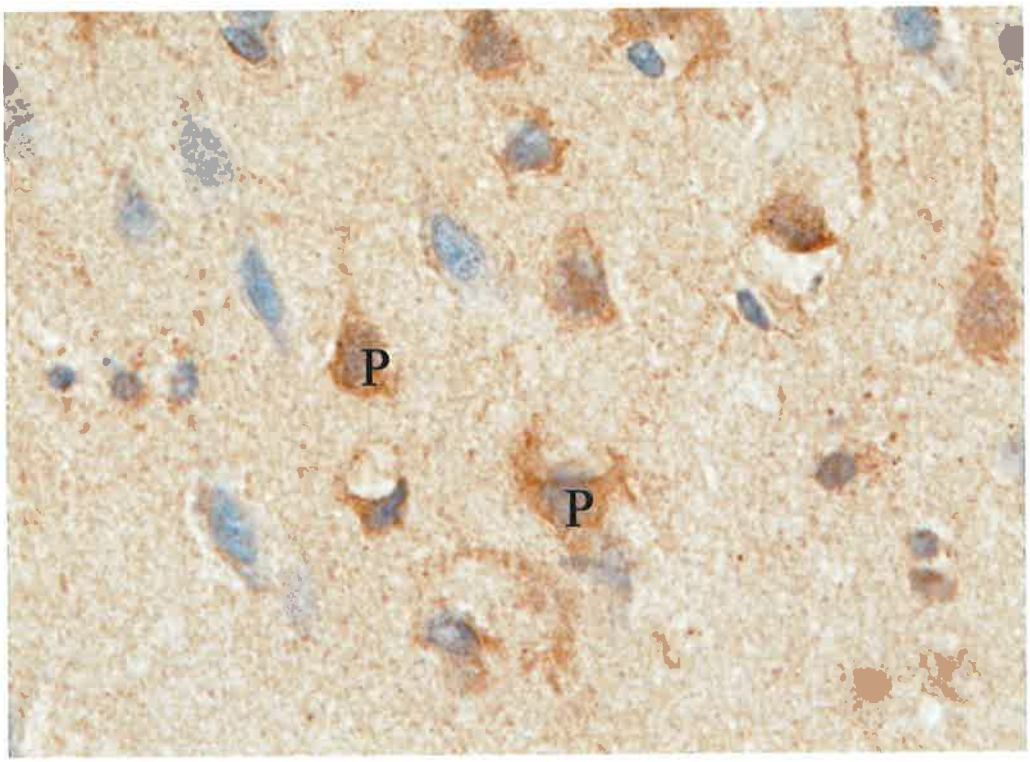


Figure 14.1 Left parietal cortex pyramidal neurones (P) adjacent to haemorrhagic contusion show intense SP granular morphology 5hr following fluid percussion injury. (x400)

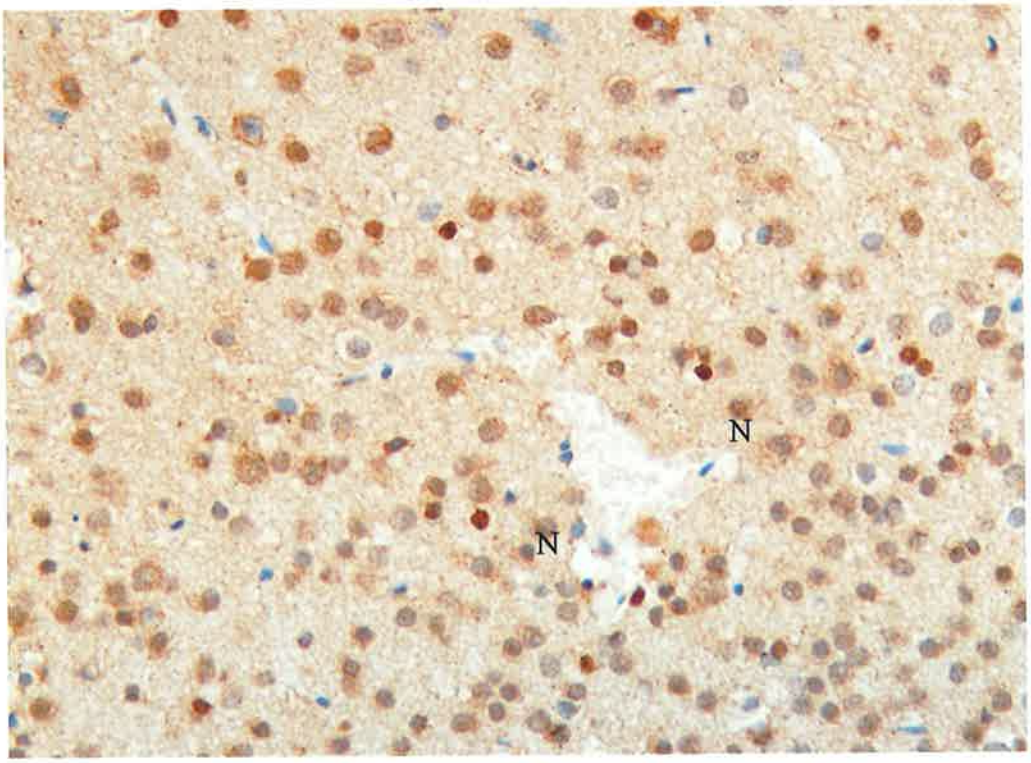


Figure 14.2 Left pyramidal non pyramidal neurones (N) adjacent to area of infarction show positive SP staining in contrast to non staining adjacent neurones.(x200)

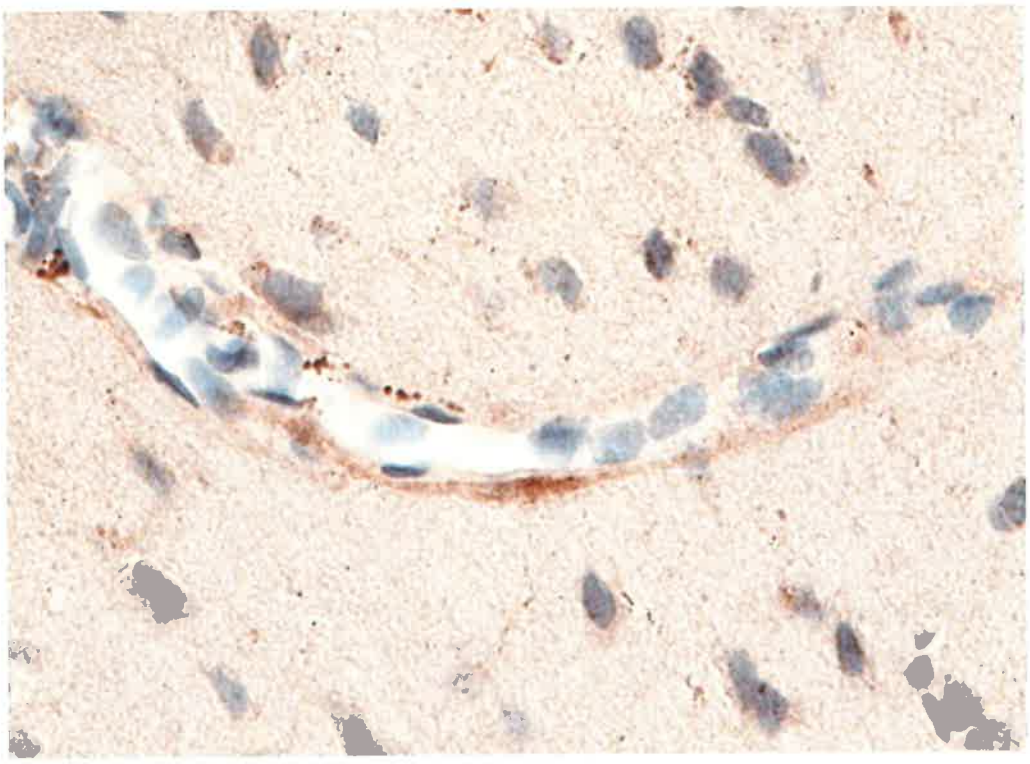
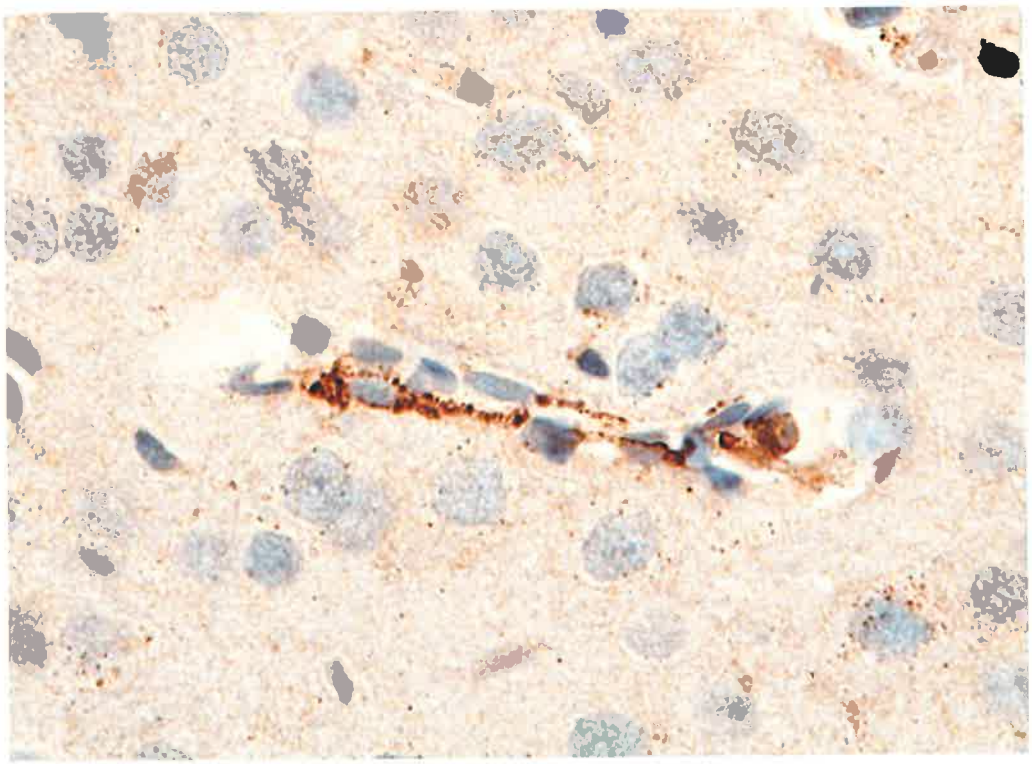


Figure 15.1 and 15.2. Left parietal cortical venules adjacent to haemorrhagic contusion show increased perivascular Sp granularity.(x400)



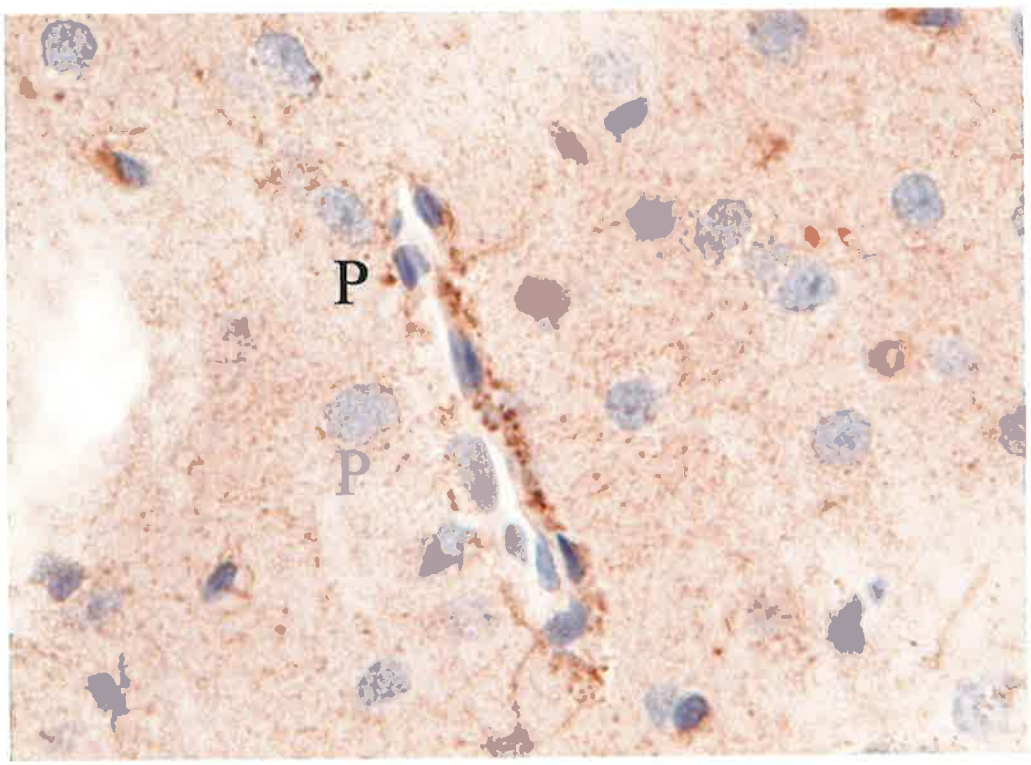


Figure 16.1 Increased SP perivascular granularity in rodent parasagittal cortex 5hr following diffuse injury and also in pyramidal neurones. (P).(x400)

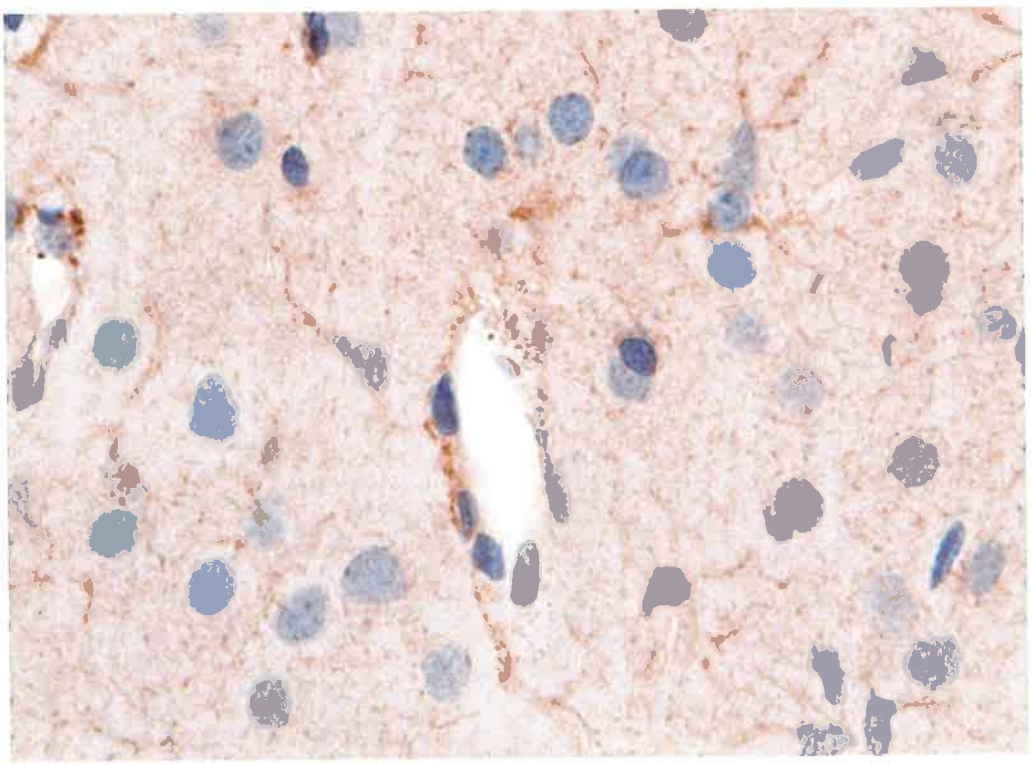


Figure 16.2 Rodent parasagittal cortical vessels 5hr following diffuse injury.(x400) .

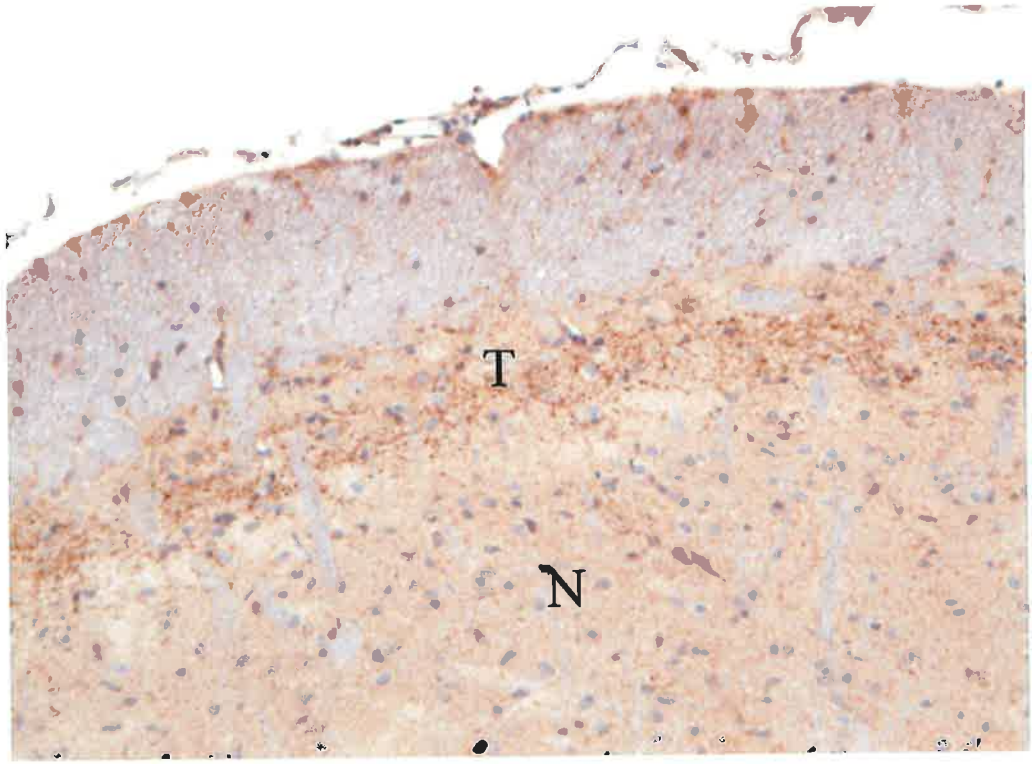


Figure 17.1 Increased SP immunoreactivity in rat caudal medulla 5hr following diffuse injury.Spinal trigeminal tract (T), nucleus (N).(x100)

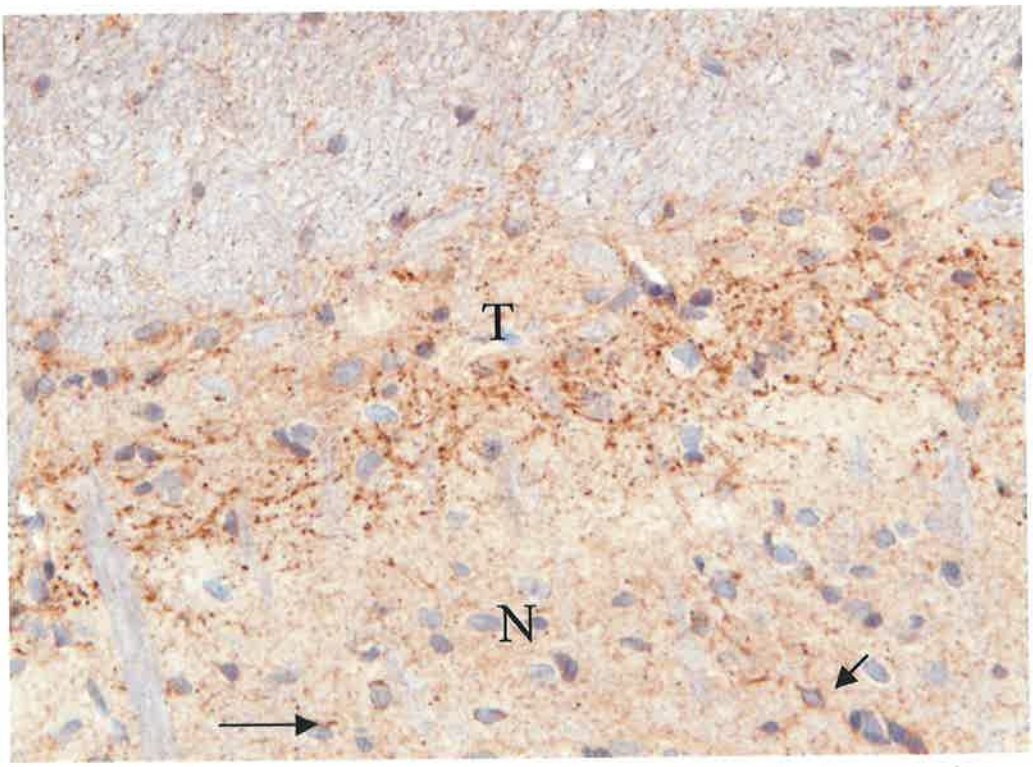


Figure 17.2 Rat spinal trigeminal nucleus (N) and tract (T) 5hr following injury. Although background staining is greater,some increased neuronal staining in the nucleus is evident (←).(x200)

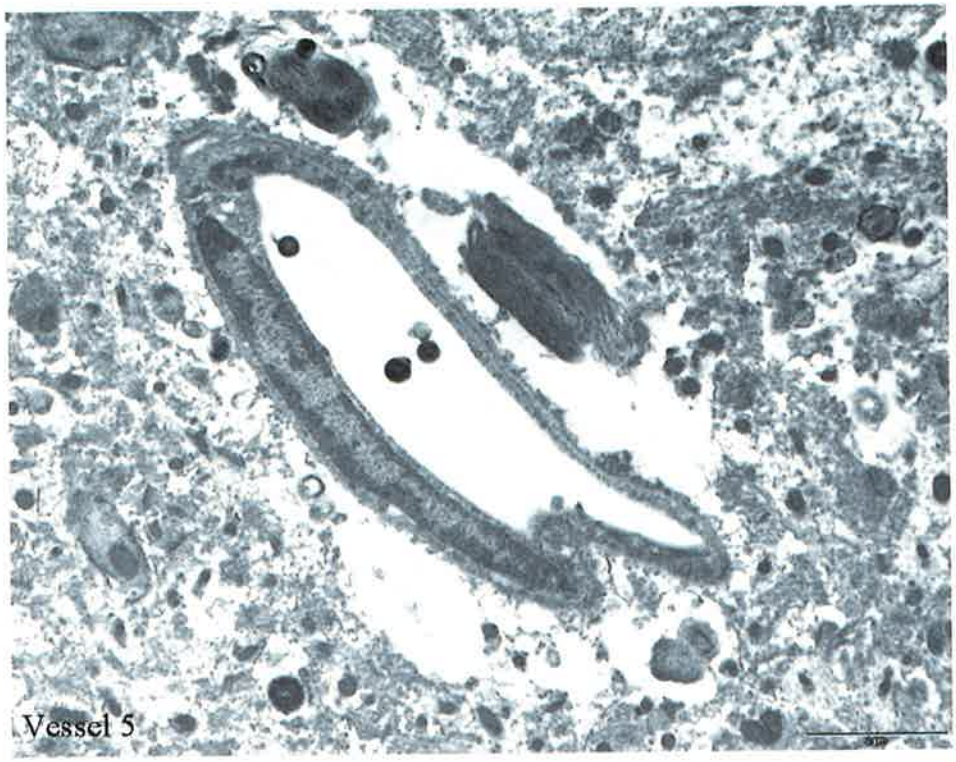


Figure 18.1 EM of rodent control parasagittal cortical vessel stained for SP.

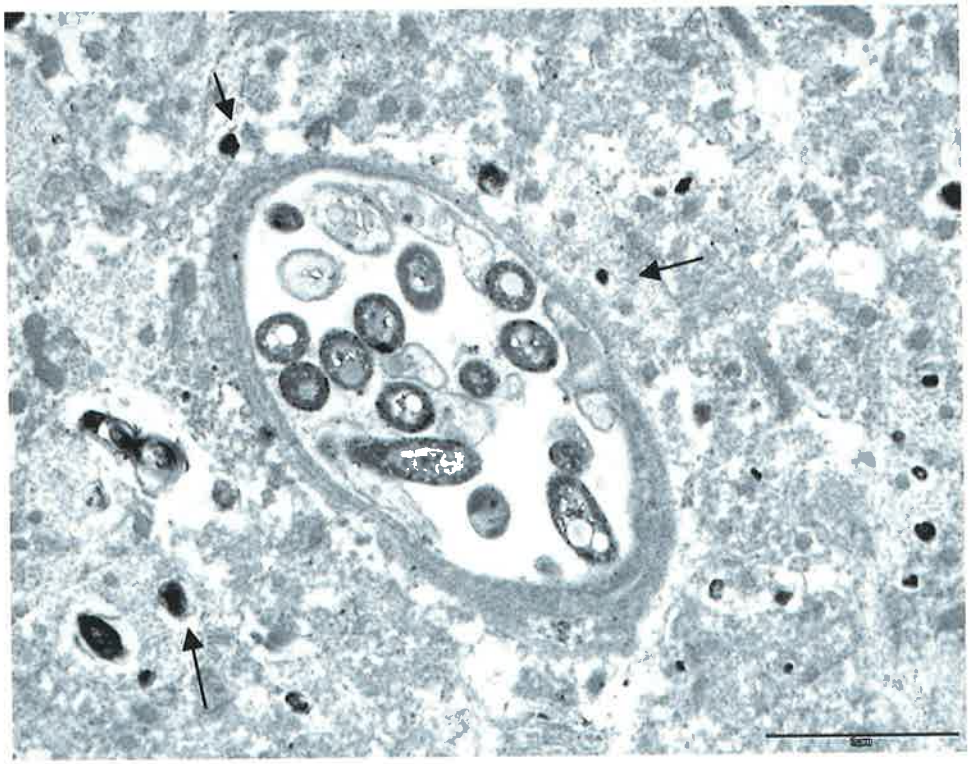


Figure 18.2 EM of rodent cortical vessel stained for SP 30 minutes following diffise impact injury.Notice the increased numbers of immunolabelled unmyelinated axons (→) compared to the control.

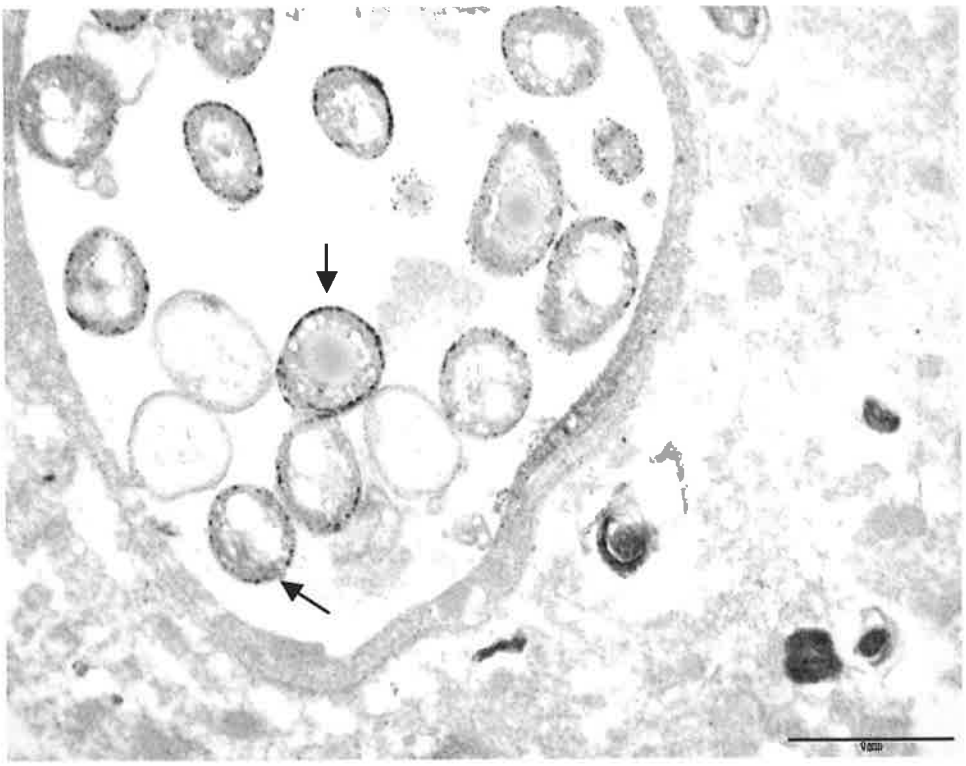


Figure 18.3 Cortical rodent vessel 30min following diffuse injury stained for SP. Stained axons are prominent. Intraluminal platelets(→) exhibit membrane staining for SP.

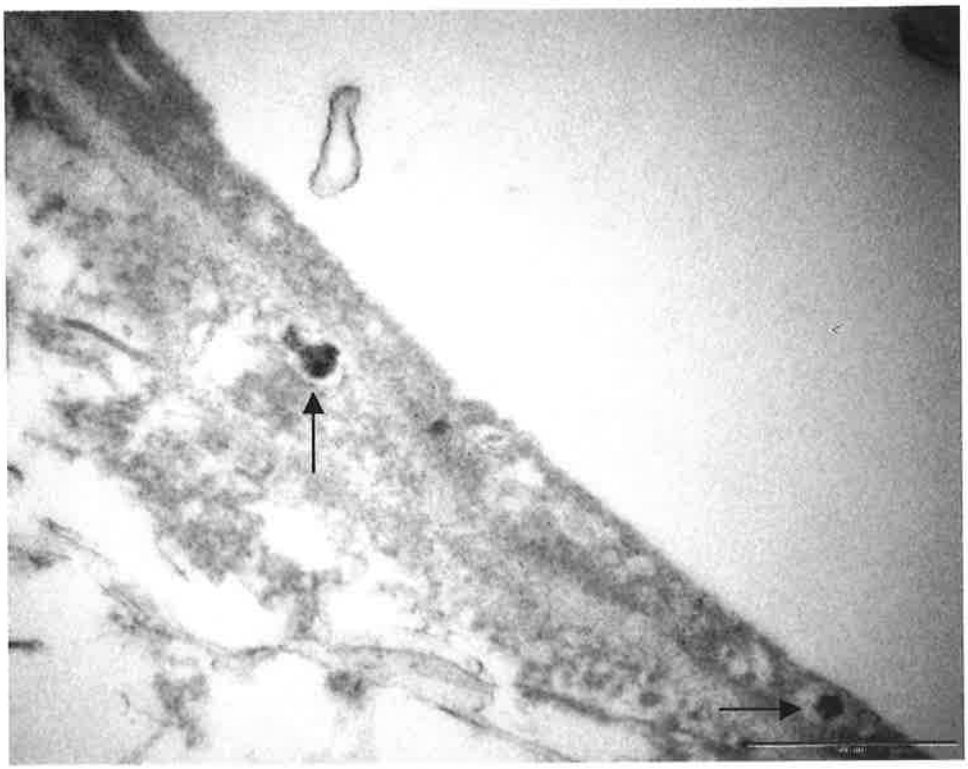


Figure 18.4. High magnification of endothelium of cortical rodent vessel stained for SP 30 minutes following diffuse injury. Small labelled axons are close to endothelium (→) but the endothelium is unstained.

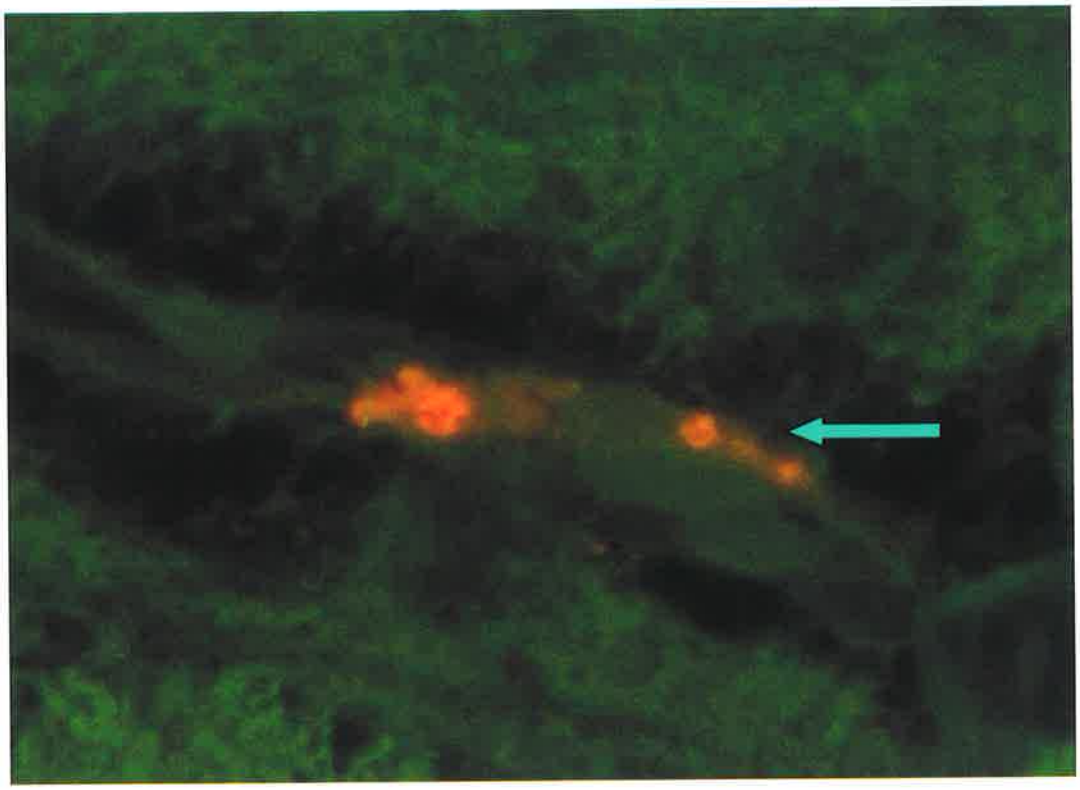
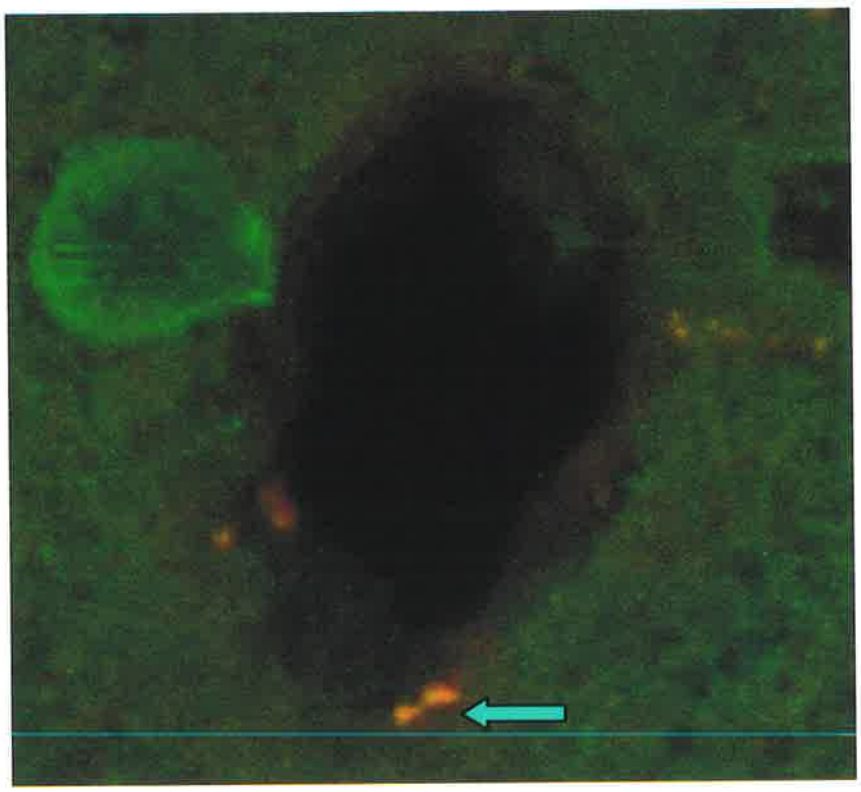


Figure 19.1 and 19.2. Confocal images of control rodent cortical vessel double labelled for PGP 9.5 (green) and SP (red) showing SP containing perivascular axons (orange) (⇐)(x600)



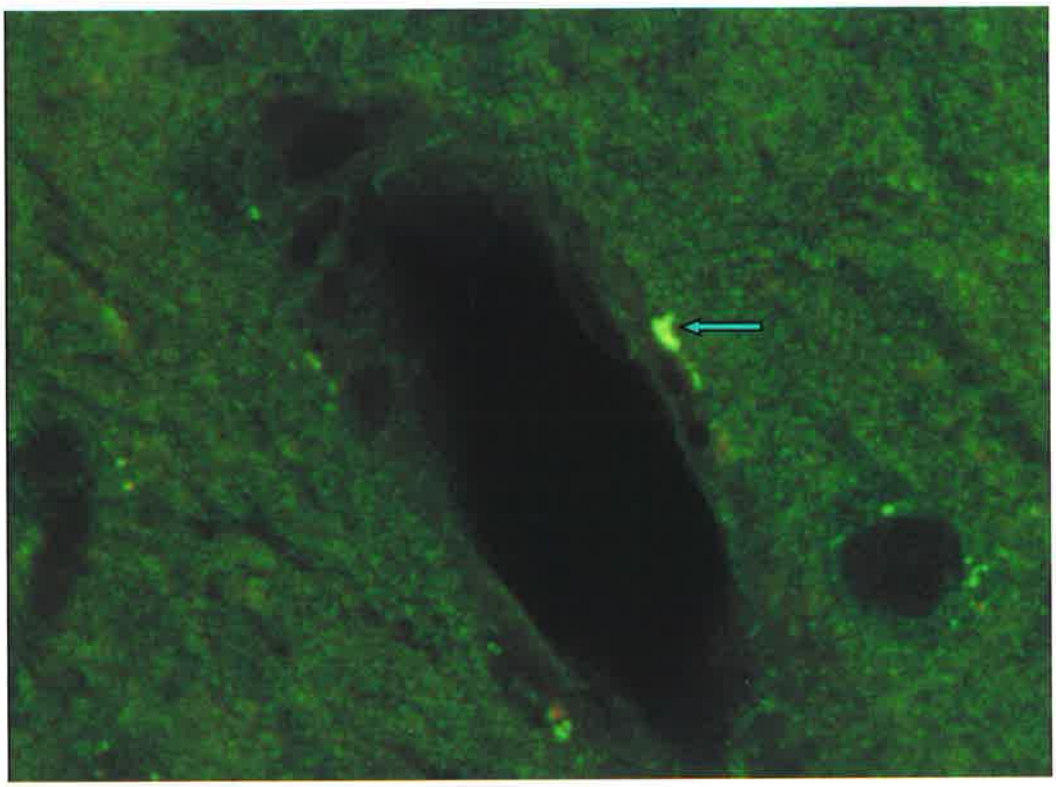


Figure 20. Confocal image of rodent cortical vessel 5hr following diffuse injury double labelled for APP (green) and SP (red) showing injured perivascular axons (yellow).(x600)

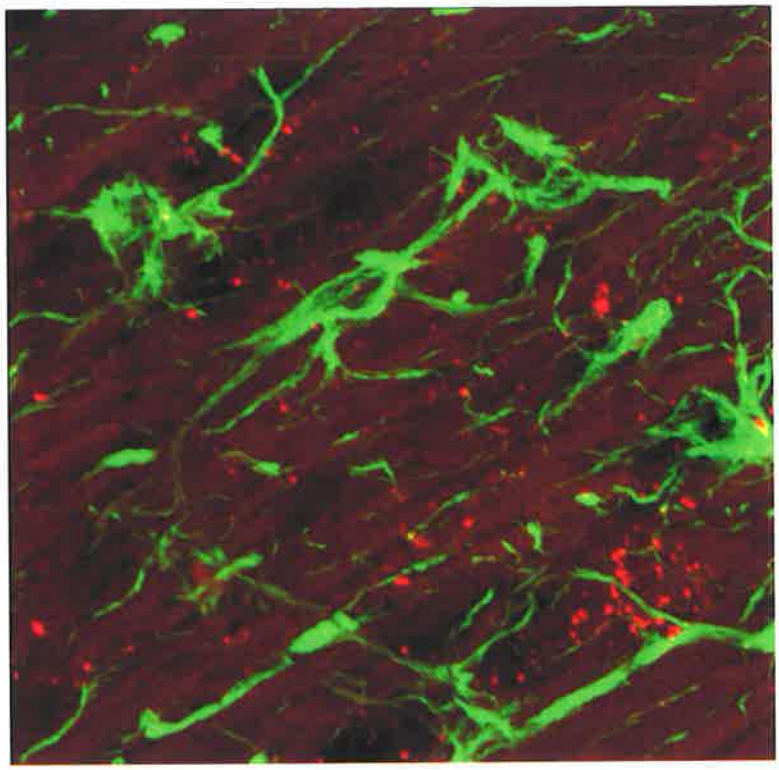


Figure 21.1 Confocal image of rodent astrocytes in deep parasagittal cortex double labelled for GFAP(green) and SP (red) 5 hours following diffuse injury showing no colocalisation.(x600)

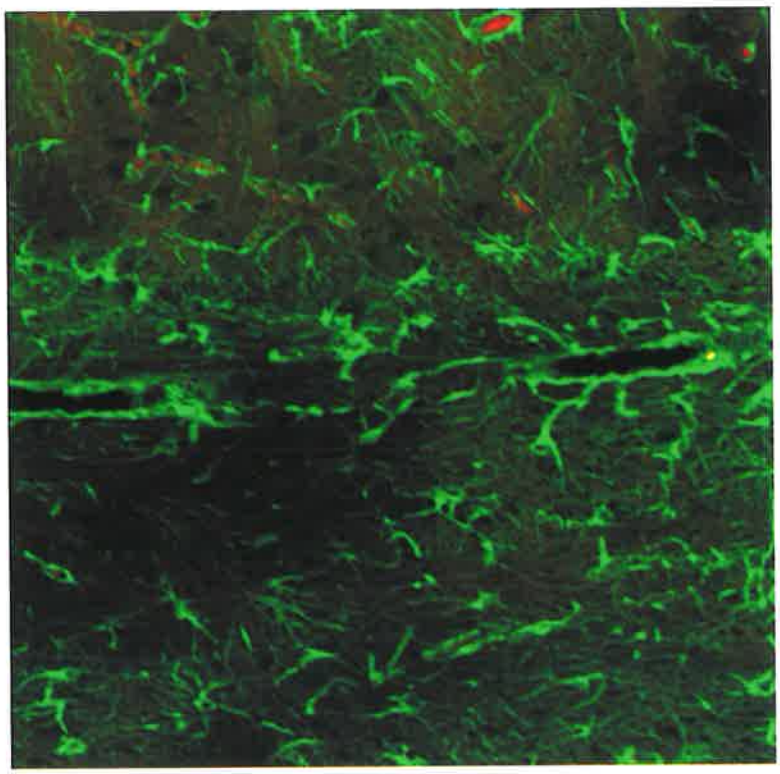


Figure 21.2 Confocal image of astrocytes in rodent corpus callosum 7days following diffuse injury double labelled for GFAP(green) and SP(red) and showing no colocalisation.(x400)

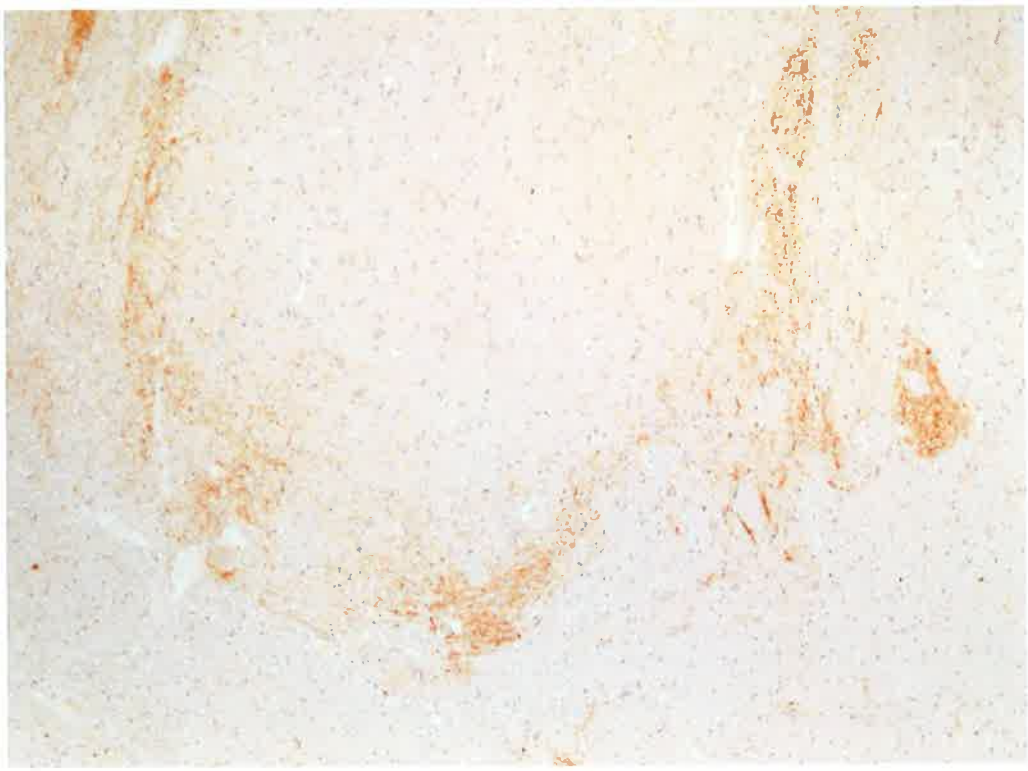


Figure 22.1. Human spinal trigeminal tract and nucleus stained for SP in caudal medulla (control).(x40)

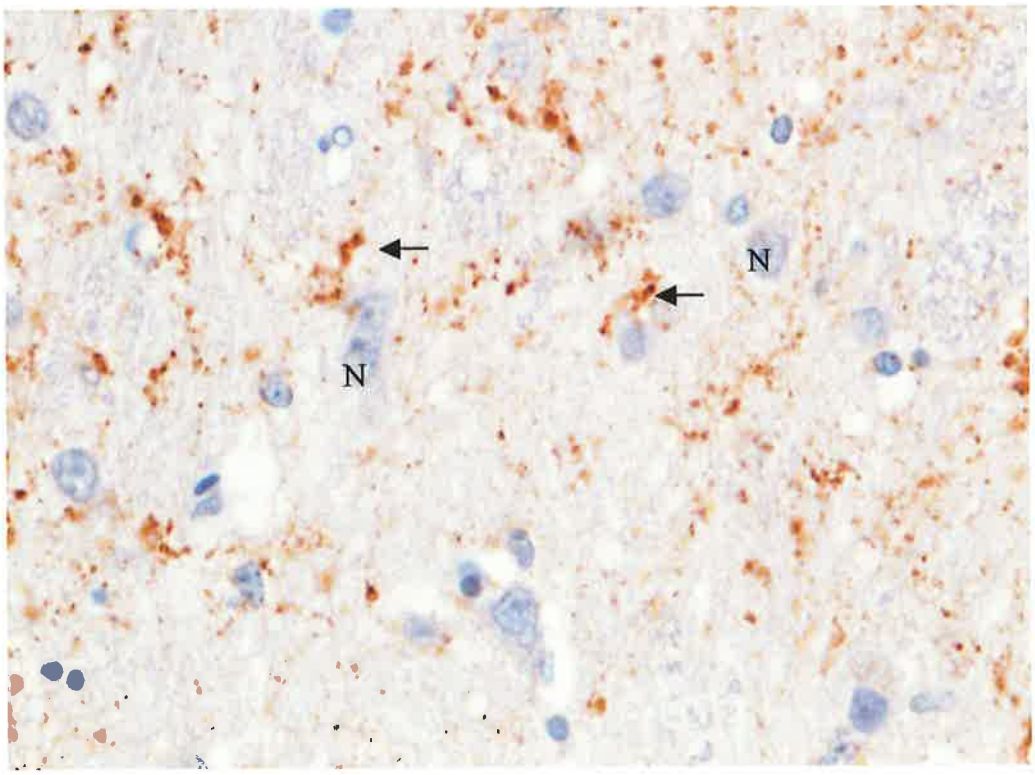


Figure 22.2. SP containing nerve terminals (←) of human spinal trigeminal tract. The neurones (N) of the nucleus do not stain for SP.(x400).

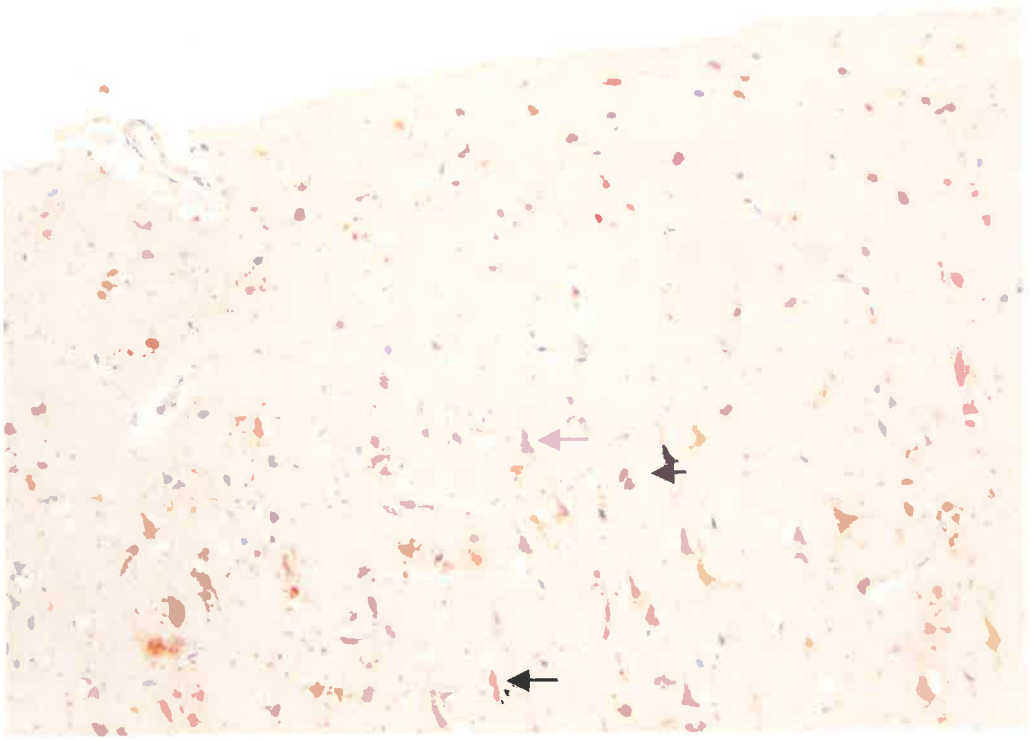


Figure 23.1 Human frontal cortex stained for SP. The pyramidal neurones are prominent (←)(x40)

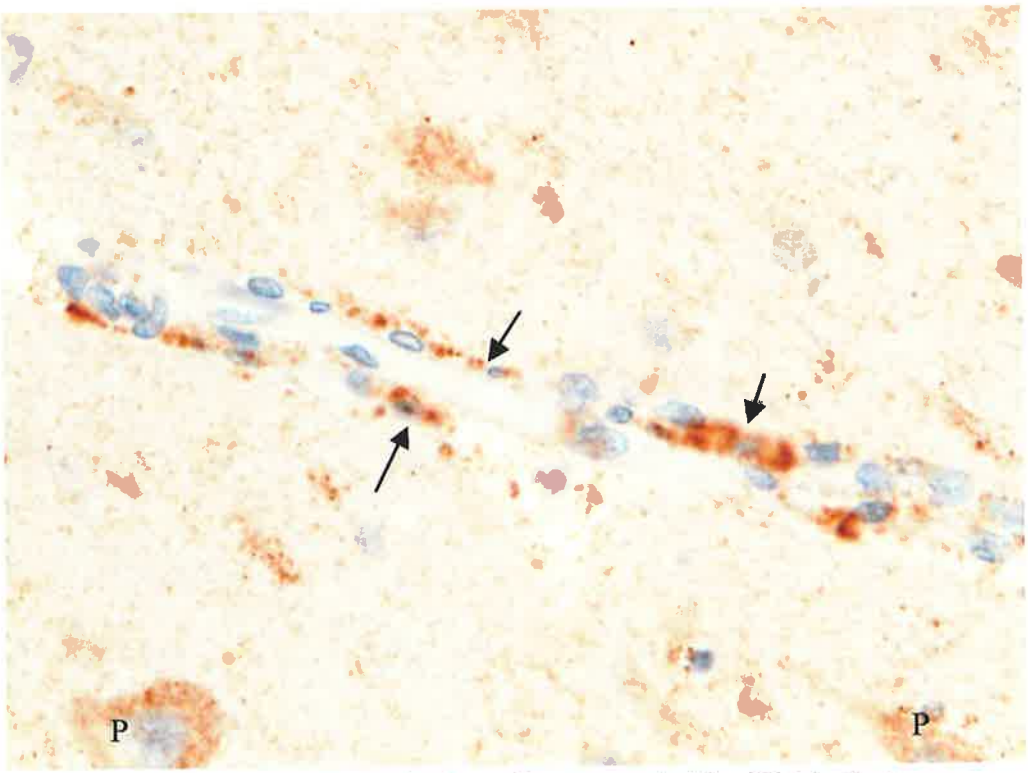


Figure 23.2. A human cortical venule surrounded by SP labelled nerve terminals (control) (←). Pyramidal neurones (P).(x400)

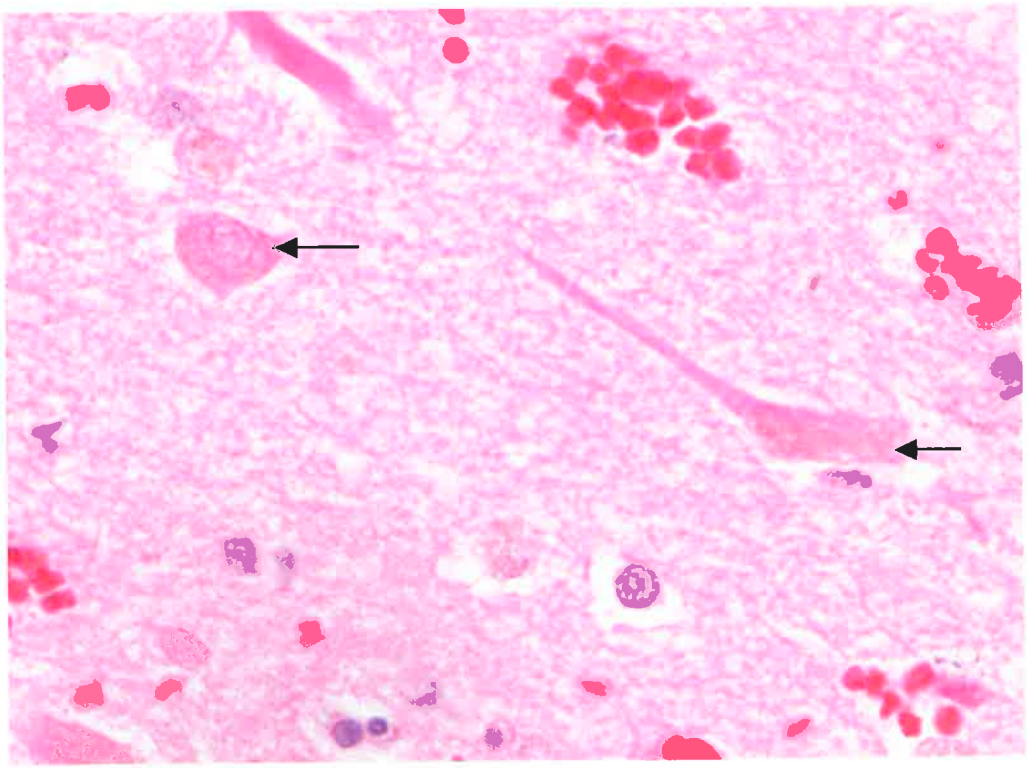


Figure 24.1 Prominent neuronal red cell change (←) in the frontal cortex of an 86 year old man with a compound depressed skull fracture with venous infarction who survived 4 days. Scattered haemorrhage is also present.(x400)

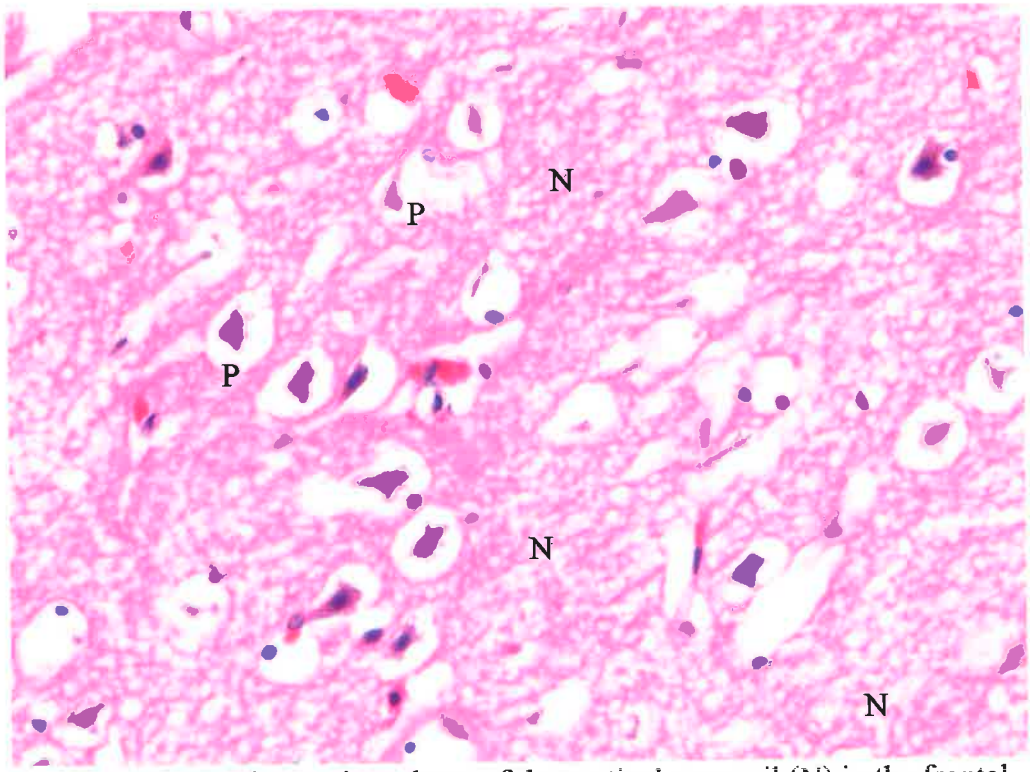


Figure 24.2 Microcytic oedema of the cortical neuropil (N) in the frontal cortex of a 13 year old girl with diffuse cerebral oedema who survived 2 days.Perineuronal fixation vacuolation (P) is also prominent.(x200)

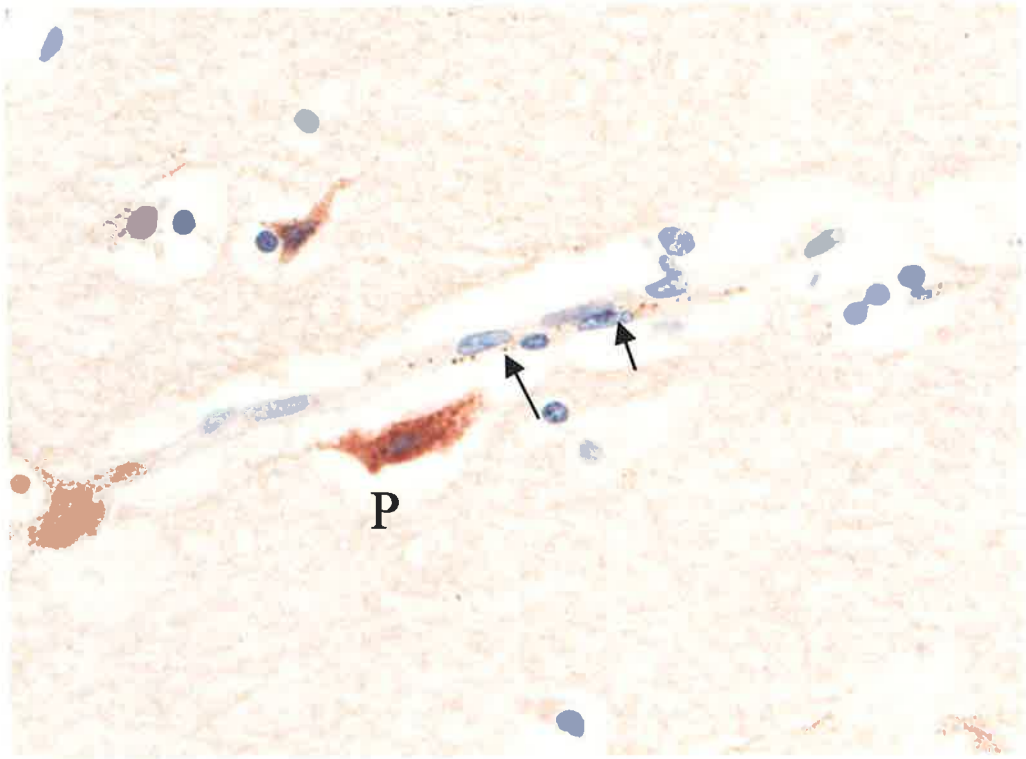


Figure 25.1 APP positive axons (→) along a cortical venule in a 21 year old who died within hours with an acute subdural haematoma. Increased neuronal APP positivity is also evident in pyramidal neurones (P).(x400)

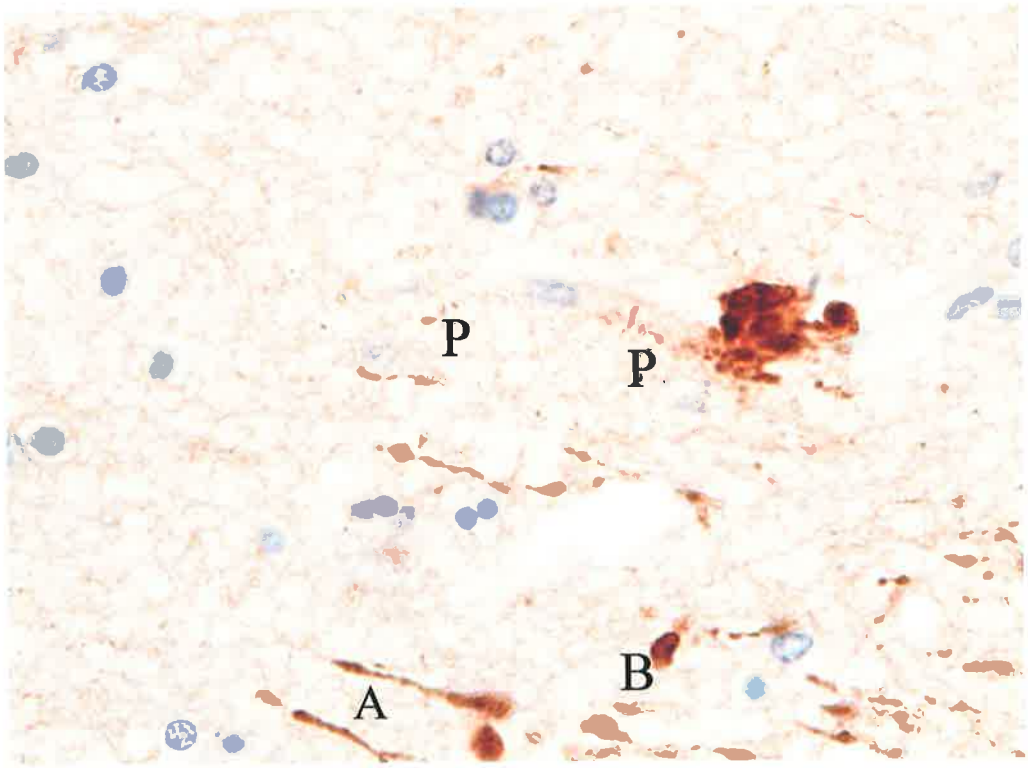


Figure 25.2 APP positive axons (A) with retraction balls (B) and perivascular axons (P) in an 18 year old patient with bilateral contusions who suvived 3days. (x400)

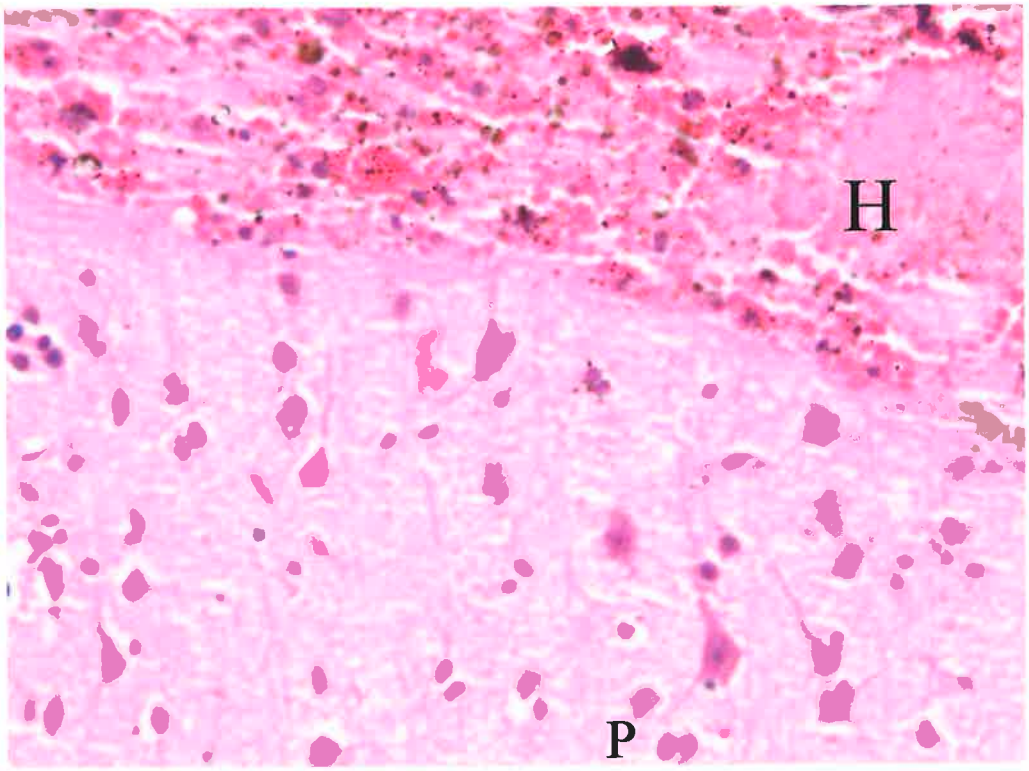


Figure 26.1 H&E section of frontal cortex underlying acute subdural haematoma (H) in a 41 year old man who survived 1day. Some pyramidal neurones (P) show red cell change.(x200).

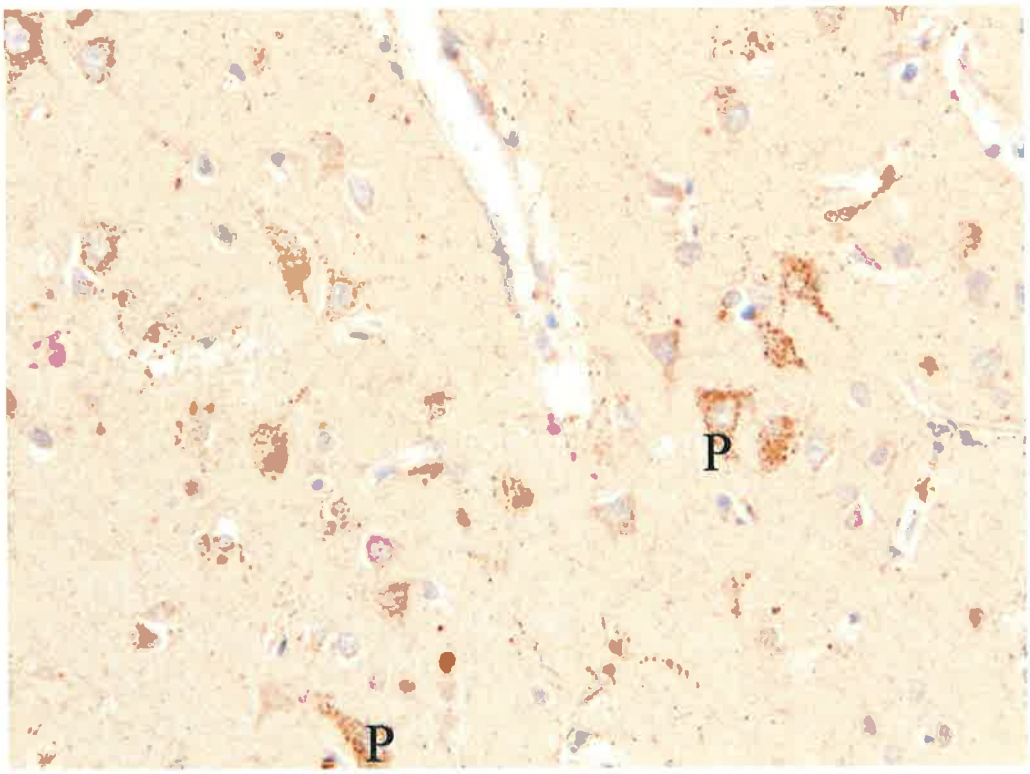


Figure 26.2 Same cortex as per 26.1 stained for SP. Notice prominence of granularity in pyramidal neurones (P).(x200).

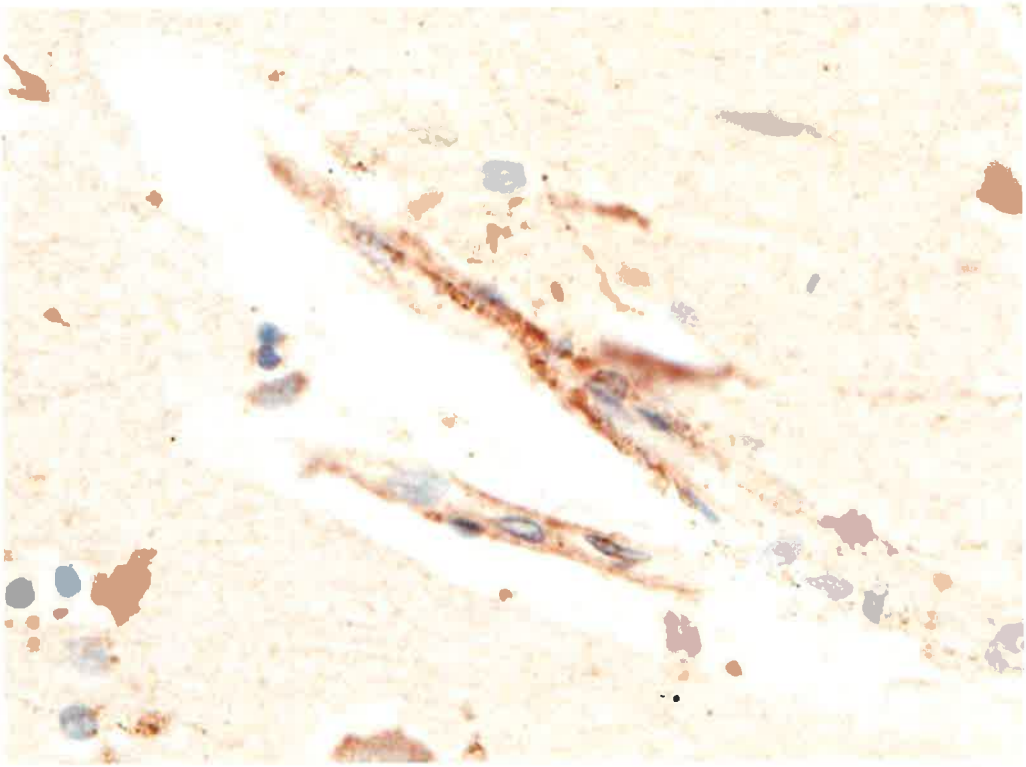


Figure 27.1 Increased perivascular SP granularity in the frontal cortex of a 36 year old with cerebral oedema who survived 1day.(x400)

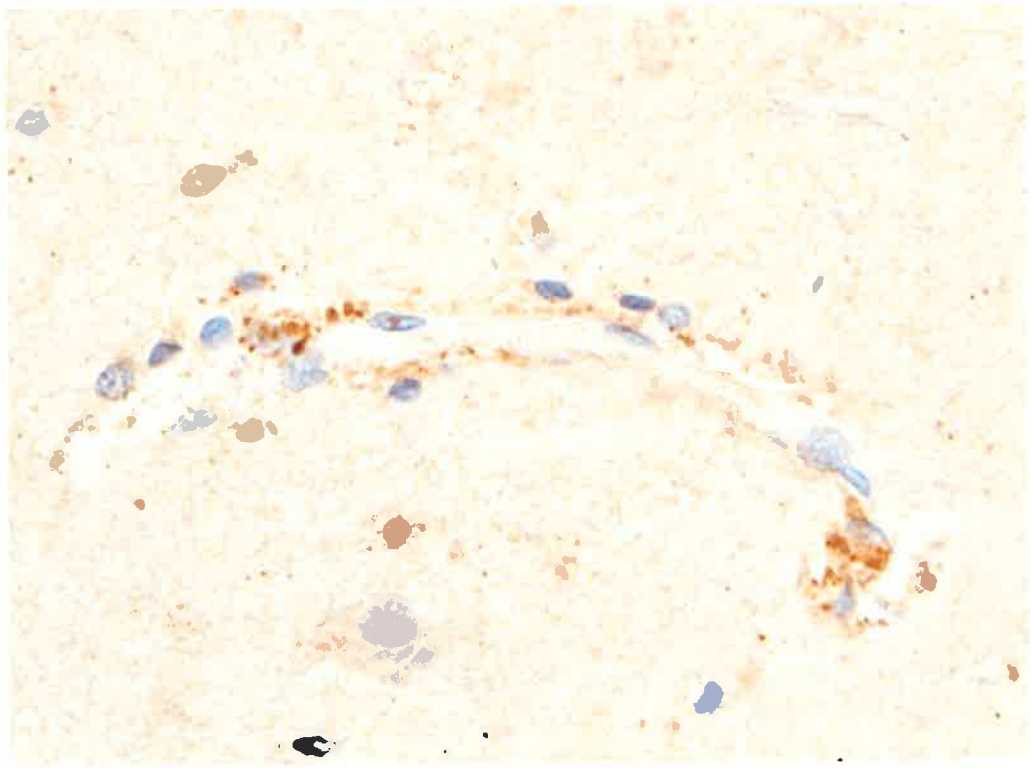


Figure 27.2 SPperivascular profile in a 21 year old with an acute subdural haematoma who died within hours.(x400)

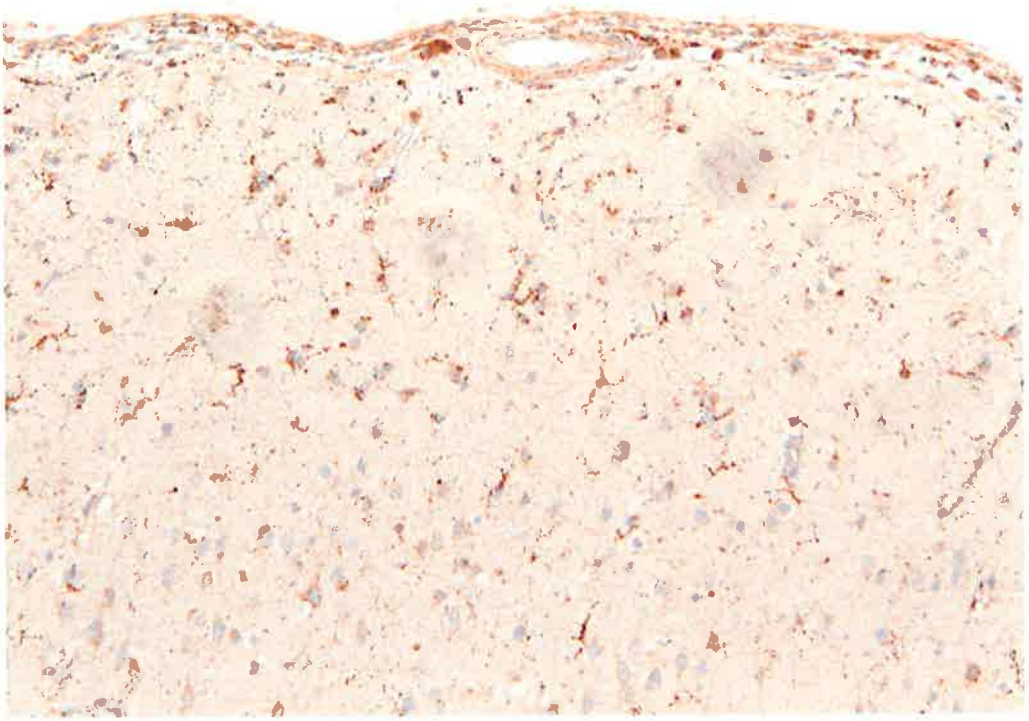


Figure 28.1 Prominent SP immunostaining in the cortex of a 23 year old with cerebral contusion who survived 6days.(x40)

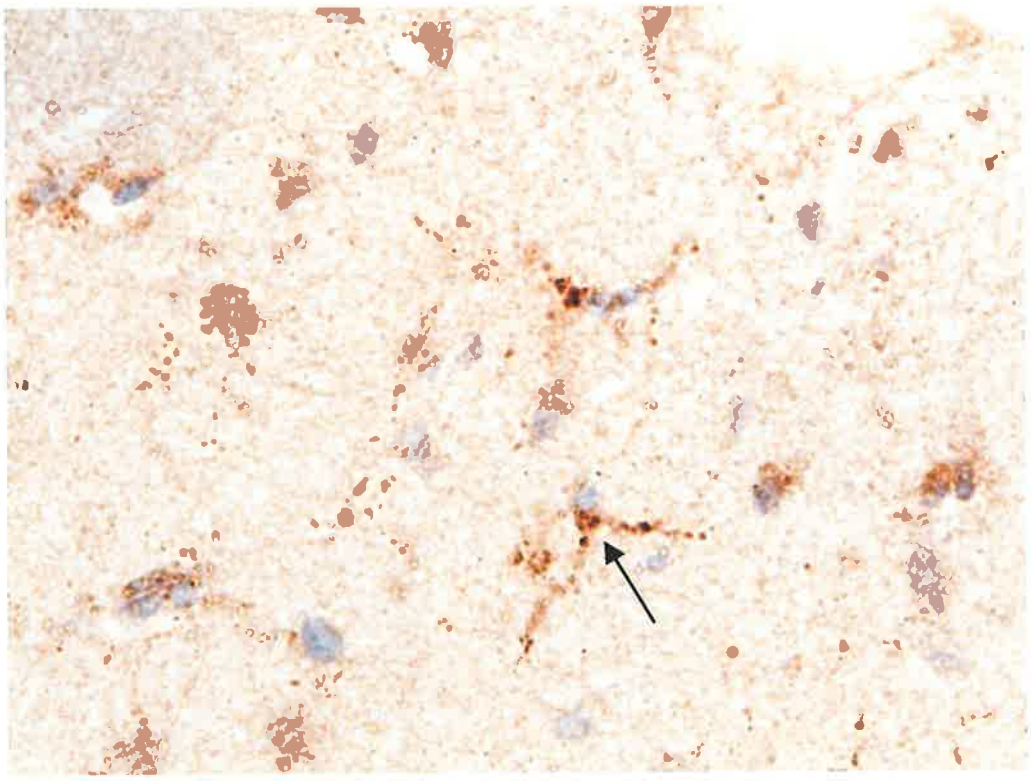


Figure 28.2 At higher power the more distinct morphology of the astrocyte is apparent (←).(x400)

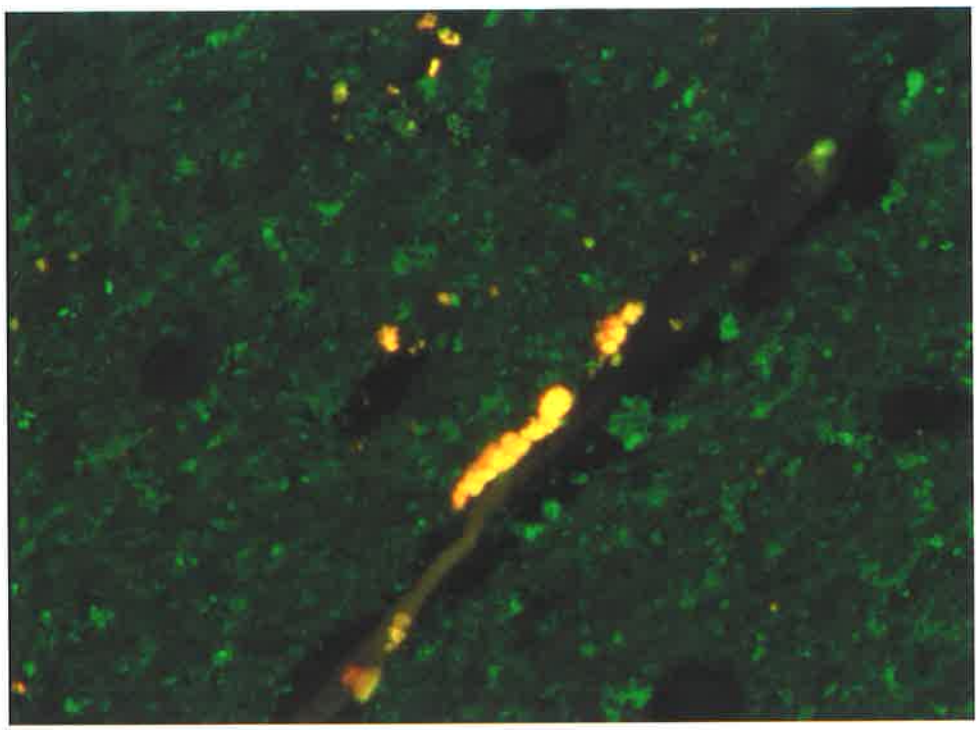


Figure 29.1 Confocal image of a cortical venule in 25 year old, double labelled for PGP 9.5 (green) and SP (red), showing intense perivascular colocalisation (yellow).(x600)

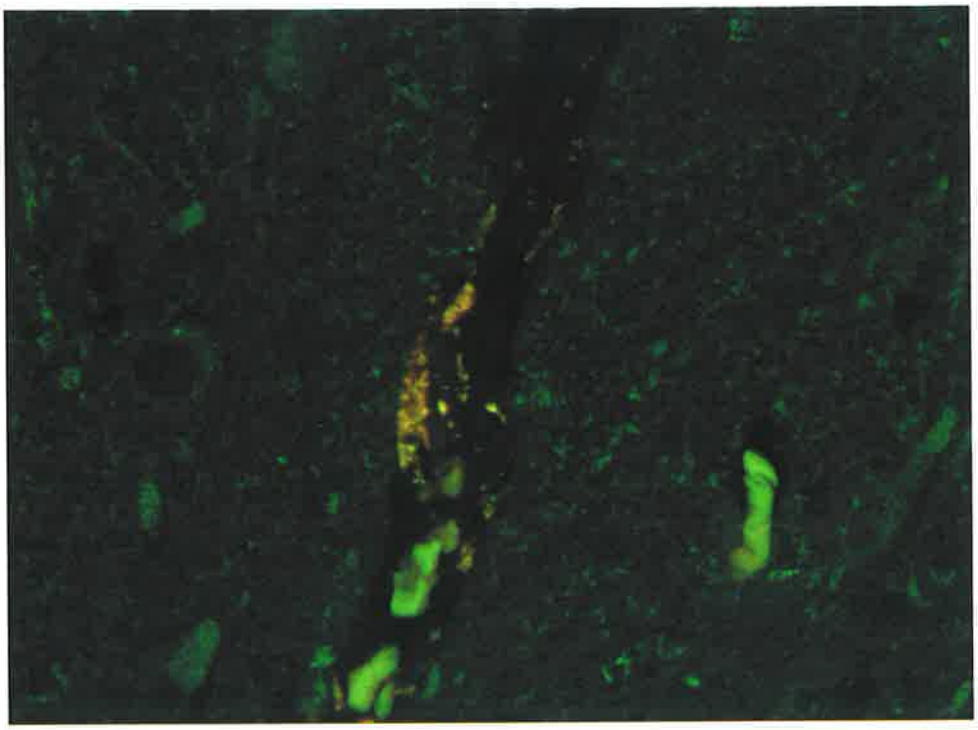


Figure 29.1 Confocal image of a cortical venule in 2 year old, double labelled for PGP 9.5 (green) and SP (red), showing perivascular colocalisation (yellow).(x600).

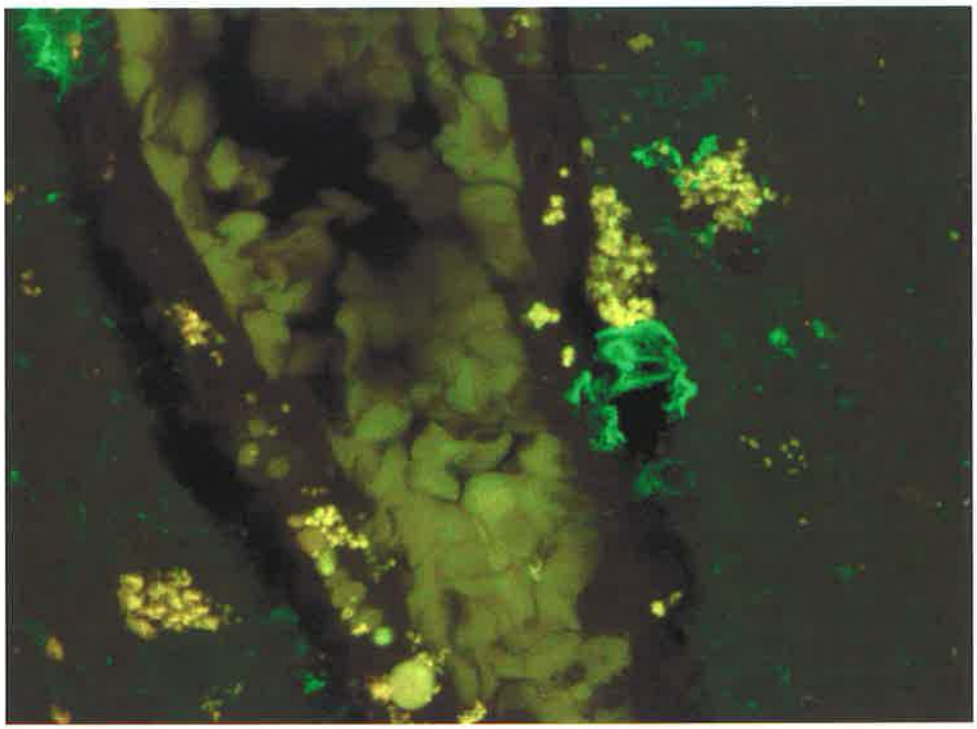


Figure 30.1. Confocal image of cortical vessel of 52 year old with acute subdural haematoma at 7 days, double labelled with PGP 9.5(green) and SP(red). The perivascular end nerve fibres and the SP granules in pyramidal neurones (P) colocalise (yellow).(x600).

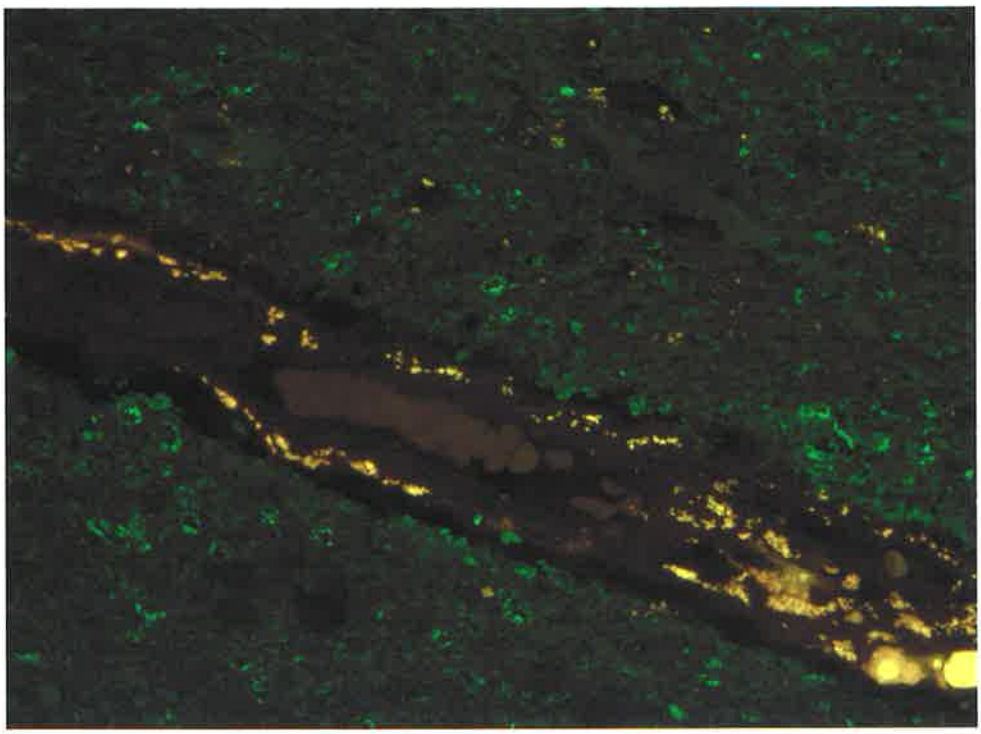


Figure 30.2.Confocal image of cortical venule of a 13 year old with diffuse cerebral oedema at 2 days, double labelled with PGP 9.5 (green) and SP(red) showing intense perivascular colocalisation (yellow).(x600).

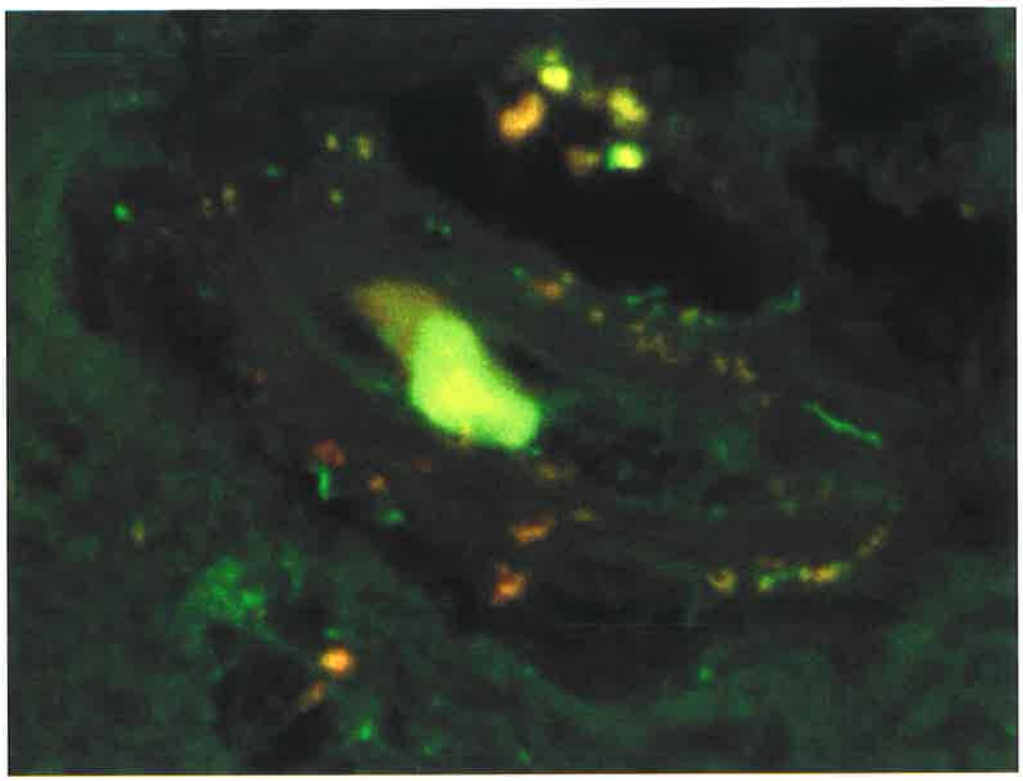


Figure 31.1. Confocal image of cortical arteriole in 41 year old with acute subdural haematoma at 1 day, double labelled with APP(green) and SP(red), showing APP positive perivascular nerve fibres (yellow).(x600)

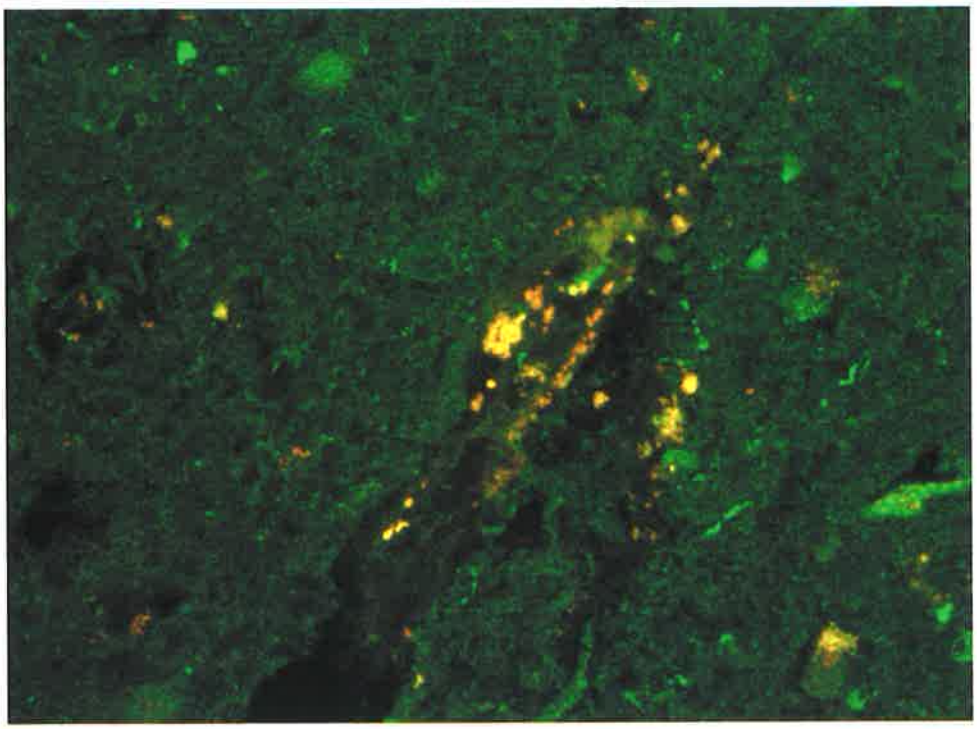


Figure 31.2. Confocal image of cortical venule in 13 year old with difuse cerebral oedema acute at 2 days, double labelled with APP(green) and SP(red), showing APP positive perivascular nerve fibres (yellow).(x600)

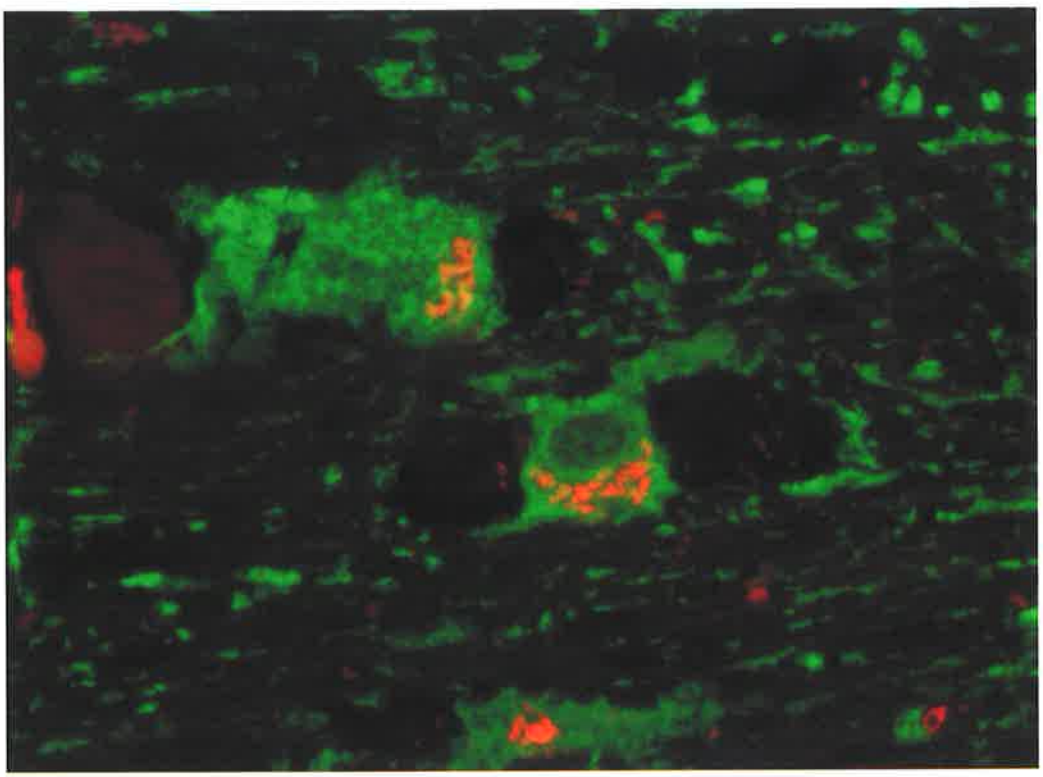


Figure 32.1. Confocal image of cortical astrocyte double labelled with GFAP(green) and SP(red) in 48 year old man (control).(x600)

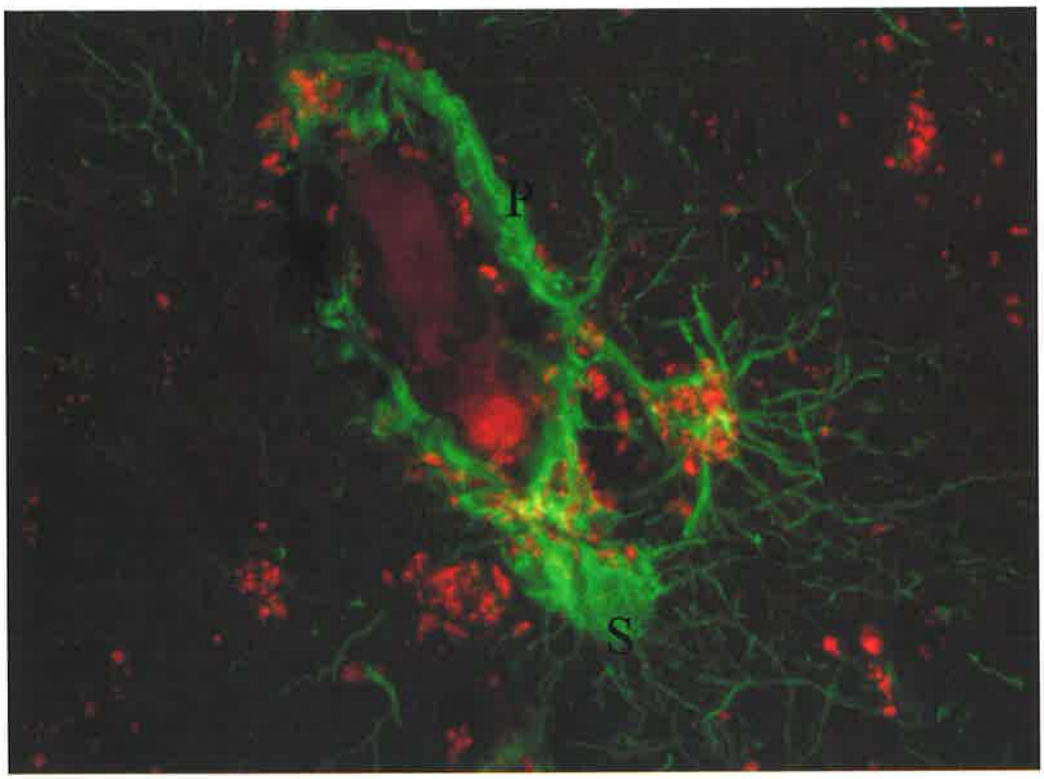
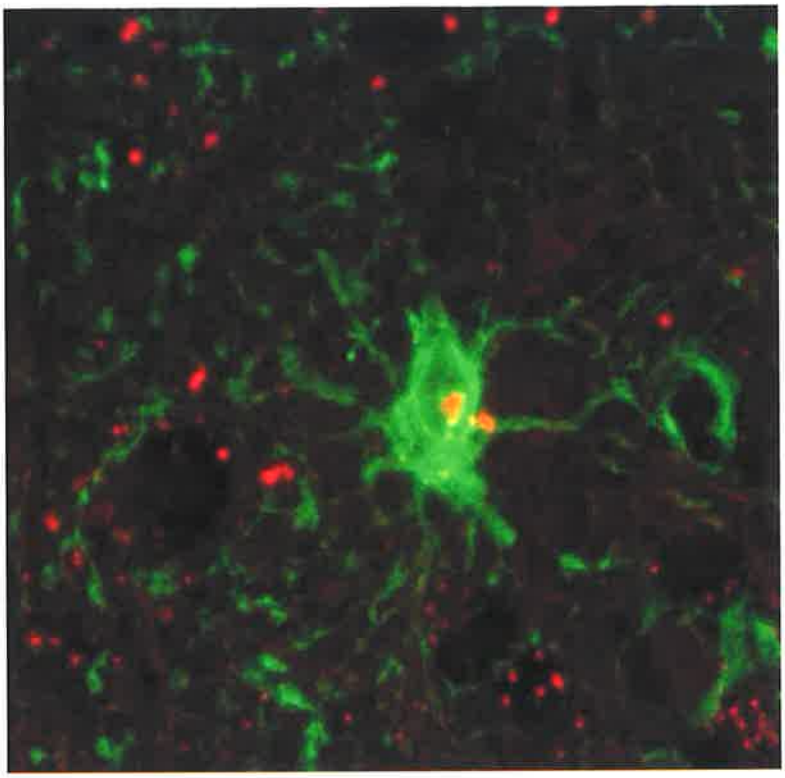
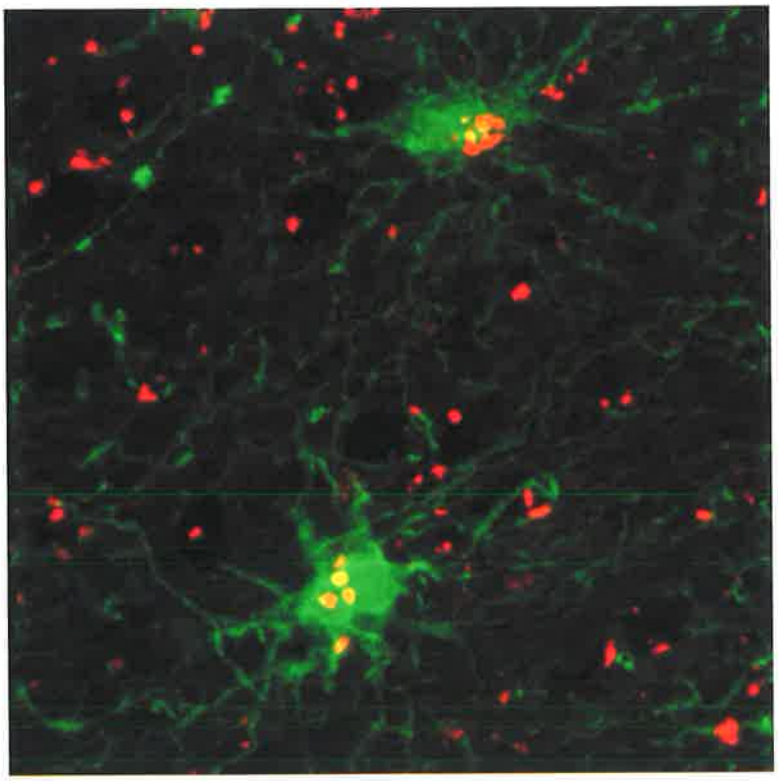
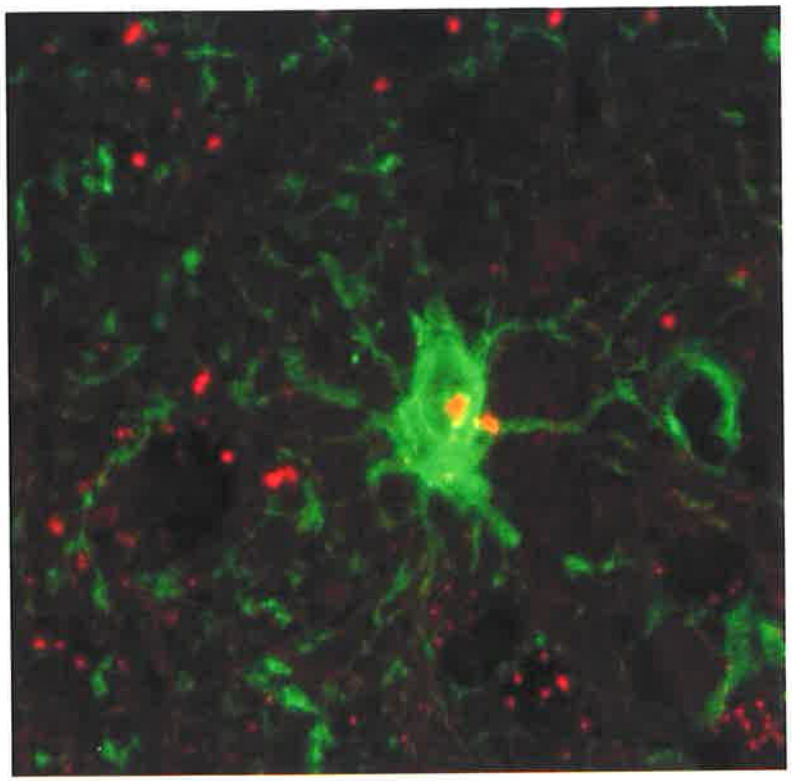


Figure 33.1.Confocal image of cortical astrocyte in 41 year old with acute subdural haematoma at 1day, double labelled for GFAP(green) and SP(red). Compared to figure the astrocyte soma (S) is enlarged and perivascular processes (P) prominent.(x600)

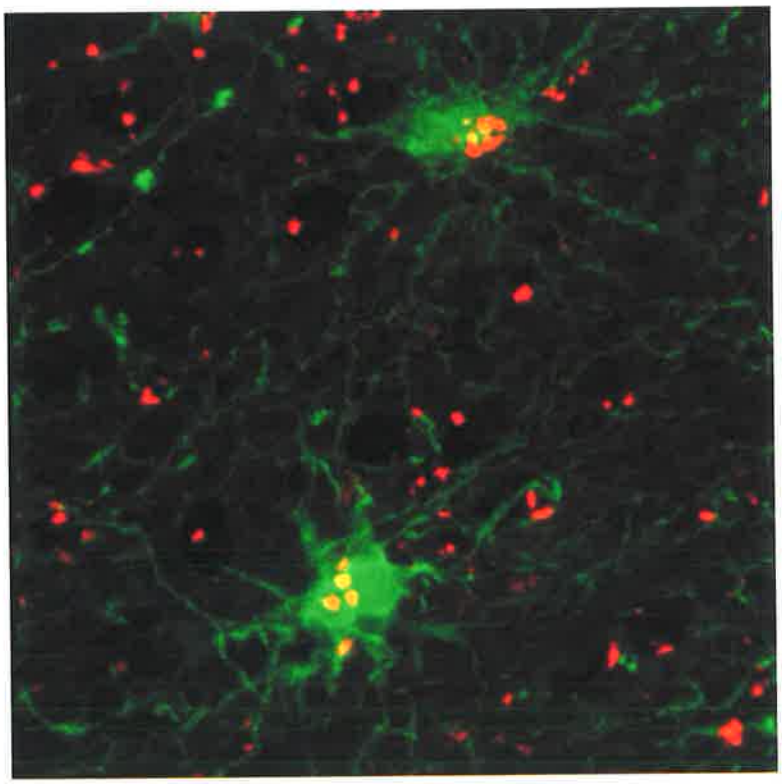


Figures 33.2 and 33.3. Confocal images of cortical astrocytes in 52 year old with acute subdural haematoma at 7 days, double labelled with GFAP(green) and SP(red). Some colocalisation is evident (orange) on the soma.(x600)





Figures 33.2 and 33.3. Confocal images of cortical astrocytes in 52 year old with acute subdural haematoma at 7 days, double labelled with GFAP(green) and SP(red). Some colocalisation is evident (orange) on the soma.(x600)



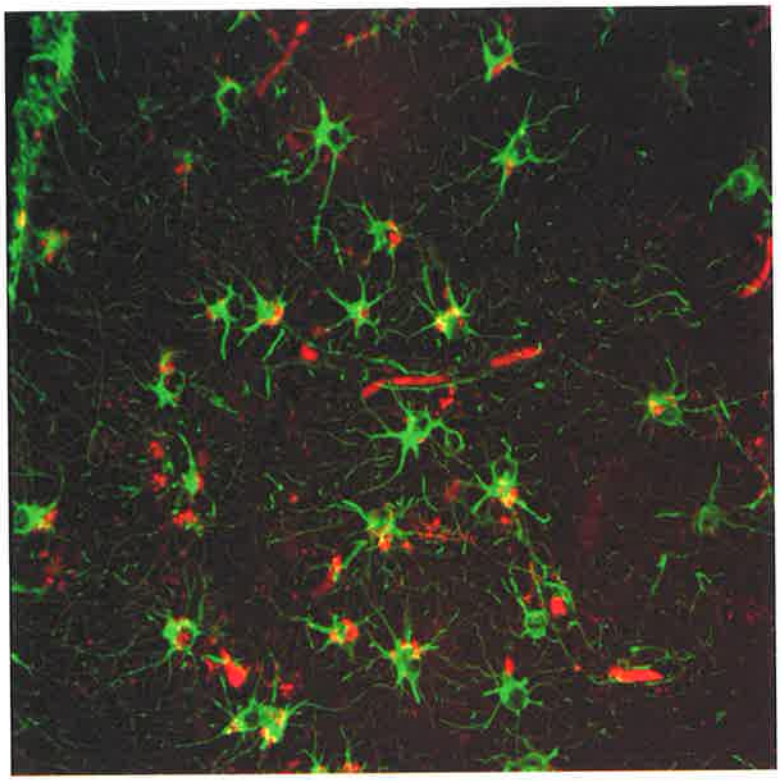


Figure 33.4. Confocal image of cortex of 23 year old with bilateral cerebral contusions at 6 days, double labelled with GFAP(green) and SP(red) showing intense astrocytosis.(x100)

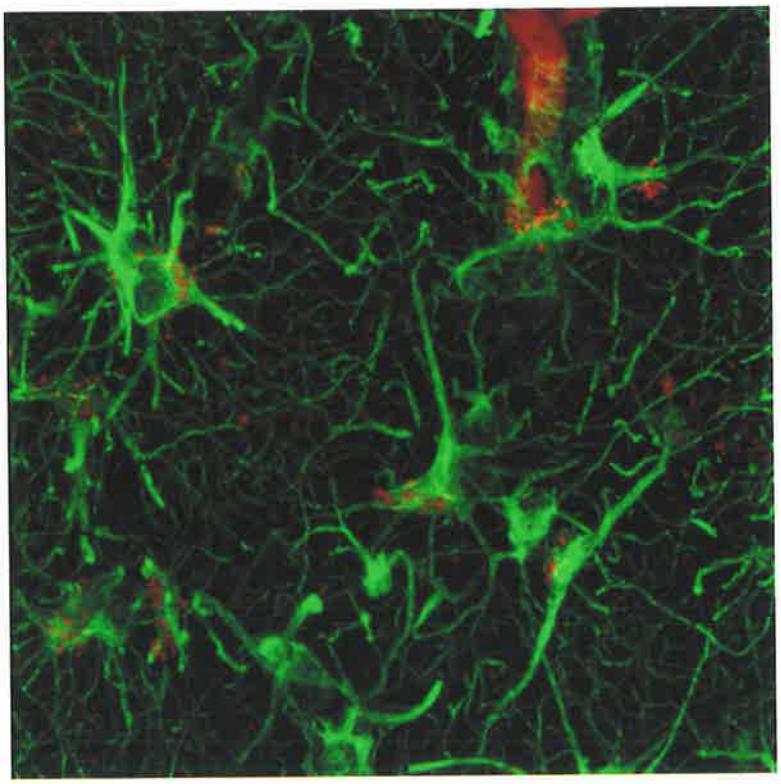


Figure 33.5.Confocal image of above case showing substance P(red) localised to the soma of astrocytes.(x600)

CHAPTER 4. DISCUSSION

4.1 Substance P Neuroanatomy.

4.1.1 History
4.1.2. Rodent CNS
4.1.2.1 Perivascular distribution
4.1.2.2 Cerebral cortical neurones
4.1.2.3 Caudal medulla
4.1.2.4 Astrocytes
4.1.3. Human CNS.
4.1.3.1 Perivascular distribution
4.2.3.2 Cerebral cortical pyramidal cells
4.2.3.3. Caudal medulla
4.2.3.4 Astrocytes

4.2 Substance P immunoreactivity following TBI.

4.2.1 Perivascular axons
4.2.1.1 Rodent
4.2.1.2 Human
4.2.2 Cerebral cortical neurones.
4.2.2.1 Rodent
4.2.2.2 Human
4.2.3 Astrocytes.
4.2.3.1 Rodent
4.2.3.2 Human
4.2.4. Nucleus tractus caudalis.
4.2.4.1 Rodent.
4.2.4.2 Human

4.3 The Neuropathology of traumatic brain injury.

4.3.1. Introduction
4.3.2. Rodent model neuropathological features
4.3.3. Comparison with human traumatic brain injury

4.4 Study limitations

4.4.1 Technical and tissue limitations.
4.4.1.1 Rodent
4.4.1.2 Human
4.4.2 Qualitative data
4.4.3 Controls, shams and variability
4.4.4 Rodent model validity

4.5 Conclusions

4.1 SP Neuroanatomy.

4.1.1 History.

Although comprehensive studies of the immunohistochemistry of SP in the central nervous system date back as early as 1975 [79, 116], there has been, more recently, a decline in interest in the detailed anatomical distribution of SP, with attention now focussed on its receptor [117]. Pardoxically this has occurred inspite of many gaps which exist in our knowledge and the availability of current technologies available for addressing these deficiencies, presenting real barriers to the forward progress and understanding of potentially clinically relevant therapies e.g.NK1 antagonists. In this thesis the immunoreactivity of SP at the peripheral and central ends of the trigeminovascular system has been studied following TBI to gain a greater understanding of the potential mechanism of action of this neuropeptide in the pathogenesis of traumatic brain injury.

4.1.2. Rodent CNS

4.1.2.1. Perivascular distribution. Although, a large number of papers have specifically characterised the existence, density, depletion and pathways of perivascular SP fibres on cerebral arteries and venous sinuses in rats and humans, little, if any work has been done on the cerebral microvasculature, the vessel of end organ perfusion, integral to the blood brain barrier and the ultimate site of CNS vascular pathology.

In this study perivascular SP immunoreactive axons, some arrayed in "end plate" like morphology and seen best in axial sections of smaller vessels and others in continuous linear profile and seen best on longitudinally cut larger venules were identified. Importantly, these profiles were more commonly identified on post capillary thin walled venules within a short distance from the endothelium. These perivascular profiles in control rodents were typically discrete.

The proximity of SP containing end terminals to endothelium suggests the possibility of an active role of this vasoactive neuropeptide on the cerebral endothelium at both the arterial, which has been better studied experimentally and venular end. Endothelium from non cerebral vasculature [110], leucocytes and platelets [170] have been shown to possess NK1 receptors and rodent brain endothelium has been shown to be able to produce SP in experimental conditions [112]. Our findings, in light of the commonly reported location of SP fibres outside the cerebral arterial muscular layer, which we also observed suggest the possibility of SP action of post capillary cerebrovenular endothelium, the locus of tachykinin induced increased vascular permeability in other vascular beds.

In this study SP could not be definitively detected in the endothelium of the rat cortical microvasculature of control animals. This observation supports the idea that the neuropeptide is chiefly stored and released from

perivascular nerves. Our findings however do not discount the possibility that small amounts of diffuse SP may bind to the endothelium

In addition some membranous binding of SP to platelets was observed in the noninjured as well as injured animals. It is well established that platelets possess membrane bound NK1 receptors [170] and further hypothesised that their tachykinin mediated release of NO and other vasoactive compounds may be important in increased vascular permeability in non CNS vascular beds [81].

Unfortunately the findings of this study were unable to verify the source of the perivascular SP axons. This has been assumed in the literature to be primary unmyelinated afferents from the trigeminal ganglion, as part of the trigeminovascular system. In support of this concept, their unmyelinated ultrastructure, observed using EM would suggest these nerves to be primary sensory afferent fibres.

4.1.2.2 Cortical neurones. Neurones positive for SP and containing PPTmRNA have been described in the rodent neocortex however their connections are generally poorly understood. Combining the data from confocal and light microscopy, this study confirmed variable but low intensity SP perikaryonal labelling of PGP 9.5 positive neocortical predominantly non pyramidal neurones in control animals. Although their processes could be occasionally identified as immunopositive, no

connections with blood vessels or other neurones could be easily identified using our methodology.

In comparison, Kanecko et al [271, 272], reported an extensive network of SP receptor positive neurones (all non pyramidal and all capable of tachykinin synthesis) in the rodent cortex in all layers except layer 1 and noted that 98% of such neurones also had GABA immunoreactivity. In addition pyramidal neurones have been identified as possessing the NK3 receptor [273]. These complementary findings suggest, as reviewed by Stumm et al [247], that SP may modulate the communication between GABA cortical neurons (inhibitory) or, acting via NK3 receptors, may activate glutaminergic cortical pyramidal neurones. Further supportive evidence for this concept comes from SP antagonist and PPT-A (preprotachykinin-A gene) deficient mice studies which have demonstrated SP to be important in the initiation and maintenance of status epilepticus.

4.1.2.3 Caudal medulla. Like many previous investigators, we could readily localise SP within presynaptic axonal boutons of the trigeminal nucleus caudalis in the rodent caudal medulla, exhibiting characteristic fibrils with intensely staining varicosities in the substantia gelatinosa or lamina 2 [79, 114, 210]. SP immunostaining of neuronal bodies within the nucleus was sparse in control animals. This distribution of SP immunostaining, complementary with that reported in the literature for

the NK1 receptor [133], appears morphologically to be homologous with SP afferent fibres entering the spinal dorsal representing the central processes and endings of primary sensory afferents, as suggested by prior investigators [133, 210].

4.1.2.4 Astrocytes. Only weak SP labelling of cortical astrocytes could be demonstrated in the control rodent and no colocalisation with GFAP. Although the existence of NK1 receptors on rodent cortical astrocytes has been demonstrated, our findings are similar to those of other investigators who have found no immunolabelling of normal astrocytes in animals [154, 247].

4.1.3. Human CNS.

4.1.3.1 Perivascular distribution. Ironically, Hokfelt et al [130] originally described "substance P containing nerve endings" in human cerebral cortex but did not identify them as perivascular fibres and wrote that " the functional significance of the results is unclear". Since then and despite the enormous work devoted to characterising the putative role of neuropeptides on the cerebral circulation, the perivascular SP anatomy of the cerebral microvasculature has been largely inferred from studies of cerebral arteries.

In this study an extensive perivascular distribution of SP nerve endings could be demonstrated in the cerebral microvasculature of controls cases in all ages (2-86 years). Although our study was not quantitative, there

did not appear to be an obvious qualitative difference in the perivascular microvascular SP, although a gradual age related decline of SP in the proximal middle cerebral artery as measured by radioimmunoassay has been noted [144].

Similar distributions and morphologies to the rodent were noted in the human tissue, and although cortical arterioles were more prominent in human tissue compared to the rodent, post capillary venules showed more SP profiles and these were closer to the endothelium. A denser innervation of cortical rather than white matter vessels was noted and this might reflect the disparity of cerebral blood flow of grey to white matter [274].

Although we did not perform EM examination of human material, SP could not be identified in human endothelial cells of the microvasculature in this study, despite its recent demonstration in the endothelium of human middle cerebral artery [149]. This difference in SP localisation, if real, may reflect an important difference in neuropeptide function in different calibre cerebral vessels. For example, SP has been shown to relax precontracted cerebral arteries and arterioles in vitro [142, 144-146, 219] and to increase cerebral blood flow via trigeminal ganglion stimulation in animals in vivo [198, 199], putatively via NK receptors on vascular smooth muscle. In contrast, other animal studies have noted ultrastructural changes in dural post capillary venules associated with

oedema following stimulation of the trigeminal ganglion, presumably related to release and action of SP at this level [177, 179].

4.1.3.2.Cerebral cortical pyramidal cells. Prominent SP staining of human cerebral cortical pyramidal cells and to a lesser extent smaller multipolar and bipolar neurones, as shown in this study, has been noted in the older literature but not emphasised [120-123]. This may be partially explained by the current tendency toward quantitative SP and NK1 receptor immunochemistry. Such studies, employing quantitative immunoreactivity [119] have suggested, like our observations, that SP immunoreactivity is comparable in frontal, temporal and hippocampal cortices. In addition NK1 receptors have been shown to be widespread in the human cortex [118], which again supports the existence of an extensive intrinsic circuit in humans. Although some variability in the intensity of pyramidal neurone immunolabelling was evident in our control cohort, it would seem reasonable to speculate that there is a potential role for SP in modulating glutaminergic pyramidal neurones in humans.

4.1.3.3 Caudal medulla. As noted by previous reviewers, a remarkable degree of similarity existed between the anatomical distribution of SP of the rodent and human brainstem in the STT [134]. Rostral to the closure of the 4th ventricle, the tract assumed a focal circular morphology, before assuming the characteristic crescentic pattern which blended into the

dorsal horns of the upper cervical cord. Although no significant differences in the immunostaining pattern of the STT were noted within the younger age groups, the impression was that the tract fibre density was less in some older patients.

4.1.3.4 Astrocytes. Human perivascular astrocytes in control cases exhibited weak SP immunoreactivity but no colocalisation with GFAP, as opposed to reactive astrocytes which exhibited more intense immunoreactivity and occasional colocalisation. The most likely interpretation of this finding, given the abundance of NK1 receptors on astrocytes, would be SP binding to membrane bound receptors. Further colocalisation studies involving triple labelling of GFAP, SP and NK1 receptors would be needed to definitively confirm this interpretation. Given the integral role of astrocytes in inflammation, the perivascular location of substance P staining astrocytes in humans suggests the possibility of a role in inflammatory conditions around the blood brain barrier, supporting data from further human studies showing SP astrocytes around multiple sclerosis plaques [156]. Furthermore, the binding of SP to astrocytes of normal morphology in patients without neurological disease may suggest an important homeostatic role including neuropeptide deactivation, autosignalling and maintenance of the blood brain barrier.

4.2 SP immunoreactivity following traumatic brain injury.

4.2.1. Perivascular axons.

4.2.1.1 Rodent. The chief new finding of this study was an apparent early increase in the number of cortical perivascular SP immunoreactive profiles following trauma that was seen in both rodent TBI models. In the impact acceleration model, electron microscopy showed an increase of perivascular immunolabelled unmyelinated axons, while confocal microscopy confirmed that the perivascular SP immunopositive profiles were axons. In addition, we observed a more diffuse pattern of SP immunoreactivity, seen particularly at 30 minutes using EM and 5hr using LM, compared to the discrete pattern of SP immunopositive granules seen in the control cases.

Both these findings could be interpreted to represent the early release of neuropeptide from its compact perivascular nerve terminals. This interpretation is consistent with previous rodent neuropeptide studies which suggest release of neuropeptides from a reservoir stored in end terminals, of which only a fraction is released following noxious stimuli. Further support for this early release comes from experimental studies of subarachnoid haemorrhage in rodents, which were characterised by a marked reduction of measurable SP (due to release) from the basilar artery, with a later upregulation of intraganglionic PPTmRNA, maximal at 2 days.

An additional explanation, suggested by the colocalisation of SP with some APP positive axons may be alterations of axonal SP transport following TBI, leading to an apparent increase in immunolabelled axons. For example nerve crush experiments have demonstrated an accumulation of SP proximal to lesions [93, 191].

Direct comparison of our experimental results with anything in the existing literature is limited because of the poor characterisation of perivascular SP axons of the cerebral microvasculature, a focus on nociceptive neuronal responses and the trend toward quantitative methods including in situ hybridisation of mRNA and antagonist studies. However, our results are complementary to and in agreement with results obtained from rodent models of cerebral ischaemia. Stumm et al [247] reported an induction of NK1 receptors on activated cerebrovenular endothelium, intraluminal leucocytes and paravascular cells following rodent middle cerebral artery occlusion maximal at 2 days. In addition they described some vessels in the area of infarct with increased SP fibres and terminals also at 2 days, which were not seen in control or sham animals. In contrast to the immediate release of neuropeptide, these findings would appear to support a role of SP in coordinating a cerebrovascular inflammatory response.

4.2.1.2. Human. Observations from the human material generally parallelled those observed in the rodent, although there was more

heterogeneity within the control and trauma cases. Explanations for this may include the difficulty of obtaining appropriate human controls (patients usually had a past history of epilepsy, which may have caused neuropeptide release) and the heterogeneity of pathological lesions and their evolution, even within the same tissue sample. In addition a reduction in the number of SP immunoreactive profiles could be observed after 3 days and particularly in the presence of evidence of tissue ischaemia and /or oedema. We also noted the regular presence of SP positive mononuclear cells, which were located within the lumen, on the endothelium and in the perivascular spaces in the human trauma material, which were not present in controls. These cells, particularly in the perivascular spaces were generally more prominent in the subacute phase and as the perivascular granules were reduced.

Overall, our human results support an early increase and subsequent reduction of perivascular SP profiles with time, consistent with neuropeptide release, especially in the presence of ongoing ischaemia or oedema. These observations corroborate the only human physiological data recording SP release measured in peripheral blood following clinical cerebral ischaemia. Bruno et al [248] showed serum SP levels were highest 12 hours following the clinical onset of cerebral ischaemia compared to 24-24 hours, which were both significantly higher than control values. As ischaemic brain injury commonly occurs early in the

course of human traumatic brain injury, similar physiological responses may well be present.

4.2.2 Cerebral cortical neurones.

4.2.2.1. Rodent. Our chief finding was that of increased granularity of SP within neurones (pyramidal and non pyramidal) following traumatic brain injury in both rodent models. Importantly SP immunoreactivity increased in cerebral cortical neurones proportionate to the degree of secondary injury. This was seen best adjacent to areas of cerebral cortical infarction or severe contusion, but also in lesser degrees underneath mild contusions and subdural haematoma, where red cell change and oedema could be identified. In addition APP positive neurones, indicative of injury, expressed increased SP immunoreactivity.

Our findings concur again with those of Stumm et al [247], who noted a marked increase in the SP immunoreactivity of lamina II,III and V pyramidal and to a lesser extent non pyramidal neurones in ischaemic tissue secondary to middle cerebral artery occlusion which was maximal at 48hours compared to sham animals. This was also associated with an increase in the PPTmRNA expression of cortical neurones from lamina II-VI, both GABA interneurons and glutaminergic pyramidal cells, indicating genomic upregulation of SP production in association with experimentally induced ischaemia.

These findings suggest upregulation of neuropeptide formation in cortical (especially pyramidal) neurones following ischaemic and traumatic brain injury characterised by ischaemia. In this setting SP, upregulated in pyramidal neurones, may enhance glutamate release and contribute to the effects of post synaptic glutamate excitation including seizures and even neurotoxicity. Similar proconvulsive effects have been demonstrated experimentally in rodents injected with kainic acid, which upregulates SP at the genomic level in rodent pyramidal hippocampal neurones [277] and which can be abolished in SP gene deficient mice and with SP antagonists [132]. Further SP itself has been directly shown to markedly increase glutamate release from hippocampal sections, initiate and maintain status epilepticus. These pathophysiological sequelae were correlated with a dramatic increase in both SP immunoreactivity and PPT-A mRNA [131]. Therapeutically SP antagonists have been shown in experimental rodent stroke models to reduce rodent stroke volume and improve neurological outcome by Yu et al [85]. Taken together with our results the possibility that SP upregulation in pyramidal neurones could have a role in glutamate induced neurotoxicity following experimental ischaemic brain injury seems a reasonable hypothesis.

4.2.2.2.Human.

Similar to the rodent, human pyramidal cerebral cortical neurones showed increased SP immunoreactivity following trauma, which was most

evident at least 24 hours following injury and persisted for up to 7 days. The main exception to this observation was in those neurones severely affected by ischaemia or surrounded by oedema, in which SP immunoreactivity was diminished at later time points. Our findings suggest that SP is upregulated following human TBI and released following ischaemia in cerebral cortical pyramidal neurones, however in situ hybridisation studies will be required to quantitate these changes. Therapeutically in spite of the failure of neuroprotective drugs targeting glutamate toxicity in human clinical trials, glutamate release has been well characterised in animal models of traumatic brain injury and human head injury patients [56, 63], particularly following ischaemic injury. While our findings offer new promise of earlier intervention, at this point a role for SP antagonists in attentuating glutamate toxicity following traumatic brain injury in humans must remain speculative.

4.2.3 Astrocytes.

4.2.3.1 Rodent. The presence of NK1 receptors on astrocytes, their capacity to synthesise SP and two reports in the animal literature [153, 154] prompted us to test the hypothesis that SP may be important in regulating the glial response to injury in experimental and human TBI. No colocalisation of SP and GFAP could be seen in any of the rodent cases examined up to 7 days. The absence of increased SP staining of astrocytes in the diffuse impact model may reflect the less severe type of

injury particularly the absence of histologically demonstrable ischaemia and paucity of axonal injury and the fact that we did not examine animals beyond 7 days. In support of these results Stumm et al [247] showed no upregulation of the NK1 receptor on activated cortical astrocytes and microglia up to 7 days following rodent middle cerebral artery occlusion. Lin et al [154] were able to demonstrate SP immunolabelling of activated hippocampal astrocytes only at 2 weeks in a gerbil carotid occlusion model of injury, while Mantyh et al [153] described high concentrations of SP binding 99 days following optic nerve section in rabbits.

4.2.3.2 Human. Unlike the rodent some human cases were associated with increased SP immunoreactivity of astrocytes, but in all but one case colocalisation of SP and GFAP could not be shown, supporting the interpretation that this pattern of SP immunolabelling at LM represents surface membrane labelling. Colocalisation was seen in the cerebral cortex of a 52 year old man 7 days following removal of a subdural haematoma.

The presence of SP astrocytic binding has not been previously described in reactive astrocytes following traumatic brain injury in humans, although SP immunolabelling has been shown in normal human infants [155] and around multiple sclerosis plaques [156].The marked astrocytosis associated with SP immunolabelling seen in a number of our human cases suggests a potential role of SP in post traumatic gliosis.

Kennedy et al [163] showed that a SP antagonist produced a marked reduction in astrocytosis and inflammatory cells, measured by the number of GFAP labelled astrocytes and inflammatory cells (lymphocytes, plasma cells and macrophages) in a mouse model of subcuratively treated African trypanosomiasis, which produces a prominent meningoencephalitis. Whether such immune hyperreactivity, as postulated to be important in this model is important in human head injury is not yet clear but the effects of SP antagonists on astrocytosis and inflammatory responses could be tested in experimental TBI.

The source of SP bound to astrocytes is not clear.

In light of these observations, speculation on the origin of the astrocytic SP is possible. The lack of colocalisation of SP and GFAP in the human cases and more prominent binding in the cortex, particularly around vessels does not support endogenous synthesis by astrocytes. The most likely source would seem to be from perivascular nerves or cortical neurones at least in the time points we observed (up to 1 week). Against this argument would be the lack of NK1 receptor upregulation for up to a week in animal studies, which would imply no increase in SP binding, at least by this mechanism. These factors may change at longer time points after injury and further studies on NK1 receptors and SP in injured human white matter are needed. It has been suggested by one group [153] that circulating inflammatory cells may be an important source of SP.

4.2.4 Nucleus tractus caudalis.

4.2.4.1. Rodent. As a large amount of the seminal work on neuropeptide release concentrated on responses in the substantia gelatinosa of the dorsal horn and later the nucleus tractus caudalis as the central ending of capsaicin sensitive primary afferent nerves, we expected this site might yield important insights into the responses of SP containing neurones following traumatic brain injury.

The rodent diffuse impact model did however show an impressive increase in SP immunoreactivity 5 hours following impact, both diffuse and neuronal, consistent with either intrinsic synthesis or more likely terminal release of SP and subsequent neuronal binding. Mantyh et al [278]and Abbadie et al [279] showed separately that NK1 receptors located on central neurones adjacent to end terminals internalised following remote noxious stimuli and that the severity of stimuli determined how many neurones internalised their receptors i.e. 3 to 5 times more neurones could be activated due to release of SP in response to more potent or more frequent stimuli. In the diffuse impact rodent model the presence of significant axonal injury in the adjacent pyramidal decussation injury was good evidence of a significant mechanical injury, that presumably could release neuropeptides.

4.2.4.2 Human. Probably the most significant negative finding of this study was that few specific changes could be identified in the human

nucleus caudalis following injury. Changes were not observed inspite of a large patient cohort, wide age spectrum and varying levels of injury. A number of reasons could be important including the brief time courses of these experimentally induced dynamic processes (a response within 5 minutes and complete normalisation within 60min), the difficulty of obtaining appropriate controls and our use of immunochemistry of the peptide rather than the receptor.

Taken together with the other findings of this study, however, these observations strongly suggest that in human traumatic brain injury, SP is released from its peripheral terminal ends. This finding is congruous with previous data demonstrating that 4 times as much neuropeptide is transported peripherally than centrally [93] and the preservation of SP mediated axonal cerebrovascular reflexes on sectioning of the trigeminal root, but not with ganglionectomy [216].

4.3 The Neuropathology of traumatic brain injury.

4.3.1 Introduction.

One of the chief methodological aims of this study was to allow comparison between the results obtained from experimental traumatic brain injury and human post mortem material. We chose two well characterised rodent models of traumatic brain injury, which possessed a number of desirable features for the purposes of this study. These included the presence of focal and diffuse injury, prior characterisation of the opening of the blood brain barrier and oedema formation (reported histologically and using MRI techniques) [37, 39, 40, 44, 45, 265, 266]. Finally NK1 antagonists have recently been reported to significantly reduce post traumatic cerebral oedema and to improve functional outcome by improving blood brain barrier permeability [86] in the rodent diffuse impact acceleration model.

4.3.2 Rodent model neuropathological features.

The impact acceleration model used in this study produced a mortality of approximatey 15%. A thin layer of parasagittal subarachnoid haemorrhage was the most consistent pathological finding, then basal subarachnoid haemorrhage with intraventricular haemorrhage occurring at 5hr. Cerebral oedema, defined as microvacuolation of the neuropil and neuronal red cell change were not present. However scattered neuronal dark cell change [267, 268], was observed in the supraventricular

parasagittal cortex under the impact. In parallel, axonal injury was present, but sparse in the corpus callosum and variably seen in the brainstem, most notably in the pyramidal decussation. Clinically these animals had a modest period of apnoa (seconds to minutes).

Our observations differ considerably from those of Foda and Marmarou [27, 28], who reported a 59% mortality from the 2m impact group, more extensive cisternal, intraventricular and brain stem haemorrhage, pink shrunken cortical neurones and pericapillary oedema. In addition a "massive" diffuse axonal injury involving numerous white matter tracts and to a lesser extent the corpus callosum were reported using a 68-kD neurofilament subunit antibody.

A number of reasons for these disparities exist. The animal weight used by the Marmarou group of 350-375gm was less than the 400-500gm weight animals used in this study and weight may effect the animals tolerance to impact. Secondly all animals with fractured skulls were excluded in this study. No resuscitation effort following impact was described in the Marmarou study, despite respiratory failure being acknowledged as the main cause of death. In a smaller study by Kallakuri et al [269], which used animals of similar weight, impact acceleration and aggressive resuscitation as in our study a mortality of 11% was recorded. Therefore it is likely that the animals in our study suffered a significantly reduced hypoxic insult compared to the Marmarou cohort. As it is well

established that hypoxic-ischaemic neurones undergo red cell change and that axonal injury is seen in ischaemic lesions [26], this may be an important reason for reduced amounts of both in our study.

The extent of axonal injury seen in our animals was less than originally reported by Foda et al and a recent study [269] which confirmed a significant number of axonal swellings in the corpus callosum using the Marmarou model. However in both of these studies axonal injury was characterised using neurofilament protein antibody and silver staining respectively and not APP, as used in this study. In contrast to the two above mentioned methods, APP stains only injured axons and background non injured axons are not stained.

Irrespective of these differences, the axonal injury pattern in our rodent impact acceleration cohort could not uniformly be described as an Adam's type 1 diffuse axonal injury involving lesions in the cerebral hemisphere white matter tracts including corpus callosum, brain stem and occasionally cerebellum [19]. Instead, our chief finding was of APP positive perivascular granules, which were prominent in the parasagittal cortex directly under impact and which colocalised with substance P. Perivascular APP immunostaining was not seen in controls and occasionally in shams.

In contrast the fluid percussion model of focal rodent injury produced a much more severe injury including frank subdural haemorrhage and

extensive haemorragic lobar contusion with mass effect, oedema, neuronal red cell change and even discrete regions of cerebral infarction.. In addition these animals had neurological deficits manifested by prolonged apnoea and a right hemiparesis following injury. Although the main neuropathological features were predominantly ipsilateral as described originally by McIntosh et al [41] involvement of the other hemisphere may be explained by variation in the proximity of the craniotomy relative to the sagittal suture, noted by Vink et al [42].
In this model marked ipsilateral corpus callosal axonal injury and sparse hemispheric, cerebellar peduncle and brainstem axonal injury were also evident. In addition APP positive perivascular granules were seen uniformly throughout the cerebral cortex with no apparent difference between injured and uninjured side suggesting a diffuse pattern of perivascular terminal axon injury.

4.3.3 Comparison with human traumatic brain injury.

The fluid percussion model more closely approximated the human pattern of axonal injury in which lesions can be found in the cerebral hemispheres, a significant amount in the corpus callosum with occasional immunopositive axons in the cerebellar peduncles and brainstem in contrast to the impact acceleration model which had sparse lesions in the corpus callosum but none regularly in the hemispheres, cerebellum or brainstem with the exception of the pyramidal decussation. As such the

axonal injury from this rodent model of impact acceleration falls well short of comparable human injury, in which extensive axonal injury has been characterised [24] in addition to our results.

The finding of perivascular APP positive nerve fibres innervating the cerebral microvasculature in the rodent models and most human sections has not been reported in the literature before nor its functional significance investigated. The frequency of this finding in the injured cortex, indeed more common than other white matter tract axons e.g.corpus callosum suggests that, at least in the impact acceleration model of injury, these smaller axons are much more sensitive to injury. Such injury could potentially contribute to a form of vascular "deafferentation", which might be important in the disturbance of autoregulation of the cerebral microvasculature following trauma. Finally mechanical injury to SP containing umyelinated axons, may be an important mechanism of post traumatic neuropeptide release.

The prevalence of secondary injury was less in the rodent models compared to the human cohort, in which neuronal red cell change and oedema were common, consistent with findings in the literature which have demonstrated ischaemic-hypoxic changes in up to 90% of patients with severe head injury who survive long enough to reach hospital [29].

In summary mild predominantly focal axonal injury was the main pathological feature of the impact acceleration model and predominantly

focal axonal injury, focal haemorrhagic contusion, ischaemia and oedema were the main features of the fluid percussion model in this study. Extrapolation to human traumatic brain injury is therefore more limited in the rodent impact acceleration model used in this study. Furthermore, the disparity in histological injury between that reported by Foda and Marmarou and the results of this study, suggest the need for caution in comparing our findings with the results of numerous studies that have used this model to derive important insights into the pathophysiology of traumatic brain injury. Such disparities have rarely been critically addressed.

In contrast the new finding of diffuse injured perivascular axons which colocalise with SP in both the rodent models and human cohort suggests that their injury may be ubiquitous in both types of traumatic brain injury.

4.4 Study limitations.

A number of important limitations need to be considered in the interpretation of the findings of this work.

4.4.1. Technical and tissue limitations.

4.4.1.1 Rodent. Although SP is well preserved in human and animal post mortem tissue following formalin fixation, ultrastructural assessment was limited by the lack of fixation with glutaraldehyde and autolysis due to the post mortem interval. While all tissues were cut and stained simultaneously, the degree of tissue preservation may have been an important determinant in the number of immunolabelled perivascular profiles. In future, selected blocks fixed in glutaraldehyde will improve the ultrastructural assessment.

Variability in the intensity of immunolabelling was minimised by staining at the same time and adhering to a rigid protocol. An extreme example of variability emerged late in the course of this study when it was discovered that if the pH of the DAB solution was not optimised to pH 7.6 the background staining of APP was markedly increased. Background staining was minimised by avoiding dehydration of tissues and copious washing of specimens.

4.4.1.2 Human. Artefact arose in the human tissue from formalin pigment and in blood vessels which were not perfusion fixed. Artefact from formalin pigment was removed by picric acid solution. Intravascular

immunolabelling was often non specific and the use of controls was often helpful.

4.4.2 Qualitative data. A fundamental limitation of the methodology used in this study was its descriptive nature. This was further compounded by the ubiquitous distribution of the neuropeptide SP throughout the central nervous system, unlike for example APP, which can be neatly quantitated in defined areas. To overcome the uncontrolled nature of human material we have used experimental models of traumatic brain injury, in which secondary insults can be avoided and time points varied.

The qualitative data of our study will require quantitative corroboration with future studies, which are being undertaken in our research group. It is to note however that almost 3 decades earlier significantly elevated rodent cortical levels of SP were measured when rodents were killed by decapitation without anaesthetic compared to freezing [137]. Contemporary techniques, now employed in our laboratory, are using ELIZA quantitation of brain homogenate SP and its reduction with time, to characterise depletion of neuropeptide following injury.

Histological quantitation was difficult in this study. For example the most relevant changes to potentially count were the number of perivascular SP granules. In the control rat there were more vessels with focal end

terminals compared to the 5hr animals where the distribution was more diffuse and difficult to quantitate.

4.4.3 Controls, shams and variability. Given the ubiquity of this neuropeptide in the CNS, it is reasonable to assume some homeostatic function and hypothesise possible pathological perturbation following injury. As such, pathological changes, unless extreme, are likely to represent shades of grey of the normal, which immunohistochemistry, with technical background variability may have difficulty distinguishing. As a result population variability of immunolabelling can be significant, even if precautions e.g. staining similar anatomical regions at the same time were taken. In an attempt to minimise these inherent problems, we tried to concentrate on the most striking pathological changes.

This problem was particularly evident in the human material, where no "true" controls exist. The fact that the selected control, often with a past history of epilepsy, died with a normal neuropathological post mortem examination does not exclude the possibility of significant neuropeptide release.

4.4.4 Rodent model validity. Although extensively used in numerous studies to characterise brain injury and test new neuroprotective drugs, a detailed neuropathological study of the impact acceleration model in our hands revealed few of the changes described in the original description of the model. In particular the axonal injury was mild and focal rather than

diffuse. Caution is required in the extrapolation of chemical studies derived from these results to human traumatic brain injury. In contrast, the less used fluid percussion model showed neuropathological changes seen in human fatal TBI and future studies, particularly if examining the effects of traumatic cerebral ischaemia or oedema might better suited using this model.

4.5 Conclusions.

This preliminary study has characterised morphological changes in SP immunoreactivity that occur following traumatic brain injury in 2 animal models and human cases. Our findings suggest that SP, stored in perivascular axons of the cerebral microvasculature is released early following injury, is reduced perivascularly in the subacute phase, has an active role in inflammation and gliosis and is markedly upregulated in pyramidal cortical neurones. Ischaemia and mechanical injury appear to be important variables associated with these responses. These primary findings complement growing evidence in the literature of cerebral neurogenic inflammation in response to noxious stimuli. While our results do not allow us to make definite conclusions regarding a role for neuropeptides in the development of cerebral oedema secondary to neurogenic inflammation, they demonstrate an abundant innervation of the cerebral microvasculature particularly post capillary venules with SP fibres and terminals, suggesting that this is a reasonable hypothesis. Further work using quantitative Western blotting, SP antagonists and immunoelectronmicroscopy is needed.

CHAPTER 5. REFERENCES

1. Narayan, R.K., et al., *Clinical trials in head injury.* J Neurotrauma, 2002. **19**(5): p. 503-57.
2. Welfare, A.I.o.H.a., *Australia's Health in 1992, The Third Biennial Report of the Australian Institute of Health and Welfare, AGPS.* 1992: Canberra.
3. Daly, K.E. and P.R. Thomas, *Trauma deaths in the south west Thames region.* Injury, 1992. **23**(6): p. 393-6.
4. Statistics, A.B.o., *ABS. Causes of death 2000.* 2000: Canberra.
5. Victoria, T.A.C., *Transport Accident Commission Information Division, Victoria. Road Safety monthly summary.* 1997: Victoria.
6. Rosner, M.J., S.D. Rosner, and A.H. Johnson, *Cerebral perfusion pressure: management protocol and clinical results.* J Neurosurg, 1995. **83**(6): p. 949-62.
7. Levin, H.S., *Neurobehavioral outcome of closed head injury: implications for clinical trials.* J Neurotrauma, 1995. **12**(4): p. 601-10.
8. Luchter, S. and M.C. Walz, *Long-term consequences of head injury.* J Neurotrauma, 1995. **12**(4): p. 517-26.
9. NSW, M.A.A.o., *Large Claims. Injuries and costs associated with claims estimated to cost over half a million dollars or more.* 1993. p. 1-17.
10. Reilly, P.L., et al., *Patients with head injury who talk and die.* Lancet, 1975. **2**(7931): p. 375-7.
11. Reilly, P.L., *Brain injury: the pathophysiology of the first hours.'Talk and Die revisited'.* J Clin Neurosci, 2001. **8**(5): p. 398-403.
12. Reilly, P.L., Bullock R., *Head Injury. Pathophysiology and management of Severe Closed Head Injury.* 1 ed. 1997, London: Chapman and Hall Medical. 478.
13. Teasdale, G.M. and D.I. Graham, *Craniocerebral trauma: protection and retrieval of the neuronal population after injury.* Neurosurgery, 1998. **43**(4): p. 723-37; discussion 737-8.
14. Povlishock, J.T. and C.W. Christman, *The pathobiology of traumatically induced axonal injury in animals and humans: a review of current thoughts.* J Neurotrauma, 1995. **12**(4): p. 555-64.
15. Blumbergs, P.C., *Changing concepts of diffuse axonal injury.* J Clin Neurosci, 1998. **5**(2): p. 123- 124.
16. Blumbergs, P.C., et al., *Topography of axonal injury as defined by amyloid precursor protein and the sector scoring method in mild and severe closed head injury.* J Neurotrauma, 1995. **12**(4): p. 565-72.
17. Panegyres, P.K., *The functions of the amyloid precursor protein gene.* Rev Neurosci, 2001. **12**(1): p. 1-39.
18. Van Den Heuvel, C., et al., *Upregulation of amyloid precursor protein and its mRNA in an experimental model of paediatric head injury.* J Clin Neurosci, 2000. 7(2): p. 140-5.
19. Adams, J.H., et al., *Diffuse axonal injury in head injury: definition, diagnosis and grading.* Histopathology, 1989. **15**(1): p. 49-59.
20. Blumbergs, P.C., et al., *Staining of amyloid precursor protein to study axonal damage in mild head injury.* Lancet, 1994. **344**(8929): p. 1055-6.
21. Blumbergs, P.C., N.R. Jones, and J.B. North, *Diffuse axonal injury in head trauma.* J Neurol Neurosurg Psychiatry, 1989. **52**(7): p. 838-41.

22. Adams, J.H., D.I. Graham, and B. Jennett, *The neuropathology of the vegetative state after an acute brain insult.* Brain, 2000. **123 (Pt 7)**: p. 1327-38.
23. Adams, J.H., et al., *Diffuse axonal injury due to nonmissile head injury in humans: an analysis of 45 cases.* Ann Neurol, 1982. **12**(6): p. 557-63.
24. Abou-Hamden, A., et al., *Axonal injury in falls.* J Neurotrauma, 1997. **14**(10): p. 699-713.
25. Koszyca, B., et al., *Widespread axonal injury in gunshot wounds to the head using amyloid precursor protein as a marker.* J Neurotrauma, 1998. **15**(9): p. 675-83.
26. Suenaga, T., et al., *Bundles of amyloid precursor protein-immunoreactive axons in human cerebrovascular white matter lesions.* Acta Neuropathol (Berl), 1994. **87**(5): p. 450-5.
27. Foda, M.A. and A. Marmarou, *A new model of diffuse brain injury in rats. Part II: Morphological characterization.* J Neurosurg, 1994. **80**(2): p. 301-13.
28. Marmarou, A., et al., *A new model of diffuse brain injury in rats. Part I: Pathophysiology and biomechanics.* J Neurosurg, 1994. **80**(2): p. 291-300.
29. Graham, D.I., J.H. Adams, and D. Doyle, *Ischaemic brain damage in fatal non-missile head injuries.* J Neurol Sci, 1978. **39**(2-3): p. 213-34.
30. Go, K.G., *The normal and pathological physiology of brain water.* Adv Tech Stand Neurosurg, 1997. **23**: p. 47-142.
31. Kimelberg, H.K., *Current concepts of brain edema. Review of laboratory investigations.* J Neurosurg, 1995. **83**(6): p. 1051-9.
32. Marmarou, A., et al., *Distinguishing between cellular and vasogenic edema in head injured patients with focal lesions using magnetic resonance imaging.* Acta Neurochir Suppl, 2000. **76**: p. 349-51.
33. Portella, G., et al., *Characterizing edema associated with cortical contusion and secondary insult using magnetic resonance spectroscopy.* Acta Neurochir Suppl, 2000. **76**: p. 273-5.
34. Pierpaoli, C., et al., *Histopathologic correlates of abnormal water diffusion in cerebral ischemia: diffusion-weighted MR imaging and light and electron microscopic study.* Radiology, 1993. **189**(2): p. 439-48.
35. Hanstock, C.C., et al., *Diffusion-weighted imaging differentiates ischemic tissue from traumatized tissue.* Stroke, 1994. **25**(4): p. 843-8.
36. Holmin, S. and T. Mathiesen, *Biphasic edema development after experimental brain contusion in rat.* Neurosci Lett, 1995. **194**(1-2): p. 97-100.
37. Ito, J., et al., *Characterization of edema by diffusion-weighted imaging in experimental traumatic brain injury.* J Neurosurg, 1996. **84**(1): p. 97-103.
38. Ayata, C. and A.H. Ropper, *Ischaemic brain oedema.* J Clin Neurosci, 2002. **9**(2): p. 113-24.
39. Barzo, P., et al., *Contribution of vasogenic and cellular edema to traumatic brain swelling measured by diffusion-weighted imaging.* J Neurosurg, 1997. **87**(6): p. 900-7.
40. Barzo, P., et al., *Biphasic pathophysiological response of vasogenic and cellular edema in traumatic brain swelling.* Acta Neurochir Suppl (Wien), 1997. **70**: p. 119-22.
41. McIntosh, T.K., et al., *Traumatic brain injury in the rat: characterization of a lateral fluid- percussion model.* Neuroscience, 1989. **28**(1): p. 233-44.

42. Vink, R., et al., *Small shifts in craniotomy position in the lateral fluid percussion injury model are associated with differential lesion development.* J Neurotrauma, 2001. **18**(8): p. 839-47.
43. van den Brink, W.A., et al., *Quantitative analysis of blood-brain barrier damage in two models of experimental head injury in the rat.* Acta Neurochir Suppl (Wien), 1994. **60**: p. 456-8.
44. Barzo, P., et al., *Magnetic resonance imaging-monitored acute blood-brain barrier changes in experimental traumatic brain injury.* J Neurosurg, 1996. **85**(6): p. 1113-21.
45. Barzo, P., et al., *Acute blood-brain barrier changes in experimental closed head injury as measured by MRI and Gd-DTPA.* Acta Neurochir Suppl (Wien), 1997. **70**: p. 243-6.
46. Shapira, Y., et al., *Blood-brain barrier permeability, cerebral edema, and neurologic function after closed head injury in rats.* Anesth Analg, 1993. **77**(1): p. 141-8.
47. Nawashiro, H., K. Shima, and H. Chigasaki, *Blood-brain barrier, cerebral blood flow, and cerebral plasma volume immediately after head injury in the rat.* Acta Neurochir Suppl, 1994. **60**: p. 440-2.
48. Sinar, E.J., et al., *Experimental intracerebral hemorrhage: effects of a temporary mass lesion.* J Neurosurg, 1987. **66**(4): p. 568-76.
49. Fukamachi, A., et al., *The incidence and developmental process of delayed traumatic intracerebral haematomas.* Acta Neurochir (Wien), 1985. **74**(1-2): p. 35-9.
50. Seelig, J.M., et al., *Traumatic acute subdural hematoma: major mortality reduction in comatose patients treated within four hours.* N Engl J Med, 1981. **304**(25): p. 1511-8.
51. Tomita, Y., et al., *The synergistic effect of acute subdural hematoma combined with diffuse traumatic brain injury on brain edema.* Acta Neurochir Suppl, 2000. **76**: p. 213-6.
52. Fujisawa, H., et al., *Focal microvascular occlusion after acute subdural haematoma in the rat: a mechanism for ischaemic damage and brain swelling?* Acta Neurochir Suppl (Wien), 1994. **60**: p. 193-6.
53. Bullock, R., et al., *Glial swelling following human cerebral contusion: an ultrastructural study.* J Neurol Neurosurg Psychiatry, 1991. **54**(5): p. 427-34.
54. Schoettle, R.J., et al., *Early polymorphonuclear leukocyte accumulation correlates with the development of posttraumatic cerebral edema in rats.* J Neurotrauma, 1990. **7**(4): p. 207-17.
55. Holmin, S., et al., *Intracerebral inflammatory response to experimental brain contusion.* Acta Neurochir, 1995. **132**(1-3): p. 110-9.
56. Bullock, R., *Excitatory amino acids following brain injury.* J Neurosurg, 1994. **80**(3): p. 595-6.
57. Walski, M. and B. Gajkowska, *The changes in the ultrastructure of the cerebrovascular junction after traumatic injury of the cerebral cortex in rats.* Neuroendocrinol Lett, 2001. **22**(1): p. 19-26.
58. Stahel, P.F., et al., *Experimental closed head injury: analysis of neurological outcome, blood-brain barrier dysfunction, intracranial neutrophil infiltration, and neuronal cell death in mice deficient in genes for pro-inflammatory cytokines.* J Cereb Blood Flow Metab, 2000. **20**(2): p. 369-80.

59. Lu, J., et al., *Cellular inflammatory response associated with breakdown of the blood- brain barrier after closed head injury in rats.* J Neurotrauma, 2001. **18**(4): p. 399-408.
60. Yokota, H., et al., *Cerebral endothelial injury in severe head injury: the significance of measurements of serum thrombomodulin and the von Willebrand factor.* J Neurotrauma, 2002. **19**(9): p. 1007-15.
61. Abbott, N.J., *Inflammatory mediators and modulation of blood-brain barrier permeability.* Cell Mol Neurobiol, 2000. **20**(2): p. 131-47.
62. Kuroda, Y., et al., *Transient glucose hypermetabolism after acute subdural hematoma in the rat.* J Neurosurg, 1992. **76**(3): p. 471-7.
63. Kawamata, T., et al., *Lactate accumulation following concussive brain injury: the role of ionic fluxes induced by excitatory amino acids.* Brain Res, 1995. **674**(2): p. 196-204.
64. Martin, N.A., et al., *Characterization of cerebral hemodynamic phases following severe head trauma: hypoperfusion, hyperemia, and vasospasm.* J Neurosurg, 1997. **87**(1): p. 9-19.
65. Fineman, I., et al., *Concussive brain injury is associated with a prolonged accumulation of calcium: a 45Ca autoradiographic study.* Brain Res, 1993. **624**(1-2): p. 94-102.
66. Hall, E.D., *Free radicals and CNS injury.* Crit Care Clin, 1989. **5**(4): p. 793-805.
67. Siesjo, B.K. and K. Katsura, *Ischemic brain damage: focus on lipids and lipid mediators.* Adv Exp Med Biol, 1992. **318**: p. 41-56.
68. Heath, D.L. and R. Vink, *Traumatic brain axonal injury produces sustained decline in intracellular free magnesium concentration.* Brain Res, 1996. **738**(1): p. 150-3.
69. Okimura, Y., et al., *Reactive astrocytes in acute stage after experimental brain injury: relationship to extravasated plasma protein and expression of heat shock protein.* J Neurotrauma, 1996. **13**(7): p. 385-93.
70. Islam, N., et al., *Detection of DNA damage induced by apoptosis in the rat brain following incomplete ischemia.* Neurosci Lett, 1995. **188**(3): p. 159-62.
71. Maas, A.I., et al., *Why have recent trials of neuroprotective agents in head injury failed to show convincing efficacy? A pragmatic analysis and theoretical considerations.* Neurosurgery, 1999. **44**(6): p. 1286-98.
72. Bullock, M.R., B.G. Lyeth, and J.P. Muizelaar, *Current status of neuroprotection trials for traumatic brain injury: lessons from animal models and clinical studies.* Neurosurgery, 1999. **45**(2): p. 207-17; discussion 217-20.
73. Vink, R. and A.J. Nimmo, *Novel therapies in development for the treatment of traumatic brain injury.* Expert Opin Investig Drugs, 2002. **11**(10): p. 1375-86.
74. von Euler, U., Gaddum, J., *An unidentified depressor substance in certain tissue extracts.* J Physiol, 1931. **1931**(72): p. 74-87.
75. Pernow, B., *Distribution of substance P in the central and peripheral nervous system.* Nature, 1953. **171**(4356): p. 746.
76. Harrison, S. and P. Geppetti, *Substance p.* Int J Biochem Cell Biol, 2001. **33**(6): p. 555-76.
77. Chang, M.M., S.E. Leeman, and H.D. Niall, *Amino-acid sequence of substance P.* Nat New Biol, 1971. **232**(29): p. 86-7.
78. Powell, D., et al., *Radioimmunoassay for substance P.* Nat New Biol, 1973. **241**(112): p. 252-4.

79. Hokfelt, T., et al., *Experimental immunohistochemical studies on the localization and distribution of substance P in cat primary sensory neurons.* Brain Res, 1975. **100**(2): p. 235-52.
80. Otsuka, M. and S. Konishi, *Release of substance P-like immunoreactivity from isolated spinal cord of newborn rat.* Nature, 1976. **264**(5581): p. 83-4.
81. Maggi, C.A., *Tachykinins and calcitonin gene-related peptide (CGRP) as co-transmitters released from peripheral endings of sensory nerves.* Prog Neurobiol, 1995. **45**(1): p. 1-98.
82. Kramer, M.S., et al., *Distinct mechanism for antidepressant activity by blockade of central substance P receptors.* Science, 1998. **281**(5383): p. 1640-5.
83. Navari, R.M., et al., *Reduction of cisplatin-induced emesis by a selective neurokinin-1- receptor antagonist. L-754,030 Antiemetic Trials Group.* N Engl J Med, 1999. **340**(3): p. 190-5.
84. Dionne, R.A., et al., *The substance P receptor antagonist CP-99,994 reduces acute postoperative pain.* Clin Pharmacol Ther, 1998. **64**(5): p. 562-8.
85. Yu, Z., et al., *Neurokinin-1 receptor antagonist SR140333: a novel type of drug to treat cerebral ischemia.* Neuroreport, 1997. **8**(9-10): p. 2117-9.
86. Vink, R., Hu X., Bennett C., Cernak I.,Nimmo A.J., *Inhibition of neurogenic inflammation improves motor and cognitive outcome following diffuse traumatic brain injury.* Rest. Neurol. Neurosci., 2000. **16**: p. 164.
87. Severini, C., et al., *The tachykinin peptide family.* Pharmacol Rev, 2002. **54**(2): p. 285-322.
88. Saria, A., *The tachykinin NK1 receptor in the brain: pharmacology and putative functions.* Eur J Pharmacol, 1999. **375**(1-3): p. 51-60.
89. Yip, J. and L.A. Chahl, *Localization of tachykinin receptors and Fos-like immunoreactivity induced by substance P in guinea-pig brain.* Clin Exp Pharmacol Physiol, 2000. **27**(11): p. 943-6.
90. Hargreaves, R., *Imaging substance P receptors (NK1) in the living human brain using positron emission tomography.* J Clin Psychiatry, 2002. **63**(Suppl 11): p. 18-24.
91. Harmar, A., J.G. Schofield, and P. Keen, *Cycloheximide-sensitive synthesis of substance P by isolated dorsal root ganglia.* Nature, 1980. **284**(5753): p. 267-9.
92. Harmar, A. and P. Keen, *Synthesis, and central and peripheral axonal transport of substance P in a dorsal root ganglion-nerve preparation in vitro.* Brain Res, 1982. **231**(2): p. 379-85.
93. Brimijoin, S., et al., *Axonal transport of substance P in the vagus and sciatic nerves of the guinea pig.* Brain Res, 1980. **191**(2): p. 443-57.
94. Gamse, R., et al., *Capsaicin applied to peripheral nerve inhibits axoplasmic transport of substance P and somatostatin.* Brain Res, 1982. **239**(2): p. 447-62.
95. Yaksh, T.L., et al., *Intrathecal morphine inhibits substance P release from mammalian spinal cord in vivo.* Nature, 1980. **286**(5769): p. 155-7.
96. Richardson, J.D. and M.R. Vasko, *Cellular mechanisms of neurogenic inflammation.* J Pharmacol Exp Ther, 2002. **302**(3): p. 839-45.
97. Kashiba, H., Y. Ueda, and E. Senba, *Systemic capsaicin in the adult rat differentially affects gene expression for neuropeptides and neurotrophin receptors in primary sensory neurons.* Neuroscience, 1997. **76**(1): p. 299-312.

98. Helme, R.D., A. Eglezos, and P.V. Andrews, *The effects of capsaicin denervation on leucocyte and complement components of the inflammatory response.* Clin Exp Neurol, 1987. **24**: p. 207-11.
99. Dray, A., *Neuropharmacological mechanisms of capsaicin and related substances.* Biochem Pharmacol, 1992. **44**(4): p. 611-5.
100. Malcangio, M., et al., *Abnormal substance P release from the spinal cord following injury to primary sensory neurons.* Eur J Neurosci, 2000. **12**(1): p. 397-9.
101. Hokfelt, T., X. Zhang, and Z. Wiesenfeld-Hallin, *Messenger plasticity in primary sensory neurons following axotomy and its functional implications.* Trends Neurosci, 1994. **17**(1): p. 22-30.
102. Wallin, J. and E. Schott, *Substance P release in the spinal dorsal horn following peripheral nerve injury.* Neuropeptides, 2002. **36**(4): p. 252-6.
103. Neubert, J.K., et al., *Inflammation-induced changes in primary afferent-evoked release of substance P within trigeminal ganglia in vivo.* Brain Res, 2000. **871**(2): p. 181-91.
104. Lam, F.Y., W.R. Ferrell, and D.T. Scott, *Substance P-induced inflammation in the rat knee joint is mediated by neurokinin 1 (NK1) receptors.* Regul Pept, 1993. **46**(1-2): p. 198-201.
105. Noguchi, K., et al., *A noxious stimulus induces the preprotachykinin-A gene expression in the rat dorsal root ganglion: a quantitative study using in situ hybridization histochemistry.* Brain Res, 1988. **464**(1): p. 31-5.
106. Schafer, M.K., et al., *Inflammation-induced upregulation of NK1 receptor mRNA in dorsal horn neurones.* Neuroreport, 1993. **4**(8): p. 1007-10.
107. Dubner, R. and G.J. Bennett, *Spinal and trigeminal mechanisms of nociception.* Annu Rev Neurosci, 1983. **6**: p. 381-418.
108. Nicoll, R.A., C. Schenker, and S.E. Leeman, *Substance P as a transmitter candidate.* Annu Rev Neurosci, 1980. **3**: p. 227-68.
109. Furness, J.B., et al., *Substance P-like immunoreactivity in nerves associated with the vascular system of guinea-pigs.* Neuroscience, 1982. **7**(2): p. 447-59.
110. Greeno, E.W., et al., *Functional neurokinin 1 receptors for substance P are expressed by human vascular endothelium.* J Exp Med, 1993. **177**(5): p. 1269-76.
111. Ho, W.Z., et al., *Human monocytes and macrophages express substance P and neurokinin-1 receptor.* J Immunol, 1997. **159**(11): p. 5654-60.
112. Cioni, C., et al., *Enhanced secretion of substance P by cytokine-stimulated rat brain endothelium cultures.* J Neuroimmunol, 1998. **84**(1): p. 76-85.
113. Ljungdahl, A., et al., *Distribution of substance P-like immunoreactivity in the central nervous system of the rat--II. Light microscopic localization in relation to catecholamine-containing neurons.* Neuroscience, 1978. **3**(10): p. 945-76.
114. Cuello, A.C., Priestley, J.V., Paxinos, G., *Substance P and enkephalin containing pathways*, in *The Rat Nervous System.* 1985, Academic Press Australia. p. 441 - 463.
115. Iritani, S., M. Fujii, and K. Satoh, *The distribution of substance P in the cerebral cortex and hippocampal formation: an immunohistochemical study in the monkey and rat.* Brain Res Bull, 1989. **22**(2): p. 295-303.
116. HoKfelt T., K.J.O., Nilsson G., Pernow B., *Substance P : Localisation in the central nervous system and in some primary sensory neurones.* Science, 1975. **190**: p. 889 - 890.

117. Ribeiro-da-Silva, A. and T. Hokfelt, *Neuroanatomical localisation of Substance P in the CNS and sensory neurons.* Neuropeptides, 2000. **34**(5): p. 256-71.
118. Tooney, P.A., G.G. Au, and L.A. Chahl, *Tachykinin NK1 and NK3 receptors in the prefrontal cortex of the human brain.* Clin Exp Pharmacol Physiol, 2000. **27**(11): p. 947-9.
119. Sutoo, D., K. Yabe, and K. Akiyama, *Quantitative imaging of substance P in the human brain using a brain mapping analyzer.* Neurosci Res, 1999. **35**(4): p. 339-46.
120. Sakamoto, N., et al., *Substance P- and enkephalin-immunoreactive neurons in the hippocampus and related areas of the human infant brain.* Neuroscience, 1987. **22**(3): p. 801-11.
121. Sakamoto, N., et al., *Evidence for the existence of substance P-like immunoreactive neurons in the human cerebral cortex: an immunohistochemical analysis.* Brain Res, 1985. **325**(1-2): p. 322-4.
122. Hokfelt, T., et al., *Immunohistochemical studies on the localization and distribution of monoamine neuron systems in the rat brain. I. Tyrosine hydroxylase in the mesand diencephalon.* Med Biol, 1976. **54**(6): p. 427-53.
123. Mai, J.K., et al., *Substance P in the human brain.* Neuroscience, 1986. **17**(3): p. 709-39.
124. Pelletier, G., R. Leclerc, and A. Dupont, *Electron microscope immunohistochemical localization of substance P in the central nervous system of the rat.* J Histochem Cytochem, 1977. **25**(12): p. 1373-5.
125. Faden, A.I., T.P. Jacobs, and C.J. Helke, *Changes in substance P and somatostatin in the spinal cord after traumatic spinal injury in the rat.* Neuropeptides, 1985. **6**(3): p. 215-25.
126. Schoenen, J., et al., *Histochemical changes of substance P, FRAP, serotonin and succinic dehydrogenase in the spinal cord of rats with adjuvant arthritis.* Life Sci, 1985. **36**(13): p. 1247-54.
127. Naftchi, N.E., et al., *Localization and changes of substance P in spinal cord of paraplegic cats.* Brain Res, 1978. **153**(3): p. 507-13.
128. Cruce, W.L., et al., *Effect of aging on the substance P receptor, NK-1, in the spinal cord of rats with peripheral nerve injury.* Somatosens Mot Res, 2001. **18**(1): p. 66-75.
129. Benoliel, R., et al., *Co-localization of N-methyl-D-aspartate receptors and substance P (neurokinin-1) receptors in rat spinal cord.* Neurosci Lett, 2000. **291**(2): p. 61-4.
130. Hokfelt, T., et al., *Immunohistochemical evidence for substance P-containing nerve endings in the human cortex.* Brain Res, 1976. **104**(1): p. 181-6.
131. Liu, H., et al., *Substance P is expressed in hippocampal principal neurons during status epilepticus and plays a critical role in the maintenance of status epilepticus.* Proc Natl Acad Sci U S A, 1999. **96**(9): p. 5286-91.
132. Zachrisson, O., N. Lindefors, and S. Brene, *A tachykinin NK1 receptor antagonist, CP-122,721-1, attenuates kainic acid-induced seizure activity.* Brain Res Mol Brain Res, 1998. **60**(2): p. 291-5.
133. Brown, J.L., et al., *Morphological characterization of substance P receptor-immunoreactive neurons in the rat spinal cord and trigeminal nucleus caudalis.* J Comp Neurol, 1995. **356**(3): p. 327-44.

134. Del Fiacco, M., et al., *Substance P in the human brainstem. Preliminary results of its immunohistochemical localization.* Brain Res, 1983. **264**(1): p. 142-7.
135. Del Fiacco, M., M.L. Dessi, and M.C. Levanti, *Topographical localization of substance P in the human post-mortem brainstem. An immunohistochemical study in the newborn and adult tissue.* Neuroscience, 1984. **12**(2): p. 591-611.
136. Nomura, H., et al., *Distribution of substance P-like immunoreactivity in the lower brainstem of the human fetus: an immunohistochemical study.* Brain Res, 1982. **252**(2): p. 315-25.
137. Kanazawa, I. and T. Jessell, *Post mortem changes and regional distribution of substance P in the rat and mouse nervous system.* Brain Res, 1976. **117**(2): p. 362-7.
138. Olszewski, J., Baxter, D., *Cytoarchitecture of the Human Brain Stem*. 1954: SK.
139. Carpenter, M.B., *Neuroanatomy*. Third Edition ed. 1985: Williams and Wilkins.
140. Crystal, H.A. and P. Davies, *Cortical substance P-like immunoreactivity in cases of Alzheimer's disease and senile dementia of the Alzheimer type.* J Neurochem, 1982. **38**(6): p. 1781-4.
141. Mauborgne, A., et al., *Decrease of substance P-like immunoreactivity in the substantia nigra and pallidum of parkinsonian brains.* Brain Res, 1983. **268**(1): p. 167-70.
142. Edvinsson, L., J. McCulloch, and R. Uddman, *Substance P: immunohistochemical localization and effect upon cat pial arteries in vitro and in situ.* J Physiol, 1981. **318**: p. 251-8.
143. Uddman, R., et al., *Perivascular substance P: occurrence and distribution in mammalian pial vessels.* J Cereb Blood Flow Metab, 1981. **1**(2): p. 227-32.
144. Edvinsson, L., et al., *Peptide-containing nerve fibers in human cerebral arteries: immunocytochemistry, radioimmunoassay, and in vitro pharmacology.* Ann Neurol, 1987. **21**(5): p. 431-7.
145. Edvinsson, L., et al., *Neurokinin A in cerebral vessels: characterization, localization and effects in vitro.* Regul Pept, 1988. **20**(3): p. 181-97.
146. Edvinsson, L., S. Rosendal-Helgesen, and R. Uddman, *Substance P: localization, concentration and release in cerebral arteries, choroid plexus and dura mater.* Cell Tissue Res, 1983. **234**(1): p. 1-7.
147. Norregaard, T.V. and M.A. Moskowitz, *Substance P and the sensory innervation of intracranial and extracranial feline cephalic arteries. Implications for vascular pain mechanisms in man.* Brain, 1985. **108**(Pt 2): p. 517-33.
148. Yamamoto, K., et al., *Overall distribution of substance P-containing nerves in the wall of the cerebral arteries of the guinea pig and its origins.* J Comp Neurol, 1983. **215**(4): p. 421-6.
149. Gorelova, E., et al., *Localisation of immunoreactive factor VIII, nitric oxide synthase, substance P, endothelin-1 and 5-hydroxytryptamine in human postmortem middle cerebral artery.* J Anat, 1996. **188 (Pt 1)**: p. 97-107.
150. Arbab, M.A., L. Wiklund, and N.A. Svendgaard, *Origin and distribution of cerebral vascular innervation from superior cervical, trigeminal and spinal ganglia investigated with retrograde and anterograde WGA-HRP tracing in the rat.* Neuroscience, 1986. **19**(3): p. 695-708.

151. Edvinsson, L., *Innervation of the Cerebral Circulation*, in *The Terminal Nerve*. 1987, Annals of the New York Academy of Sciences: New York. p. 334- 348.
152. Torrens, Y., et al., *Substance P receptors in primary cultures of cortical astrocytes from the mouse.* Proc Natl Acad Sci U S A, 1986. **83**(23): p. 9216-20.
153. Mantyh, P.W., et al., *Substance P receptor binding sites are expressed by glia in vivo after neuronal injury.* Proc Natl Acad Sci U S A, 1989. **86**(13): p. 5193-7.
154. Lin, R.C., *Reactive astrocytes express substance-P immunoreactivity in the adult forebrain after injury.* Neuroreport, 1995. **7**(1): p. 310-2.
155. Michel, J.P., et al., *Substance P-immunoreactive astrocytes related to deep white matter and striatal blood vessels in human brain.* Brain Res, 1986. **377**(2): p. 383-7.
156. Kostyk, S.K., N.W. Kowall, and S.L. Hauser, *Substance P immunoreactive astrocytes are present in multiple sclerosis plaques.* Brain Res, 1989. **504**(2): p. 284-8.
157. Martin, F.C., et al., *Substance P stimulates IL-1 production by astrocytes via intracellular calcium.* Brain Res, 1992. **599**(1): p. 13-8.
158. Kincy-Cain, T. and K.L. Bost, *Substance P-induced IL-12 production by murine macrophages.* J Immunol, 1997. **158**(5): p. 2334-9.
159. Too, H.P., D.R. Marriott, and G.P. Wilkin, *Preprotachykinin-A and substance P receptor (NK1) gene expression in rat astrocytes in vitro.* Neurosci Lett, 1994. **182**(2): p. 185-7.
160. Lai, J.P., et al., *Detection of substance P and its receptor in human fetal microglia.* Neuroscience, 2000. **101**(4): p. 1137-44.
161. Luber-Narod, J., R. Kage, and S.E. Leeman, *Substance P enhances the secretion of tumor necrosis factor-alpha from neuroglial cells stimulated with lipopolysaccharide.* J Immunol, 1994. **152**(2): p. 819-24.
162. Kennedy, P.G., et al., *Clinical and neuroinflammatory responses to meningoencephalitis in substance P receptor knockout mice.* Brain, 2003. **126**(Pt 7): p. 1683-90.
163. Kennedy, P.G., et al., *A substance P antagonist, RP-67,580, ameliorates a mouse meningoencephalitic response to Trypanosoma brucei brucei.* Proc Natl Acad Sci U S A, 1997. **94**(8): p. 4167-70.
164. Campos, M.M. and J.B. Calixto, *Neurokinin mediation of edema and inflammation.* Neuropeptides, 2000. **34**(5): p. 314-22.
165. Hughes, S.R., T.J. Williams, and S.D. Brain, *Evidence that endogenous nitric oxide modulates oedema formation induced by substance P.* Eur J Pharmacol, 1990. **191**(3): p. 481-4.
166. Beattie, D.T., D.K. McNeil, and H.E. Connor, *The influence of neurokinins and calcitonin gene-related peptide on cerebral blood flow in anaesthetized guinea-pigs.* Neuropeptides, 1993. **24**(6): p. 343-9.
167. Edvinsson, L., *Functional role of perivascular peptides in the control of cerebral circulation.* Trends in Neurosci., 1985. **8**: p. 126 - 131.
168. Hafstrom, I., et al., *Substance P activates and modulates neutrophil oxidative metabolism and aggregation.* J Rheumatol, 1989. **16**(8): p. 1033-7.
169. Serra, M.C., et al., *Effect of substance P on superoxide anion and IL-8 production by human PMNL.* Immunology, 1994. **82**(1): p. 63-9.

170. Ohlen, A., et al., *Substance P activates leukocytes and platelets in rabbit microvessels.* Blood Vessels, 1989. **26**(2): p. 84-94.
171. Matsuda, H., et al., *Substance P induces granulocyte infiltration through degranulation of mast cells.* J Immunol, 1989. **142**(3): p. 927-31.
172. Cao, T., N.P. Gerard, and S.D. Brain, *Use of NK(1) knockout mice to analyze substance P-induced edema formation.* Am J Physiol, 1999. **277**(2 Pt 2): p. R476-81.
173. Nakagawa, N., H. Sano, and I. Iwamoto, *Substance P induces the expression of intercellular adhesion molecule-1 on vascular endothelial cells and enhances neutrophil transendothelial migration.* Peptides, 1995. **16**(4): p. 721-5.
174. Quinlan, K.L., et al., *Substance P activates coincident NF-AT- and NF-kappa B-dependent adhesion molecule gene expression in microvascular endothelial cells through intracellular calcium mobilization.* J Immunol, 1999. **163**(10): p. 5656-65.
175. Newbold, P. and S.D. Brain, *An investigation into the mechanism of capsaicin-induced oedema in rabbit skin.* Br J Pharmacol, 1995. **114**(3): p. 570-7.
176. Ziche, M., et al., *Substance P induces migration of capillary endothelial cells: a novel NK-1 selective receptor mediated activity.* Life Sci, 1991. **48**(2): p. PL7-11.
177. Dimitriadou, V., et al., *Ultrastructural evidence for neurogenically mediated changes in blood vessels of the rat dura mater and tongue following antidromic trigeminal stimulation.* Neuroscience, 1992. **48**(1): p. 187-203.
178. Ghabriel, M.N., et al., *Substance P-induced enhanced permeability of dura mater microvessels is accompanied by pronounced ultrastructural changes, but is not dependent on the density of endothelial cell anionic sites.* Acta Neuropathol (Berl), 1999. **97**(3): p. 297-305.
179. Markowitz, S., K. Saito, and M.A. Moskowitz, *Neurogenically mediated leakage of plasma protein occurs from blood vessels in dura mater but not brain.* J Neurosci, 1987. **7**(12): p. 4129-36.
180. Gazelius, B., E. Brodin, and L. Olgart, *Depletion of substance P-like immunoreactivity in the cat dental pulp by antidromic nerve stimulation.* Acta Physiol Scand, 1981. **111**(3): p. 319-27.
181. Bayliss, W.M., *On the origin from the spinal cord of the vaso-dilator fibres of the hind limb and on the nature of these fibres.* J Physiol (Lond), 1901. **26**: p. 173-209.
182. Black, P.H., *Stress and the inflammatory response: A review of neurogenic inflammation.* Brain Behav Immun, 2002. **16**(6): p. 622-53.
183. Alves, R.V., et al., *Receptor subtypes involved in tachykinin-mediated edema formation.* Peptides, 1999. **20**(8): p. 921-7.
184. Lembeck, F., J. Donnerer, and L. Bartho, *Inhibition of neurogenic vasodilation and plasma extravasation by substance P antagonists, somatostatin and [D-Met2, Pro5]enkephalinamide.* Eur J Pharmacol, 1982. **85**(2): p. 171-6.
185. Frode-Saleh, T.S., J.B. Calixto, and Y.S. Medeiros, *Analysis of the inflammatory response induced by substance P in the mouse pleural cavity.* Peptides, 1999. **20**(2): p. 259-65.
186. Kuo, H.P., et al., *Lipopolysaccharide enhances neurogenic plasma exudation in guinea-pig airways.* Br J Pharmacol, 1998. **125**(4): p. 711-6.

187. Schaible, H.G., et al., *Release of immunoreactive substance P in the spinal cord during development of acute arthritis in the knee joint of the cat: a study with antibody microprobes.* Brain Res, 1990. **529**(1-2): p. 214-23.
188. Mantyh, P.W., et al., *Receptor endocytosis and dendrite reshaping in spinal neurons after somatosensory stimulation.* Science, 1995. **268**(5217): p. 1629-32.
189. Liu, H., P.W. Mantyh, and A.I. Basbaum, *NMDA-receptor regulation of substance P release from primary afferent nociceptors.* Nature, 1997. **386**(6626): p. 721-4.
190. Bowden, J.J., et al., *Direct observation of substance P-induced internalization of neurokinin 1 (NK1) receptors at sites of inflammation.* Proc Natl Acad Sci U S A, 1994. **91**(19): p. 8964-8.
191. Brodin, E., et al., *Substance P in trigeminal nerve endings: occurrence and release.* Acta Physiol Scand, 1981. **111**(4): p. 501-3.
192. Awawdeh, L., et al., *Quantitative analysis of substance P, neurokinin A and calcitonin gene-related peptide in pulp tissue from painful and healthy human teeth.* Int Endod J, 2002. **35**(1): p. 30-6.
193. Morita-Tsuzuki, Y., J.E. Hardebo, and E. Bouskela, *Interaction between cerebrovascular sympathetic, parasympathetic and sensory nerves in blood flow regulation.* J Vasc Res, 1993. **30**(5): p. 263-71.
194. Branston, N.M., *The physiology of the cerebrovascular parasympathetic innervation.* Br J Neurosurg, 1995. **9**(3): p. 319-29.
195. Erzurumlu, R.S. and H.P. Killackey, *Efferent connections of the brainstem trigeminal complex with the facial nucleus of the rat.* J Comp Neurol, 1979. **188**(1): p. 75-86.
196. O'Connor, T.P. and D. van der Kooy, *Pattern of intracranial and extracranial projections of trigeminal ganglion cells.* J Neurosci, 1986. **6**(8): p. 2200-7.
197. Suzuki, N., J.E. Hardebo, and C. Owman, *Origins and pathways of cerebrovascular nerves storing substance P and calcitonin gene-related peptide in rat.* Neuroscience, 1989. **31**(2): p. 427-38.
198. Goadsby, P.J., et al., *Stimulation of an intracranial trigeminally-innervated structure selectively increases cerebral blood flow.* Brain Res, 1997. **751**(2): p. 247-52.
199. Goadsby, P.J. and J.W. Duckworth, *Effect of stimulation of trigeminal ganglion on regional cerebral blood flow in cats.* Am J Physiol, 1987. **253**(2 Pt 2): p. R270-4.
200. Mejia, J.A., et al., *Effects of neuropeptide Y, calcitonin gene-related peptide, substance P, and capsaicin on cerebral arteries in man and animals.* J Neurosurg, 1988. **69**(6): p. 913-8.
201. Edvinsson, L., et al., *Reduced levels of calcitonin gene-related peptide-like immunoreactivity in human brain vessels after subarachnoid haemorrhage.* Neurosci Lett, 1991. **121**(1-2): p. 151-4.
202. Edvinsson, L., et al., *Involvement of perivascular sensory fibers in the pathophysiology of cerebral vasospasm following subarachnoid hemorrhage.* J Cereb Blood Flow Metab, 1990. **10**(5): p. 602-7.
203. Nozaki, K., H. Kikuchi, and N. Mizuno, *Changes of calcitonin gene-related peptide-like immunoreactivity in cerebrovascular nerve fibers in the dog after experimentally produced subarachnoid hemorrhage.* Neurosci Lett, 1989. **102**(1): p. 27-32.

204. Liu-Chen, L.Y., et al., *Co-localization of retrogradely transported wheat germ agglutinin and the putative neurotransmitter substance P within trigeminal ganglion cells projecting to cat middle cerebral artery.* J Comp Neurol, 1984. **225**(2): p. 187-92.
205. Liu-Chen, L.Y., M.R. Mayberg, and M.A. Moskowitz, *Immunohistochemical evidence for a substance P-containing trigeminovascular pathway to pial arteries in cats.* Brain Res, 1983. **268**(1): p. 162-6.
206. Mayberg, M.R., N.T. Zervas, and M.A. Moskowitz, *Trigeminal projections to supratentorial pial and dural blood vessels in cats demonstrated by horseradish peroxidase histochemistry.* J Comp Neurol, 1984. **223**(1): p. 46-56.
207. Saito, K., L.Y. Liu-Chen, and M.A. Moskowitz, *Substance P-like immunoreactivity in rat forebrain leptomeninges and cerebral vessels originates from the trigeminal but not sympathetic ganglia.* Brain Res, 1987. **403**(1): p. 66-71.
208. Salt, T.E., R. Morris, and R.G. Hill, *Distribution of substance P-responsive and nociceptive neurones in relation to substance P-immunoreactivity within the caudal trigeminal nucleus of the rat.* Brain Res, 1983. **273**(2): p. 217-28.
209. Allen, G.V. and S.P. Pronych, *Trigeminal autonomic pathways involved in nociception-induced reflex cardiovascular responses.* Brain Res, 1997. **754**(1-2): p. 269-78.
210. Cuello, A.C., M. Del Fiacco, and G. Paxinos, *The central and peripheral ends of the substance P-containing sensory neurones in the rat trigeminal system.* Brain Res, 1978. **152**(3): p. 499-500.
211. Samsam, M., et al., *Depletion of substance P, neurokinin A and calcitonin gene-related peptide from the contralateral and ipsilateral caudal trigeminal nucleus following unilateral electrical stimulation of the trigeminal ganglion; a possible neurophysiological and neuroanatomical link to generalized head pain.* J Chem Neuroanat, 2001. **21**(2): p. 161-9.
212. Uddman, R., et al., *Neuropeptide expression in the human trigeminal nucleus caudalis and in the cervical spinal cord C1 and C2.* Cephalalgia, 2002. **22**(2): p. 112-6.
213. Pioro, E.P., J.T. Hughes, and A.C. Cuello, *Loss of substance P immunoreactivity in the nucleus of the spinal trigeminal tract after intradural tumour compression of the trigeminal nerve.* Neurosci Lett, 1985. **58**(1): p. 7-12.
214. Atalay, B., et al., *Transcorneal stimulation of trigeminal nerve afferents to increase cerebral blood flow in rats with cerebral vasospasm: a noninvasive method to activate the trigeminovascular reflex.* J Neurosurg, 2002. **97**(5): p. 1179-83.
215. Adachi, T., et al., *Cutaneous stimulation regulates blood flow in cerebral cortex in anesthetized rats.* Neuroreport, 1990. **1**(1): p. 41-4.
216. Moskowitz, M.A., et al., *Postocclusive cerebral hyperemia is markedly attenuated by chronic trigeminal ganglionectomy.* Am J Physiol, 1989. **257**(5 Pt 2): p. H1736-9.
217. Macfarlane, R., et al., *Chronic trigeminal ganglionectomy or topical capsaicin application to pial vessels attenuates postocclusive cortical hyperemia but does not influence postischemic hypoperfusion.* J Cereb Blood Flow Metab, 1991. **11**(2): p. 261-71.

218. Moskowitz, M.A., et al., *Trigeminalectomy modifies pial arteriolar responses to hypertension or norepinephrine.* Am J Physiol, 1988. **255**(1 Pt 2): p. H1-6.
219. Edvinsson, L., et al., *Antagonism by (D-Pro2, D-Trp7,9)-substance P of the cerebrovascular dilatation induced by substance P.* Acta Physiol Scand, 1982. **116**(4): p. 411-6.
220. Davis, K.D. and J.O. Dostrovsky, *Activation of trigeminal brain-stem nociceptive neurons by dural artery stimulation.* Pain, 1986. **25**(3): p. 395-401.
221. Ebersberger, A., et al., *Recordings from brain stem neurons responding to chemical stimulation of the subarachnoid space.* J Neurophysiol, 1997. **77**(6): p. 3122-33.
222. Messlinger, K., A. Ebersberger, and H.G. Schaible, *Release of immunoreactive substance P in the brain stem upon stimulation of the cranial dura mater with low pH - inhibition by the serotonin (5-HT1) receptor agonist CP 93,129.* Br J Pharmacol, 1998. **125**(8): p. 1726-32.
223. Schaible, H.G., et al., *Release of immunoreactive substance P in the trigeminal brain stem nuclear complex evoked by chemical stimulation of the nasal mucosa and the dura mater encephali--a study with antibody microprobes.* Neuroscience, 1997. **76**(1): p. 273-84.
224. Strassman, A., et al., *Response of brainstem trigeminal neurons to electrical stimulation of the dura.* Brain Res, 1986. **379**(2): p. 242-50.
225. Shepheard, S.L., et al., *Comparison of the effects of sumatriptan and the NK1 antagonist CP- 99,994 on plasma extravasation in Dura mater and c-fos mRNA expression in trigeminal nucleus caudalis of rats.* Neuropharmacology, 1995. **34**(3): p. 255-61.
226. Moussaoui, S.M., et al., *Inhibition of neurogenic inflammation in the meninges by a non-peptide NK1 receptor antagonist, RP 67580.* Eur J Pharmacol, 1993. **238**(2-3): p. 421-4.
227. Nozaki, K., P. Boccalini, and M.A. Moskowitz, *Expression of c-fos-like immunoreactivity in brainstem after meningeal irritation by blood in the subarachnoid space.* Neuroscience, 1992. **49**(3): p. 669-80.
228. Strittmatter, M., et al., *[Substance P, somatostatin and monoaminergic transmitters in the cerebrospinal fluid of patients with chronic idiopathic trigeminal neuralgia].* Schmerz, 1996. **10**(5): p. 261-8.
229. Moskowitz, M.A., *The neurobiology of vascular head pain.* Ann Neurol, 1984. **16**(2): p. 157-68.
230. Goadsby, P.J., L. Edvinsson, and R. Ekman, *Vasoactive peptide release in the extracerebral circulation of humans during migraine headache.* Ann Neurol, 1990. **28**(2): p. 183-7.
231. Goadsby, P.J., L. Edvinsson, and R. Ekman, *Release of vasoactive peptides in the extracerebral circulation of humans and the cat during activation of the trigeminovascular system.* Ann Neurol, 1988. **23**(2): p. 193-6.
232. Lambert, G.A., et al., *Suppression by eletriptan of the activation of trigeminovascular sensory neurons by glyceryl trinitrate.* Brain Res, 2002. **953**(1-2): p. 181-8.
233. Goadsby, P.J. and Y. Knight, *Inhibition of trigeminal neurones after intravenous administration of naratriptan through an action at 5-hydroxy-tryptamine (5-HT(1B/1D)) receptors.* Br J Pharmacol, 1997. **122**(5): p. 918-22.
234. Edvinsson, L., *Calcitonin gene-related Peptide (CGRP) in cerebrovascular disease.* ScientificWorldJournal, 2002. **2**(5): p. 1484-90.

235. Roon, K.I., et al., *No acute antimigraine efficacy of CP-122,288, a highly potent inhibitor of neurogenic inflammation: results of two randomized, double-blind, placebo-controlled clinical trials.* Ann Neurol, 2000. **47**(2): p. 238-41.
236. Maggi, C.A., *The effects of tachykinins on inflammatory and immune cells.* Regul Pept, 1997. **70**(2-3): p. 75-90.
237. Holmin, S., et al., *Intracerebral inflammation after human brain contusion.* Neurosurgery, 1998. **42**(2): p. 291-8; discussion 298-9.
238. Holmin, S. and T. Mathiesen, *Intracerebral administration of interleukin-1beta and induction of inflammation, apoptosis, and vasogenic edema.* J Neurosurg, 2000. **92**(1): p. 108-20.
239. Toulmond, S. and N.J. Rothwell, *Interleukin-1 receptor antagonist inhibits neuronal damage caused by fluid percussion injury in the rat.* Brain Res, 1995. **671**(2): p. 261-6.
240. Tehranian, R., et al., *Improved recovery and delayed cytokine induction after closed head injury in mice with central overexpression of the secreted isoform of the interleukin-1 receptor antagonist.* J Neurotrauma, 2002. **19**(8): p. 939-51.
241. Catalan, R.E., et al., *Substance P stimulates translocation of protein kinase C in brain microvessels.* Biochem Biophys Res Commun, 1989. **164**(2): p. 595-600.
242. Souza, D.G., et al., *Role of tachykinin NK receptors on the local and remote injuries following ischaemia and reperfusion of the superior mesenteric artery in the rat.* Br J Pharmacol, 2002. **135**(2): p. 303-12.
243. Milner, P., et al., *Ultrastructural localisation of substance P and choline acetyltransferase in endothelial cells of rat coronary artery and release of substance P and acetylcholine during hypoxia.* Experientia, 1989. **45**(2): p. 121-5.
244. Ralevic, V., et al., *Substance P is released from the endothelium of normal and capsaicin-treated rat hind-limb vasculature, in vivo, by increased flow.* Circ Res, 1990. **66**(5): p. 1178-83.
245. Cao, T., et al., *Neurokinin-1 receptor agonists are involved in mediating neutrophil accumulation in the inflamed, but not normal, cutaneous microvasculature: an in vivo study using neurokinin-1 receptor knockout mice.* J Immunol, 2000. **164**(10): p. 5424-9.
246. Kramer, J.H., T.M. Phillips, and W.B. Weglicki, *Magnesium-deficiency-enhanced post-ischemic myocardial injury is reduced by substance P receptor blockade.* J Mol Cell Cardiol, 1997. **29**(1): p. 97-110.
247. Stumm, R., et al., *Adaptive plasticity in tachykinin and tachykinin receptor expression after focal cerebral ischemia is differentially linked to gabaergic and glutamatergic cerebrocortical circuits and cerebrovenular endothelium.* J Neurosci, 2001. **21**(3): p. 798-811.
248. Bruno, G., et al., *The role of substance P in cerebral ischemia.* Int J Immunopathol Pharmacol, 2003. **16**(1): p. 67-72.
249. Yamanouchi, H., S. Takashima, and L.E. Becker, *Correlation of astrogliosis and substance P immunoreactivity in the brainstem of victims of sudden infant death syndrome.* Neuropediatrics, 1993. **24**(4): p. 200-3.
250. Castro-Obregon, S., et al., *A ligand-receptor pair that triggers a non-apoptotic form of programmed cell death.* Cell Death Differ, 2002. **9**(8): p. 807-17.

251. Karakucuk, E.I., et al., *Endogenous neuropeptides in patients with acute traumatic head injury. II: Changes in the levels of cerebrospinal fluid substance P, serotonin and lipid peroxidation products in patients with head trauma.* Neuropeptides, 1997. **31**(3): p. 259-63.
252. Herbert, M.K. and P. Holzer, *[Why are substance P(NK1)-receptor antagonists ineffective in pain treatment?].* Anaesthesist, 2002. **51**(4): p. 308-19.
253. Kingery, W.S., M.F. Davies, and J.D. Clark, *A substance P receptor (NK1) antagonist can reverse vascular and nociceptive abnormalities in a rat model of complex regional pain syndrome type II.* Pain, 2003. **104**(1-2): p. 75-84.
254. Mansikka, H., et al., *Nerve injury-induced mechanical but not thermal hyperalgesia is attenuated in neurokinin-1 receptor knockout mice.* Exp Neurol, 2000. **162**(2): p. 343-9.
255. Pearson, J., L. Brandeis, and A.C. Cuello, *Depletion of substance P-containing axons in substantia gelatinosa of patients with diminished pain sensitivity.* Nature, 1982. **295**(5844): p. 61-3.
256. Tsuchiya, M., et al., *Anti-emetic activity of the novel nonpeptide tachykinin NK(1) receptor antagonist ezlopitant (CJ-11,974) against acute and delayed cisplatin- induced emesis in the ferret.* Pharmacology, 2002. **66**(3): p. 144-52.
257. Rupniak, N.M. and M.S. Kramer, *Discovery of the antidepressant and anti-emetic efficacy of substance P receptor (NK1) antagonists.* Trends Pharmacol Sci, 1999. **20**(12): p. 485-90.
258. Rupniak, N.M., *New insights into the antidepressant actions of substance P (NK1 receptor) antagonists.* Can J Physiol Pharmacol, 2002. **80**(5): p. 489-94.
259. Stockmeier, C.A., et al., *Neurokinin-1 receptors are decreased in major depressive disorder.* Neuroreport, 2002. **13**(9): p. 1223-7.
260. Santarelli, L., et al., *Behavioral and physiologic effects of genetic or pharmacologic inactivation of the substance P receptor (NK1).* J Clin Psychiatry, 2002. **63**(Suppl 11): p. 11-7.
261. Conley, R.K., et al., *Substance P (neurokinin 1) receptor antagonists enhance dorsal raphe neuronal activity.* J Neurosci, 2002. **22**(17): p. 7730-6.
262. Shibata, K., D.M. Haverstick, and M.J. Bannon, *Tachykinin gene expression in rat limbic nuclei: modulation by dopamine antagonists.* J Pharmacol Exp Ther, 1990. **255**(1): p. 388-92.
263. Paxinos G, W.C., *The rat brain in stereotactic coordinates*. 4th ed. 1998.
264. Eng, L.F., R.S. Ghirnikar, and Y.L. Lee, *Glial fibrillary acidic protein: GFAP-thirty-one years (1969-2000).* Neurochem Res, 2000. **25**(9-10): p. 1439-51.
265. Beaumont, A., et al., *Secondary insults worsen blood brain barrier dysfunction assessed by MRI in cerebral contusion.* Acta Neurochir Suppl, 2002. **81**: p. 217-9.
266. Beaumont, A., et al., *The permissive nature of blood brain barrier (BBB) opening in edema formation following traumatic brain injury.* Acta Neurochir Suppl, 2000. **76**: p. 125-9.
267. Cammermeyer, J., *Is the solitary dark neuron a manifestation of postmortem trauma to the brain inadequately fixed by perfusion?* Histochemistry, 1978. **56**(2): p. 97-115.
268. Cammermeyer, J., *The importance of avoiding "dark" neurones in experimental neuropathology.* Acta Neuropathol (Berl), 1961. **1**: p. 245-270.

269. Kallakuri, S., et al., *The effect of varying impact energy on diffuse axonal injury in the rat brain: a preliminary study.* Exp Brain Res, 2003. **148**(4): p. 419-24.
270. Povlishock, J.T., *Traumatically induced axonal injury: pathogenesis and pathobiological implications.* Brain Pathol, 1992. **2**(1): p. 1-12.
271. Kaneko, T., et al., *Morphological and chemical characteristics of substance P receptor- immunoreactive neurons in the rat neocortex.* Neuroscience, 1994. **60**(1): p. 199-211.
272. Kaneko, T., et al., *Characterization of neocortical non-pyramidal neurons expressing preprotachykinins A and B: a double immunofluorescence study in the rat.* Neuroscience, 1998. **86**(3): p. 765-81.
273. Ding, Y.Q., et al., *Localization of the neuromedin K receptor (NK3) in the central nervous system of the rat.* J Comp Neurol, 1996. **364**(2): p. 290-310.
274. Edvinsson, L., Krause,D., *Cerebral Blood Flow and Metabolism.* 2nd ed. 2002, Philadelphia: Lippincott, Williams and Wilkins.
275. Linnik, M.D., et al., *Subarachnoid blood and headache: altered trigeminal tachykinin gene expression.* Ann Neurol, 1989. **25**(2): p. 179-84.
276. Santicioli, P., C.A. Maggi, and A. Meli, *Functional evidence for the existence of a capsaicin-sensitive innervation in the rat urinary bladder.* J Pharm Pharmacol, 1986. **38**(6): p. 446-51.
277. Brene, S., et al., *Kainic acid-mediated increase of preprotachykinin-A messenger RNA expression in the rat hippocampus and a region-selective attenuation by dexamethasone.* Neuroscience, 1992. **50**(3): p. 611-8.
278. Mantyh, P.W., *Neurobiology of substance P and the NK1 receptor.* J Clin Psychiatry, 2002. **63**(Suppl 11): p. 6-10.
279. Abbadie, C., et al., *Inflammation increases the distribution of dorsal horn neurons that internalize the neurokinin-1 receptor in response to noxious and non-noxious stimulation.* J Neurosci, 1997. **17**(20): p. 8049-60.

TABLE 1. SUMMARY OF EXPERIMENTAL ANIMALS USED

INJURY	SURVIVAL	NUMBER	LM	EM
CONTROL		4	3	1
SHAM (DI)	5hr	4	2	2
SHAM (FP)	5hr	1	1	
DI	30min	1		1
DI	5hr	4	3	1
DI	1 day	3	3	
DI	2 days	3	3	
DI	3 days	3		
DI	7 days	3		
FP	5 hr	2	2	

DI diffuse impact LM light microscopy
FP fluid percussion EM electron microscopy

TABLE 2. NEUROPATHOLOGY OF EXPERIMENTAL ANIMALS. HE.

INJURY (N)	SURV	OED	HAEM	RED CELL	INFARCT	DARK CELL
CONTROL 3		N	N	N	N	N
SHAM (DI) 3	5hr	N	N	N	N	N
SHAM (FP) 1	5hr	N	N	N	N	N
DI 3	5hr	N	SUBARACHNOID, INTRAVENTRICULAR	N	N	PARASAGITTAL CORTEX
DI 3	1day	N	SUBARACHNOID	N	N	N
DI 3	2day	N	SUBARACHNOID HAEMORRHAGE	N	N	N
DI 3	3day	N	N	N	N	N
DI 3	7day	N	N	N	N	N
FP 2	5hr	Y	L PARIETAL CONTUSION, SDH	Y	L HEMISPHERE, TECTUM	Y

TABLE 3. NEUROPATHOLOGY OF EXPERIMENTAL ANIMALS. APP IMMUNOPOSITIVITY.

INJURY (N)	SURV	AXONS	PERIVASCULAR	NEURONES
CONTROL 3		N	N	N
SHAM (DI) 3	5hr	N	N	N
SHAM (FP) 1	5hr	N	N	Y
DI 3	5hr	CORPUS CALLOSUM, PONS, MEDULLA	++	++
DI 3	1day	CORPUS CALLOSUM, MEDULLA	+	+
DI 3	2day	MEDULLA	+	+
DI 3	3day	N	+	+
DI 3	7day	MEDULLA	+	+
FP 2	5hr	CORPUS CALLOSUM, PONS, MEDULLA	+	+

DI diffuse impact, FP fluid percussion, Surv – survival, Oed – oedema, Haem – Haemorrhage, N = number

TABLE 4. NEUROPATHOLOGY OF EXPERIMENTAL ANIMALS. SP POSITIVITY

INJURY (N)	SURV	PERIVASCULAR	PYRAMIDAL	GLIAL
CONTROL 3		DISCRETE	-	-
SHAM (DI) 3	5hr	DISCRETE	-	-
SHAM (FP) 1	5hr	DISCRETE	-	-
DI 3	5hr	DIFFUSE ++	+	-
DI 3	1day	DIFFUSE +	+	-
DI 3	2day	DIFFUSE +	+	-
DI 3	3day	DIFFUSE +	-	-
DI 3	7day	DIFFUSE	-	-
FP 2	5hr	DIFFUSE ++	+	-

DI – diffuse impact
FP – fluid percussion
N = number

TABLE 5. SUMMARY OF HUMAN POST MORTEM CASES.

CATEGORY	CASE NO	AGE/SEX	PATHOLOGY	SURVIVAL	REGION
					MEDULLA CORTEX
CONTROL	24/03	2M			M
	176/02	14M			M,C
	52/02	18M			M,C
	09/03	27M			M,C
	20/03	36F			M,C
	04/03	32F			M
	56/02	45F	CEREBELLAR ATROPHY		
	71/02	48F			M,C
	65/01	52F	DOWN'S SYNDROME		
	82/01	82M			
MILD TBI	96/94	87F	CONCUSSION	3/7	M
	306/86	81M	CONCUSSION	Hours	M
	233/94	59F	CONCUSSION	Hours	M
	160/93	89F	CONCUSSION	2/7	M
	220/92	59F	CONCUSSION	4/52	M
PAEDIATRIC	26/87	3F	CEREBRAL OEDEMA	0	M
	75/01	9F	ASDH/SAH	Hours	C
	424/86	2.5M	CONTUSION	Hours	M
	648/83	0.3F	CONTUSION	Hours	M
	61/96	2.5M	MCA INFARCT	Hours	M
	145/01	13F	CEREBRAL OEDEMA	2/7	M,C
	367/89	2.5F	CONTUSION	2/7	M
	696/86	3M	CONTUSION	7/7	M
	79/85	0.5F	SUBDURAL HAEMATOMA	7/52	M
SEVERE TBI	67/00	29M	MEDULLARY CONTUSION	0	M
	263/00	96F	MEDULLARY CONTUSION	0	M
	73/01	21	SUBDURAL HAEMATOMA	0	M,C
	111/01	36	CEREBRAL OEDEMA	1/7	M,C
	76/01	41	SUBDURAL HAEMATOMA	1/7	M,C
	75/02	86	SUBDURAL HAEMATOMA	1/7	M,C
	230/94	18M	CONTUSION	2/7	M
	166/96	31M	INTRACEREBRAL HAEMATOMA	2/7	M
	36/01	34	CEREBRAL OEDEMA	2/7	M,C
	142/00	18M	CONTUSION,SDH	3/7	M,C
	35/01	57	SUBDURAL HAEMATOMA	3/7	M,C
	133/99	22M	INFARCTION	3/7	M,C
	275/01	86M	SDH/CONTUSION	4/7	M,C
	139/02	23	CONTUSION/SAH	6/7	M,C
	248/01	52	SUBDURAL HAEMATOMA	7/7	M,C

TABLE 6. THE NEUROPATHOLOGY OF HUMAN CORTICAL LESIONS – HE PROFILE.

CASE	LESION	SURV	OED	HAEM	RED CELL CHANGE
75/01N4	SDH	0	-	+	+
N9	SDH		+W	-	+
73/01N4	SDH	0	-	-	-
N6	-		+W	-	-
76/01N1	SDH/CONT	1/7	-	+	+
N5	SDH		-	-	+
75/02N2	SDH/CONT	1/7	+C	+	+
N10	-		-	+	+
111/01N5	OEDEMA	1/7	+C	+	+
N6	SDH		++C	-	+
145/01N1	CONT/OED	2/7	+C/W	+	+
N3	-		-	+	+
36/01N5	OEDEMA	2/7	++C	-	++ thrombosed vessels
N10	-		+C	-	+
142/00N1	CONT	3/7	+C	+	+
N6	SDH		-	+	+
133/99N4	NECROSIS	3/7	+C	+	+ *thrombosed vessels
N3	CONT		+C/W	+	+
35/01N5	-	3/7	-	-	+
N7	SDH		-	-	+
275/01N2	SDH/CONT	4/7	+W	++	+
N12	ICH		-	++	+
139/02N5	-	6/7	-	+	+
N8	SAH		-	-	-
248/01N4	SDH	7/7	-	-	+
N6	-		-	-	+

SDH – subdural haematoma, Cont – contusion, W – white matter, C – cortex, SAH – subarachnoid haemorrhage, + presence of, - absence of.

TABLE 7. NEUROPATHOLOGY OF HUMAN CORTICAL LESIONS. APP AXONAL POSITIVITY.

CASE	LESION	SURV	PERIVASCULAR	AXONS
75/01N4	SDH	0	+	+
N9	SDH		+	+
73/01N4	SDH	0	+	+
N6	-		+	+
76/01N1	SDH/CONT	1/7	+	+
N5	SDH		+	+
75/02N2	SDH/CONT	1/7	+	+
N10	-		+	+
111/01N5	OEDEMA	1/7	+	+
N6	SDH		+	+
145/01N1	CONT/OED	2/7	+	+
N3	-		+	+
36/01N5	OEDEMA	2/7	-	+
N10	-		-	+
142/00N1	CONT	3/7	+	+
N6	SDH		+	+
133/99N4	NECROSIS	3/7	-	-
N3	CONT		-	-
35/01N5	-	3/7	+	+
N7	SDH		+	+
275/01N2	SDH/CONT	4/7	+	+
N12	ICH		+	+
139/02N5	-	6/7	+	+
N8	SAH		+	+
248/01N4	SDH	7/7	+	-
N6	-		+	-

+ - presence of , - absence of , Cont = contusion, SAH – subarachnoid haemorrhage, ICH – intracerebral haemorrhage, SDH – subdural haematoma, Oed - oedema

TABLE 8. NEUROPATHOLOGY OF HUMAN CASES – SP

CASE	LESION	SURV	PERIVASCULAR	GLIAL	NEURONAL
75/01N4	SDH	0	++	N	+
N9	SDH		+	N	N
73/01N4	SDH	0	++	N	+
N6	-		++	N	N
76/01N1	SDH/CONT	1/7	++	+	+
N5	SDH		++	+	+
75/02N2	SDH/CONT	1/7	N	N	+
N10	-		N	N	+
111/01N5	OEDEMA	1/7	-	N	+
N6	SDH		N	N	+
145/01N1	CONT/OED	2/7	N	+	+
N3	-		-	+	+
36/01N5	OEDEMA	2/7	-	+	+
N10	-		-	N	-
142/00N1	CONT	3/7	++	N	+
N6	SDH		+	N	+
133/99N4	NECROSIS	3/7	-	N	-
N3	CONT		-	N	-
35/01N5	-	3/7	N	N	-
N7	SDH		N	N	+
275/01N2	SDH/CONT	4/7	-	N	-
N12	ICH		+	N	-
139/02N5	-	6/7	N	+	++
N8	SAH		+	N	+
248/01N4	SDH	7/7	N	+	++
N6	-		+	+	+

N = same as compared to control case, + increased, - reduced
Cont = contusion, SAH – subarachnoid haemorrhage, ICH – intracerebral haemorrhage, SDH – subdural haematoma, Oed - oedema

APPENDIX 1a. HAEMATOXYLIN/EOSIN STAIN.

1. Dewax slides in xylene (2x2min) and dehydrate in graded alcohol (2x2 min).
2. Place in haematoxylin for 5 minutes
3. Wash in water
4. Place in acid alcohol (4 dips)
5. Wash in water
6. Place in lithium carbonate (4 dips)
7. Wash in water
8. Place in eosin for 1 minute
9. Dehydrate in graded alcohol and 2 changes of xylene.
10. Mount and coverslip.

APPENDIX 1b. WEIL'S STAIN.

1. As per 1 above
2. Place in 4% iron alum and 2% alcoholic haematoxylin for 1 hr
3. Differentiate sections with 4% iron alum
4. Wash in water
5. Differentiate sections in borax ferricyanide
6. Wash in water
7. As per 9 and 10 above.

APPENDIX 2. SUMMARY OF ANTIBODIES AND FLUOROCHROMES.

Immunochemistry was performed with the following primary antibodies and conjugated with the following fluorochromes

Antibody/Fluorochrome	Dilution*	Retrieval Solution
1. APP mouse monoclonal (Boehringer 22C11)	1/10 000	Citrate
2. Substance P goat polyclonal (Santa Cruz)	1/2000	EDTA
3. GFAP rabbit polyclonal (Dako)	1/1000	EDTA
4. PGP 9.5 mouse monoclonal (UltraClone)	1/20 000	Citrate
5. Alexa 546 donkey antigoat (Molecular probes)	1/500	
6. FITC donkey antigoat (Jackson)	1/200	

N.B. dilutions decrease 10 fold for double immunolabelling

FITC – fluorescein isothiocyanate

APPENDIX 3. STREPTAVIDIN-BIOTIN TECHNIQUE FOR PARAFFIN/VIBRATOME SECTIONS.

1. Mount sections on silane coated slides and dry at 60°C for 20 mins

2. De wax in xylene (2 changes of 2 min) and take to absolute alcohol (2 changes)

3. [If prominent formalin pigment – immersion into picric acid solution for 15 minutes]. Immerse in methanol 500ml and 8.3ml H2O2 for 30 minutes to block endogenous peroxidase activity.

4. Rinse in PBS buffer (ph 7.4) 2 changes of 3 min

5. Apply appropriate pretreatment (boiling for 10 minutes in citrate or EDTA depending on primary antibody) for optimum antigen retrieval. Allow to cool to below 50°C.

6. Rinse in PBS buffer (2x3min)

7. Circle section with PAP pen and incubate with 3% Normal Horse Serum (NHS) for 30min.

8. Drain NHS and apply primary antibody overnight in humidifed chamber

9. Rinse in PBS buffer (2x3 min)

10. Incubate with secondary antibody for 30 min (Vector biotinylated secondary used at 1/250)

11. Rinse in PBS buffer (2x3 min)

12. Incubate with streptavidin peroxidase for 60 min (Pierce streptavidin tertiary used at 1/1000)

13. Rinse in PBS buffer (2x3 min)

14. Apply diaminobenzidene (DAB), H2O2 (pH 7.65-7.7) to obtain optimum staining at approximately 7 minutes

15. Rinse in PBS buffer (2x3 min)

16.Light counterstain in Mayer's haematoxylin

17.Dehydrate, clear and mount.

N.B. For immunolabelling of vibratome sections for EM steps 1,2,5,16,17 were ommitted and sections were washed in PBS buffer for 3x10min instead

N.B. For confocal microscopy, fluorescent staining was performed at step 10, then following PBS washing, slides were mounted using an aqueos mounting solution with antifade.

APPENDIX 4. TISSUE PROCESSING FOR TRANSMISSION ELECTRON MICROSCOPY.

1. fixation in paraformaldehyde 4% following immunolabelling
2. wash in 0.2M phosphate buffer, 2 x 15 minutes
3. post fixation in 1% OsO4 in 0.1M phosphate buffer for 2 hours
4. wash in 0.2M phosphate buffer; 15 minutes
5. dehydrate
   30% alcohol 15 min
   50% " "
   60% " "
   70% " "
   80% " "
   90% " "
   100% " "

   100% CuSO4, 3 x 30 minutes
6. Propylene oxide, 2 x 30 minutes
7. Infiltrate 2:1 propylene oxide:resin – overnight
   1:2 propylene oxide: resin 9am – 4pm
   Pure resin 4pm
8. Embed next day
9. Polymerise at 60°C for 24 hours.
10. Thin sections are placed onto copper grids for viewing under electron microscope.

APPENDIX 5. THE HUMAN SPINAL TRIGEMINAL TRACT BY LEVEL. WEIL'S STAIN AND SP IMMUNOSTAIN.

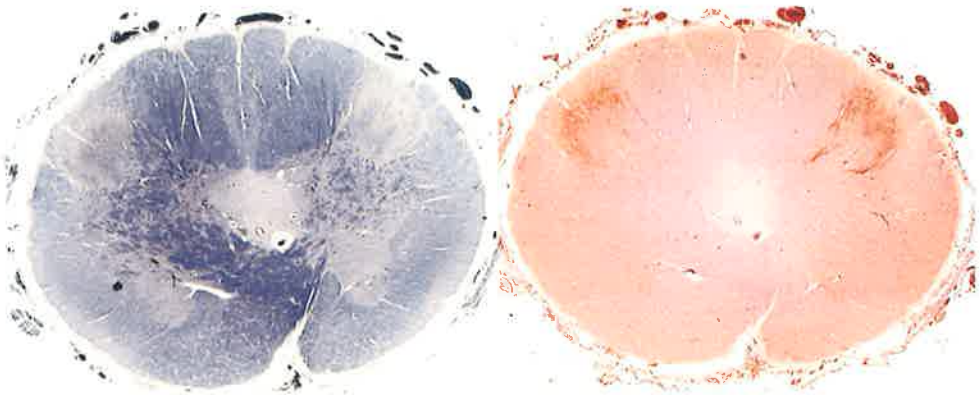
1. Rostral open medulla.

2. Transitional medulla.

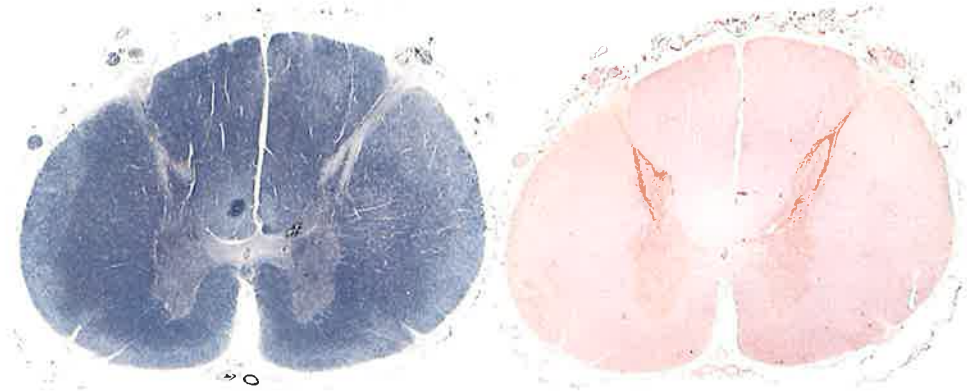
3. Closed Medulla, inferior to inferior olivary nucleus.

4. Medulla at pyramidal decussation

5. **Craniocervical junction**



6. Cervical cord



APPENDIX 6. AUTOPSY SUMMARY OF HUMAN CASES.

1. Mild head Injury.

96/94. 87F.

1. Gliding contusion right frontal lobe
2. Petechial haemorrhage corpus callosum (trivial)

306/86. 81 M.

1. Recent striate haemorrhage right parasagittal cortex.
2. Old lacunar infarct pons
3. Small haemorrhages left external capsule and fornices
4. Hypertensive vascular disease.
5. No APP nor ischaemic change.

233/94. 59F.

1. Trace of subarachnoid haemorrhage

160/93. 89F.

1. Minimal subarachnoid haemorrhage

220/92. 59F.

1. Mild cerebral swelling.

2. Paediatric.

26/87. 3F.

1.Cerebral contusions
2. Corpus callosum haemorrhage
3. Disruption of upper cervical spinal cord – obvious atlanto occipital dislocation with surrounding haemorrhage and spinal cord compression.
4. Neuronal red cell change medulla

75/01. 9F.

1. Large left fungus hernia
2. Subarachnoid haemorrhage
3. Left intracerebral haemorrhage with intraventricular extension
4. Left transtentorial herniation with secondary brain stem haemorrhage
5. Hypoxic ischaemic damage

6. Old lobular infarction right cerebellum

424/86. 2.8M.

1.laceration/ contusion of left cerebellum
2. petechial haemorrhage of right dentate nucleus and medulla
3. Axonal injury medulla

648/83. 0.3M.

1. Haemorrhages in the right parieto-occipital region, third ventricle and inferior orbital surface of frontal lobe.
2. Macroscopic tears of the corpus callosum.
7. SAH over left cerebral hemisphere
8. No sign of cerebral herniation

61/96. 2.75M.

1.Traumatic cerebral SAH
2. Laceration left parietal lobe
3. Scattered cortical contusions (small)
4. Petechial haemorrhage of corpus callosum.
5. Early infarction of L MCA territory.

145/01. 13 F.

1. Fungus hernia of frontal lobes
2. Contusions of right frontal lobe
3. Laceration and contusion of temporal lobes
4. Haemorrhagic indentation right uncus
5. Diffuse axonal injury
6. Hypoxic ischaemic encephalopathy.

367/89. 2.5 F.

1.cerebral contusions and lacerations
2. white matter petechiae
3. brain swelling with raised intracranial pressure.
4. Subependymal haemorrhage in pons

696/86. 3 M.

1. Severe laceration and haemorrhagic necrosis of inferior frontal lobes.
2. Ruptured corpus callosum
3. Small haemorrhages of central grey matter and rostral brain stem
4. Bilateral tonsilar necrosis.
5. Normal pons and medulla.
6. Extensive APP positivity in medulla

7. No ischaemic change
8. Congestion, vacuolation, stretched neurones, microhaemorrhages medulla

79/85. 0.6F.

1.Old SAH with haemosiderin over surface and base of brain
2. infarction of right thalamus and subtotal posterior cerebral artery R>L
3. brownish discoloration in right inferior colliculus
4. Neuronal red cell change in medulla

3. Severe TBI

67/00. 28M.

1. Raised intracranial pressure with tonsillar necrosis
2. Medullary contusion.
3. Contusions of left frontal, temporal and occipital lobes
4. Petechial haemorrhages within corpus callosum
5. Diffuse axonal injury
6. Extensive ischaemic injury- left anterior, left posterior and right middle cerebral arteries.
7. Hypoxic encephalopathy

263/00. 96 F.

1.Severe medullary and upper cervical cord contusion.
2. Traumatic cerebral SAH
3. Evidence of old SAH over left temporal lobe.
4. Widespread APP positive axons medulla
5. Supependymal haemorrhage at obex

73/01. 21 M

1. Cerebral laceration right frontal and left temporal lobes
2. Microscopic contusion right parietal, calcarine and temporal lobes
3. Extensive microscopic petechial haemorrhage midbrain and pons

111/01. 38 M.

1. Subarachnoid haemorrhage
2. Cerebral contusions
3. Gliding contusions
4. Bilateral tentorial and tonsillar herniation
5. Extensive brain haemorrhage

76/ 01. 40 M.

1. Compression deformity of right cerebral hemispherc consistent with right acute subdural haematoma
2. Right uncal, right subfalcine and cerebellar tonsillar herniation
3. Bilateral haemorrhagic infarcts in posterior cerebral artery territories.

75/02. 9 M.

1. Subarachnoid haemorrhage
2. Cerebral contusions
3. Gliding contusions
4. Petechial haemorrhage left and right corpus striatum
5. Petechial haemorrhages midbrain, pons, medulla, upper cervical

230/94. 18M.

1.Raised ICP with tonsillar herniation and bilateral foci of uncal haemorrhagic necrosis.
2.Cortical contusions involving frontal and temporal lobes.
3.Macroscopic evidence of DAI with haemorrhagic necrosis of corpus callosum and right dorsolateral quadrant.
4.Bilateral gliding contusions.
5.Petechial haemorrhages of central white matter of frontal and temporal lobes
6.Petechial haemorrhages of central grey matter.
7.Focal haemorrhagic infarction of left frontal lobe.

166/96. 30 M.

1. Extensive subarachnoid haemorrhage
2. Traumatic right basal ganglionic haemorrhage and adjacent disrupted perforator
3. Scattered small petechial haemorrhages in right central white matter.
4. C4/C5 fracture

36/01. 34 F.

1. Right subdural haematoma
2. Subarachnoid haemorrhage
3. Right cerebral hemispheric swelling
4. Secondary pontine haemorrhage and midbrain compression

142/00. 18M.

1.SAH over cerebral cortex
2. contusions of the left frontal, left temporal, left parietal and left occipital cortex
3. petechial haemorrhages within the white matter of the same and the corpus callosum
4. haemorrhage within pons.
5. haemorrhage within left occipital horn.

6. Red cell change, pyramids stretched rostral medulla
7. Axonal injury rostral medulla

135/01. 56 F.

1. Right subdural haemorrhage
2. Right supracallosal herniation, right tentorial herniation, cerebellar herniation
3. Left and right frontal, right temporal contusions
4. Secondary brain stem haemorrhages

133/99. 22 M.

1. L middle cerebral artery infarct
2. L anterior cerebral artery infarct
3. Cortical contusions of inferior frontal and temporal lobes
4. Scattered haemorrhages of central grey and white matter
5. Left hemispheric swelling with evidence of left transtentorial herniation
6. Patchy subarachnoid haemorrhage
7. APP positive axons in rostral medulla and pyramids
8. Neuronal red cell change rostral medulla

275/ 01. 87 M.

1. Laceration of left temporal and frontal lobes associated with cortical venous thrombosis and subarachnoid haemorrhage
2. Left Sylvian fissure haematoma
3. Left intracerebral haemorrhage
4. Right intraventricular haemorrhage
9. Multifocal axonal injury

139/02. 23 F.

1. Subarachnoid haemorrhage
2. Cerebral contusions
3. Gliding contusions and corpus callosum injury
4. Focal pontine haemorrhage
5. Diffuse axonal injury
10. Extensive hypoxic ischaemic injury

248/01. 52 F.

1. Left acute subdural haemorrhage
2. Subarachnoid haemorrhage
3. Left cerebral hemispheric swelling with supracallosal and tenorial herniation
4. Secondary pontine haemorrhage