“Traumatic brain injury with particular reference to diffuse traumatic axonal injury subpopulations”

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Dedication

'This Thesis is dedicated to the memory of my father, who died on May 15th, 2004. Gone, but not forgotten'

Declaration

I hereby declare that the work presented in this thesis is my own, except where stated. This work has not been submitted for any other degree or professional qualification.

Omer Hussain Al-Hasani

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Abbreviations

ABC	Avidin-biotin complex
AD	Alzheimer's disease
ANOVA	Analysis Of Variance
AOI	Area of interest
Apo E	Apolipoprotein E
Aß	Amyloid ß-protein
β-APP	Beta-amyloid precursor protein
BBB	Blood-brain barrier
BDPs	Breakdown products
Ca^{2+}	Calcium
CMSP	Calpain-mediated spectrin proteolysis
CO	Carbon monoxide
CSF	Cerebrospinal fluid
DAB	3,3'-diaminobenzidine
DAI	Diffuse axonal injury
DIC	Disseminated intravascular coagulation
DVI	Diffuse vascular injury
EDH	Extradural haemorrhage
EDTA	Ethylenediamine-tetraacetic acid
GCS	Glasgow Coma Scale
GFAP	Glial Fibrillary Acidic Protein
H&E	Haematoxylin and eosin
HIER	Heat-induced epitope retrieval
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
IAT	Impaired axonal transport
ICH	Intracerebral haemorrhage
ICP	Intracranial pressure
IHC	Immunohistochemistry
IMT	Intravascular microthrombosis

MAP	Mitogen-activated protein
MAPs	Microtubule-associated proteins
MPT	Membrane permeability transition
Na^+	Sodium
NAD	Nicotinamide deaminase
NCIPC	National Center for Injury Prevention and Control
NF	Neurofilament
NF-160	Neurofilament 160 kD subunit
NF-200	Neurofilament 200 kD subunit
NF-68	Neurofilament 68 kD subunit
NFC	Neurofilament compaction
NFH	Heavy neurofilament subunit
NFL	Light neurofilament subunit
NFM	Medium neurofilament subunit
NFs	Neurofilaments
NINDS	National Institute of Neurological Disorders and Stroke
Nmnat1	Nicotinamide mononucleotide adenyltransferase 1
NSE	Neuron-Specific Enolase
OR	Odds-ratio
PAP	Peroxidise-antiperoxidase
PIER	Protease-induced epitope retrieval
RTA	Road traffic accidents
SAH	Subarachnoid haemorrhage
SBDPs	Spectrin breakdown products
SDH	Subdural haemorrhage
TAI	Traumatic axonal injury
TBI	Traumatic brain injury
TCI	Total contusion index
WHO	World Health Organization
Wld^S	*Slow Wallerian degeneration* mutant mouse

Abstract

Traumatic brain injury (TBI) remains an important cause of morbidity and mortality within society. TBI may result in both focal and diffuse brain injury. Diffuse traumatic axonal injury (TAI) is an important pathological substrate of TBI, and can be associated with a range of clinical states, ranging from concussion through to death, the clinical severity being associated with a number of factors related to the injury.

A retrospective study was conducted using 406 cases with TBI, from the archive of the Academic Department of Pathology (Neuropathology) University of Edinburgh, during the period from1982 and 2005. This cohort was sequential and provided a unique description of the range of pathologies associated with fatal TBI within the Edinburgh catchment area. All the data was collected on a proforma and analysed to provide a description of the incidence in the injury patterns among the Edinburgh cohort.

This cohort was then used to provide cases to try and critically assess the mechanisms of axonal injury in TBI. A study was undertaken to investigate TAI in an experimental model of non-impact head injury in a gyrencephalic mammalian model (piglet model) and in human autopsy materials using immunohistochemical analysis of a range of antibodies, and to define the distribution of axonal injury with flow and neurofilament markers in TAI. A further objective was to examine the expression of β-APP as an indicator of impaired axonal transport, three neurofilament markers targeting NF-160, NF-200, and the phosphorylated form of the neurofilament heavy chain (NFH), in different anatomical regions of piglet and human brains. The double immunofluorescence labelling method was then employed to investigate the hypothesis of co-localisation between β-APP and each one of the previous neurofilament markers.

The animal studies showed significant differences in NF-160 between sham and injured 3-5 days old piglet cases (6 hour survival) and between 3-5 days sham and injured, when stained with SMI-34 antibody. In 4 weeks old piglet cases (6 hour survival), immunoreactivity of β-APP was significantly higher in injured than control. No other significant differences for any of the antibodies were noted, based on age, velocity, and survival time. Human results suggested that the brainstem had a higher level of β-APP and NF-160 than the corpus callosum and internal capsule. Co-localisation of β-APP with NFs was not a consistent feature of TAI in piglet and human brains, suggesting that markers of impaired axonal transport and neurofilament accumulation are sensitive to TAI, but may highlight different populations involved in the evolution of TAI.

1. Introduction

1.1 Traumatic brain injury

1.1.1 Epidemiology of traumatic brain injury

The human brain is highly vulnerable to injury which can compromise the quality of life through profound cognitive and neurobehavioral dysfunction (Dash et al., 2010). Traumatic brain injury (TBI) is an overwhelming and major global health problem, and is one of the most important causes of morbidity and mortality in both industrialized and developing countries (Park et al., 2008). The death rate related to TBI in people less than 35 years of age is 3.5 times more than that of both cancer and heart disease (Lewin, 1991). Estimates by the World Health Organization (WHO) indicate 57 million people internationally have been hospitalised with one or more TBIs (Murray and Lopez, 1996).

In the United States, recent statistics from the National Center for Injury Prevention and Control (NCIPC) reveal that approximately 1.4 million cases of TBI were reported each year from 1995 through 2001 (NCIPC, 2006). Of those patients, about 1.1 million (80%) were treated and discharged from emergency departments, 235,000 (17%) were hospitalized, and 50,000 (3.6%) died from their injuries. As part of their findings, their analysis showed that the leadings causes of TBI were falls (28%), motor vehicle crashes (20%), struck by or against events (19%), and assaults (11%) respectively. A different study showed that at the beginning of 2005, 3.17 million people in the United States were living with permanent disability as a consequence of TBI (Zaloshnja et al., 2008).

In the United Kingdom, more than 1 million patients attend hospital each year suffering from head injury (Kay and Teasdale, 2001). Based on the Glasgow Coma Scale (GCS) (Teasdale and Jennett, 1974), about 90% of this group have a mild head injury, 5% have moderate, and 5% severe head injury (mild GCS 15-13, moderate 12-9, severe 8 or less). Approximately 20% were admitted to hospital for observation, while 5% were transferred to specialist neurological care. Most serious injuries result from road traffic accidents (collisions) (RTA), but most head injuries follow a fall (40%) or an assault (20%).

Mild head injury (acute GCS 13-15) is associated with a higher than expected incidence of disability (Glasgow Outcome Scale (GOS) moderate or severe disability) at one year post injury (Thornhill et al., 2000). Our understandings of the chronic effects of repetitive head injury are evolving, and there has been a recently described condition known as chronic traumatic encephalopathy (McKee et al., 2009). The relationship between a single head injury and subsequent neurodegeneration is complex, but studies do suggest that neurodegenerative disease is more common amongst survivors of a single head injury, particularly males. Meta-analysis has been used to review case-control studies. Mortimer et al (1991) studied 7 case-control studies and reported a relative risk of developing Alzheimer's disease (AD) of 1.82 for head injury with loss of consciousness. The relative risk, however, only reached significance for males. Fleminger et al (2003) studied 15 case-control studies and showed an odds-ratio (OR) of 1.58. Again, however, this study showed that the association between head injury and AD was only significant for males (males OR 2.26, females OR 0.92). The reason for this male predominance may reflect an inherent predisposition to risk taking in males or may be attributed to

participation in particular occupational and leisure activities which put the individual at higher risk for sustaining TBI, or (Yates et al., 2006).

1.1.2 Classification of injury

There is no single classification of TBI which completely encompasses all the clinical, pathological and cellular/molecular features of this complex process. The current status of classification systems for TBI was reviewed in a workshop convened by the National Institute of Neurological Disorders and Stroke (NINDS), with support from the Brain Injury Association of America, the Defense and Veterans Brain Injury Center and the National Institute of Disability and Rehabilitation Research (Saatman et al., 2008). Head injuries can be classified clinically, physically or pathologically. A number of clinical classifications such as the Brussells Coma Grades, Grady Coma Grades, Innsbruck Coma Scale and the FOUR score scale have been developed over the years (Brihaye et al., 1978; Fleischer et al., 1976; Gerstenbrand and Lucking, 1970; Wijdicks et al., 2005). However, the most widely used neurological injury severity scale is the 15-point Glasgow Coma Scale (GCS) (Teasdale & Jennett, 1974), although it is recognised that this scale is of limited value in paediatric evaluation as well as in cases with mild head injury. Generally, brain injury is classified as severe with GCS of ≤ 8, moderate with GCS of 9-12, or mild with GCS of 13-15. Physical classification of TBI includes impact (contact) and inertial loading (non-contact) injuries. Impact injury results from contact between the head and an object whereas the latter arises from the movement of the brain within the skull. The type and severity of injury can be predicted by the magnitude and direction of each type or combination of loading forces (Gennarelli et al., 1985). Penetrating and blast mechanisms of brain injury

have also been described (Saatman et al., 2008). These injuries are generally seen in firearm injuries and in a military or terrorist situation. There has been a major recent shift in TBI research towards blast-related TBI, mostly due to increased military funding in this field.

Pathological classifications can be pathophysiological, describing primary and secondary injuries, or anatomical, based on focal or diffuse injuries. In one single case it is not uncommon to see both focal and diffuse injuries (Smith et al., 2003). The traditional division into primary and secondary brain damage remains usefull since time of injury onset has serious consequences for neuroprotection treatment approaches (Davis, 2000). Throughout this thesis the anatomical pathological classification will be used, describing focal and diffuse injuries.

The focal lesions can be further subdivided into skull fractures, haematomas and contusions, whereas the diffuse injuries can be sub-classified into ischaemic brain damage, traumatic axonal injury/ diffuse vascular injury and brain swelling (Table 1.1) (Smith, 2005). The differences between traumatic and ichaemic axonal injury are illustrated in figure 1.1.

The descriptor focal brain injury is defined as the damage to a localized region of the brain, but the diffuse term refers to widespread injury to either white or grey matter, or both (Graham et al., 2000). Focal brain injury occurs due to direct impact, while the diffuse type might take place in the absence of direct contact as a consequence of rapid acceleration / deceleration of the head leading to shearing forces spread throughout the brain (Smith et al., 2003).

Table 1.1: Classification of traumatic brain injury. Adapted from (Smith, 2005).

Focal	Diffuse
Scalp lacerations	Global ischaemic injury
Skull fractures	Traumatic axonal injury/ diffuse vascular injury
Contusions /lacerations	Brain swelling
Intracranial haemorrhage	
Focal lesions secondary to raised intracranial pressure	

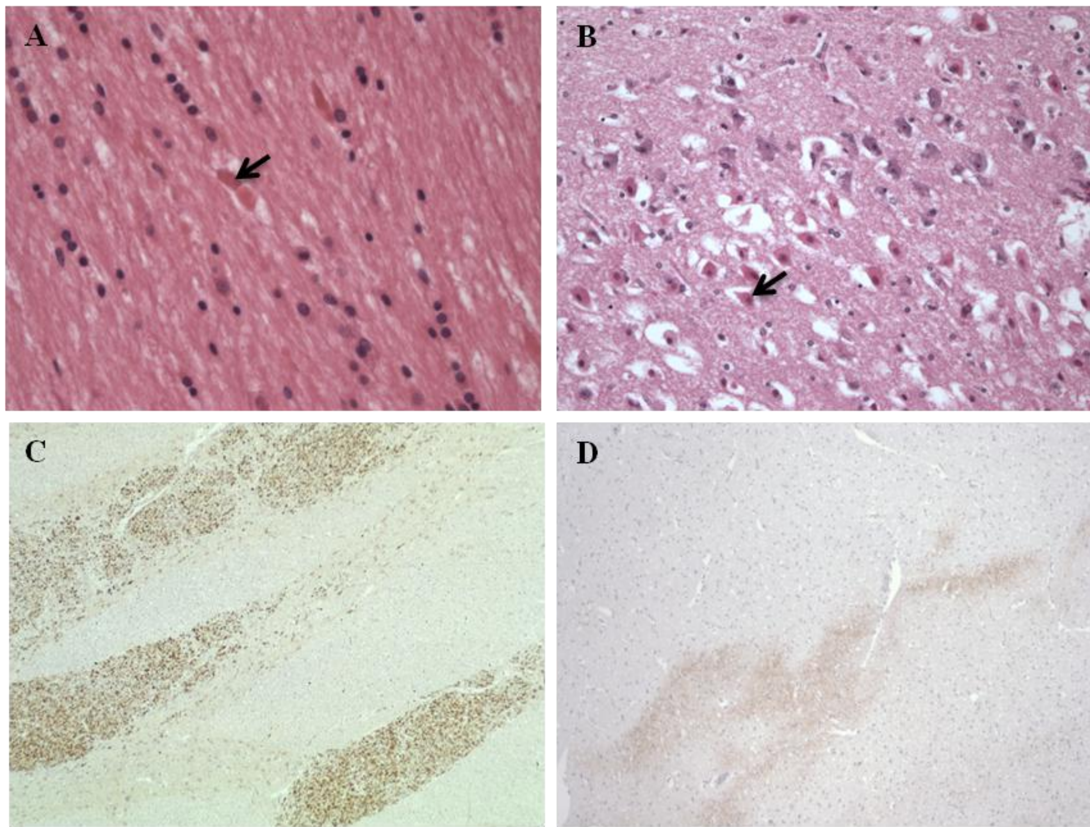


Figure 1.1: Microscopic differentiation between traumatic and ischaemic axonal injury. H and E staining images demonstrate the axonal spheroid and ischaemic cell changes associated with trauma (A) and ischaemia (B) consecutively. Different patterns of β-APP accumulation are demonstrated immunohistochemically in trauma (C) and ischaemia (D). Images provided by Dr Colin Smith.

1.1.3 Pathology associated with brain injury

1.1.3.1 Soft tissue injuries

The initial injuries sustained by the scalp when the head is either struck by an object or strikes the ground are lacerations, contusions or abrasions (DiMaio and DiMaio, 2001). Lacerations may provide clues to the site of head impact and can be a source of blood loss due to the great vascularity of the scalp (Graham and Gennarelli, 2000).

1.1.3.2 Skull fractures

The incidence of skull fracture is associated with the severity of the head injury (Graham and Gennarelli, 2000). Skull fractures are found in 25% of fatal head injuries at autopsy (Osborn et al., 2002). The direction, location and force of the impact producing the injury may be shown by the pattern of a skull fracture. Fracture lines usually radiate from the impact point into the base of the skull and connect with cranial nerve foramina. The presence of interlacing fracture lines is indicative of multiple blows. A range of skull fractures have been described according to the patterns they display including linear, depressed, hinge and ring fractures (Hardman and Manoukian, 2002).

1.1.3.3 Contusions

A cortical contusion is essentially a bruise of the surface of the brain and the overlying pia matter remains intact. A cerebral contusion is indicative of a contact head injury (Finnie and Blumbergs, 2002). Different types of contusions have been identified and associated with different biomechanical forces (Freytag and Lindenberg, 1957; Lindenberg and Freytag, 1960). Contusions typically involve the crest of gyri, but may extend through the cortex into the subcortical white matter

(Davis, 2000). They are typically distributed in the frontal poles; the orbital surfaces of the frontal lobes; the temporal poles; the lateral and inferior surfaces of the temporal lobes; and the cortex above and below the sylvian fissure (Adams et al., 1980a).

Contusions are categorized into different patterns including coup, contre-coup, herniation, fracture and gliding contusions. Coup and contre-coup injuries have a different pathogenesis, but both may involve the frontal and temporal lobes. Coup contusions occur under the impact site following a forward fall striking the forehead. Contre-coup contusions are found in brain tissue directly opposite to the impact site, being seen in the frontal or temporal lobes following a backwards fall striking the occiput (Gennarelli and Meaney, 1996). Coup and contre-coup contusions involve frontal and temporal lobes due to the bony anatomy of the anterior and middle cranial fossae. Fracture contusions are found in brain tissue lying beneath the fracture site. When the parahippocampal gyri and cerebellar tonsils are forced against the tentorium and foramen magnum at the time of injury, herniation contusions occur. Herniation contusions may also been seen around tissue which herniates through a surgical site such as a craniotomy. Gliding contusions have been defined as haemorrhagic lesions located in the parasagittal white matter, often associated with deep hemispheric traumatic haematoma (Lindenberg and Freytag, 1960; Adams.J. 1986).

The depth and extent of contusions in various parts of the brain can be assessed by a contusion index (Adams et al., 1985). This is discussed in more detail in the Materials and Methods section. A similar index, although more detailed and not limited to assessment of contusions, has also been proposed (Rayan et al., 1994).

1.1.3.4 Lacerations

A laceration can be defined as a mechanical tear of brain parenchyma, with disruption of the pia. Such tears are found in the same sites as contusions but they need greater force to occur. Lacerations invariably appear around penetrating or perforating wounds and are most commonly seen adjacent to fracture lines (Hardman and Manoukian, 2002). Although lacerations may be associated with skull fractures this is not a requirement (Adams and Graham, 1976). Microscopic or large haematomas can be produced by lacerations, and the combination of extensive laceration with underlying parenchymal haemorrhage may be associated with subdural and subarachnoid haemorrhages, forming a burst lobe. The site of bleeding is reported to be from overlying cortical veins and brain parenchyma. A burst lobe typically involves the temporal lobe, but it also occurs in the frontal lobes (Smith, 2005).

1.1.3.5 Intracranial haemorrhage

Intracranial haemorrhages have been classified into epidural, also known as extradural (EDH), subdural (SDH), subarachnoid (SAH) and intracerebral (ICH). They are the most common cause of clinical deterioration in patients who experience a lucid interval, “talk and die” or “talk and deteriorate after injury” (Bullock and Teasdale, 1990). The clinical signs associated with EDH and SDH are related to the size /volume of the lesion, the anatomical location, and the rapidity with which the haematoma develops. EDH and SDH may be accompanied with a minor head injury wherein concussion can be either present or absent. A “lucid interval” has been documented in about 30% of patients (Jamieson and Yelland, 1968; Kvarnes and Trumpy, 1978; Reale et al., 1984). Concussion refers to an immediate, usually

reversible episode of brain dysfunction after traumatic brain injury (Bazarian et al., 2006).

Extradural (epidural) Haematoma: Extradural haematomas are accumulations of blood between the calvarium and the dura matter (Figure 1.1). Such a haemorrhage is typically due to blunt force trauma causing a torn blood vessel, which is usually associated with skull fracture (Smith and Graham, 2005). A number of clinical and autopsy studies of head injury have documented the incidence of EDH. Clinically, EDH has been reported in 0.2% to 6% in all head injuries (Galbraith, 1973; Jamieson and Yelland, 1968; Kvarnes and Trumpy, 1978; Weinman and Muttucumaru, 1969) and in 9% among cases with severe TBI (Seelig et al., 1984). In fatal blunt force head injury, the incidence has been reported as between 5% and 15% (Cordobes et al., 1981; Freytag, 1963). The incidence has been revealed to be the highest in fatal cases with a fracture of the skull, being found in 22% (Freytag, 1963).

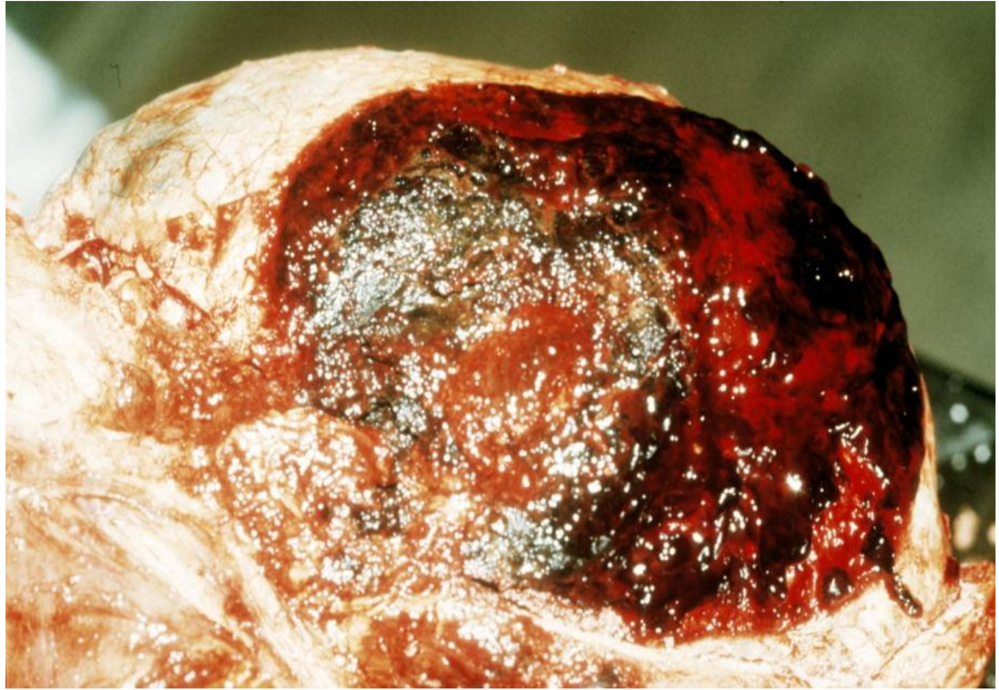


Figure 1.2: Extradural haematoma at autopsy. Image provided by Dr Colin Smith.

EDHs are seen in frontal and occipital regions in about 20%-30%, but they are most commonly found overlying the convexity in relation to the temporo-parietal area (Graham et al., 2002). They have been reported in the posterior fossa in about 3% of all EDHs found at autopsy (Lindenberg, 1971). Cases with EDH have a significant association with fracture of the squamous temporal bone in almost 50%, causing the underlying middle meningeal artery or vein to be injured. The bleeding will strip the dura forming a circumscribed ovoid blood clot that progressively indents and flattens the adjacent brain. Other blood vessels can be damaged including the anterior meningeal artery, superior sagittal sinus, occipital meningeal artery and transverse or sigmoid sinuses. The involvement of the internal carotid artery has been also reported (Cooper, 2000).

Subdural Haematoma: Subdural haematomas form when bleeding occurs in the subdural space (Figure 1.2) and result most commonly after traumatic head injury, or occasionally due to non-traumatic causes (Smith and Graham, 2005). SDH can be caused traumatically by inertial loading (acceleration/deceleration) and no contact is required. The incidence of SDH has been reported in 5% of all head injuries (Echlin et al., 1956; Jamieson and Yelland, 1972) and in 9% to 22% of severe head injuries (Duhaime et al., 1994; Henderson et al., 2001). There are three types of SDH including acute, subacute and chronic; these are classified according to the time interval between injury and onset of symptoms.

SDHs have been described as simple or complicated. This is based on the absence or presence of related parenchymal injury. Simple SDH had a mortality of 20% while complicated SDH was associated with mortality of more than 50% (Jamieson and Yelland, 1972). In patients with an enlarged haematoma, nausea,

vomiting and headache, followed by a reducing level of consciousness, may develop. This can also be associated with other focal neurological signs such as an ipsilateral fixed dilated pupil or ipsilateral motor deficit (Bullock and Teasdale, 1990). The signs and symptoms of chronic SDH are frequently insidious in onset and the diagnosis of this haemorrhage can be difficult to make since a history of head injury is often uncertain (Cooper, 2000).

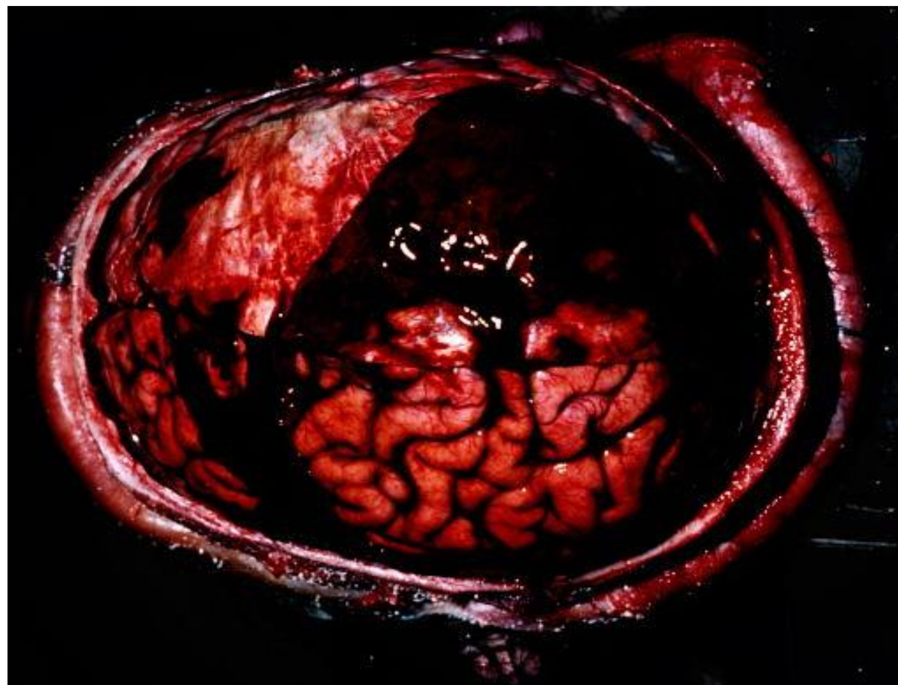


Figure 1.3: Subdural haematoma identified at autopsy. Image provided by Dr Colin Smith.

Acute SDH typically presents clinically within 72 hours of the head injury (Hernesniemi, 1979; Rosenorn and Gjerris, 1978; Talalla and Morin, 1971) although most present clinically within hours. Haematomas that become symptomatic between 3 and 21 days are considered subacute (Jamieson and Yelland, 1972). Autopsy studies have reported an incidence of acute SDHs in between 20% and 63% of cases (Adams et al., 1980; Freytag, 1963). Acute SDH can result from a ruptured bridging vein causing what is called pure SDH. It may also occur due to contusions associated with damage to cortical veins or arteries in the overlying leptomeninges (Smith and Graham, 2005).

Chronic SDH is thought to occur after an episode of minor head injury (Svien and Gelety, 1964). However, there was no history of trauma in 25% to 50% of cases (Marshall et al., 1983). The association of chronic SDH with intra-axial malignancy has been reported (Alimehmeti and Locatelli, 2002; Popovic et al., 1994). Alcohol abuse is common in chronic SDH cases (Stroobandt et al., 1995). In this situation, the SDH might be a result of an increased bleeding diathesis associated with liver disease and thrombocytopenia. Imaging studies have demonstrated that most acute SDH resolve without the formation of a chronic SDH, although an episode of acute haemorrhage is clearly needed to initiate such lesion (Dolinskas et al., 1979). The mechanism of enlargement is still uncertain in those cases that progress to a chronic SDH, although the evidence suggests that fragile new blood vessels within the evolving haematoma are susceptible to bleeding resulting in repeated episodes of haemorrhage enlarging the overall lesion (Markwalder, 1981; Yamashima and Yamamoto, 1984).

Subarachnoid Haemorrhage: Subarachnoid Haemorrhage (SAH) is the presence of blood in the subarachnoid space. There are several different causes such as trauma and rupture of an aneurysm (Herrmann and Zabramski, 2007). In cases with head injuries, SAH has been reported to be a common autopsy finding (Freytag, 1963; Lindenberg and Freytag, 1970; Tatsuno and Lindenberg, 1974) although rarely it is clinically significant. There is an association between SAH and contusions, although it has no clinical significance (Graham and Smith, 2001).

Primary traumatic SAH is a rare entity which is the result of a single blow to the side of the head resulting in damage to the vertebral arteries. Clinically traumatic SAH is often associated with sudden collapse, and alcohol excess is often involved (Gray et al 1999).

Intracerebral Haematoma: Intracerebral haemaorrhages are seen most frequently in the frontal and temporal lobes, and were described in 15% of fatal head injuries in one series (Freytag, 1963). Autopsy studies have reported an incidence of up to 40% (Clifton et al., 1981; Graham, 1996). They were seen less often with EDH alone or combined with SDH, but they were concomitant in about 20% of SDH cases. Superficial intracerebral haematomas are most likely associate with extensive contusional injury. However, impacts of greater force, such as road traffic accidents, can cause more deeply seated haematomas which occur in regions of maximal acceleration-induced brain injury (Graham and Smith, 2001).

The mechanism of intracranial haematomas seems to be a complex pathophysiologic process, with alcohol intoxication being an important contributing factor (Hardman and Manoukian, 2002). Alcohol appears to enhance bleeding by altering platelet function, coagulation or membrane-bound enzymes (Cowan, 1975;

Flamm et al., 1977). In addition, traumatic coagulaopathy has been known to be initiated after mechanical injury in TBI (Stein et al., 2005). Traumatic coagulaopathy has been established to be a type of disseminated intravascular coagulation (DIC), which can occur through the release of tissue thromboplastin into the circulation from injured brain tissue (Gando et al., 1999; Hoots, 1996). It has been confirmed that this process leads to the stimulation of intravascular microthrombosis (IMT) in the brain (Stein et al., 2004). The incidence of DIC has been reported in several studies, being present in up to 76% of cases following head injury (Kumura et al., 1987; Miner et al., 1982; Olson et al., 1989; Pondaag, 1979). DIC is seen most commonly in patients with acute SDH or parenchymal contusions (Kumura et al., 1987). The ε4 allele of the apolipoprotein E (Apo E) gene has been suggested to have a possible a role in traumatic coagulopathy since variations exist amongst patients with similar types of injury (Stein et al., 2005).

1.1.3.6 Brain injury secondary to raised intracranial pressure

The skull and dura are known to form a relatively fixed space. Intracranial pressure (ICP) is defined by the intracranial contents comprising brain, blood and cerebrospinal fluid (CSF) (Little, 2008). A principle known as the Monroe-Kellie doctrine has stated that an increase in the volume of one of these components must result in a decrease in the others (Ropper and Brown, 2005). So, ICP will begin to increase if the volume of one of these components continues to increase.

Normal ICP is dependent on age and body position, and in a supine healthy adult, between 7 and 15 mm Hg (Steiner and Andrews, 2006). In the vertical posture, it has a negative value with an approximate mean of -10 mm Hg, but not exceeding -

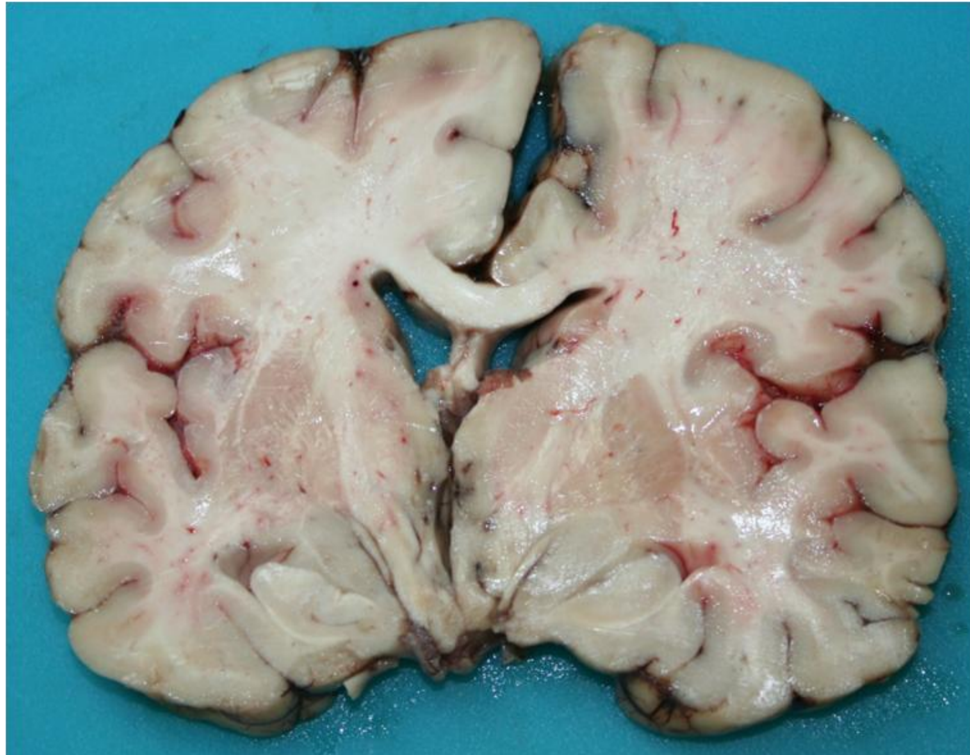
15 mm Hg (Czosnyka and Pickard, 2004). In term children, 3-7 mm Hg is considered normal, whereas in infants values between 1.5 and 6 are quoted (Dunn, 2002).

A rise in ICP may be precipitated as a result of a number of intracranial pathologies including mass lesions, accumulation of CSF, vascular congestion and cerebral oedema. The pathology of raised ICP is thought to be a function of the causative pathology. Its consequences might include localized deformation of the brain, a reduction in CSF volume, shift of the brain and associated herniation. Brain herniation may extend under the falx cerebri, damaging the cingulate gyrus (subfalcine or supracallosal hernia); under the tentorium cerebelli damaging the parahippocampal gyrus /medial temporal lobe (tentorial or uncal hernia); and through the foramen magnum, damaging the tonsil of the cerebllum (tonsillar hernia) (Barlow and Stewart, 2007).

A supracallosal hernia may lead to ischaemia of parasagittal cortex in addition to pressure necrosis of the cingulate gyrus as a consequence of compressing the pericallosal arteries. Tentorial hernia may cause medial occipital cortical infarction as a result of compressing the posterior cerebral artery, and caudal displacement and elongation of the rostral brainstem. Clinically a tentorial hernia may manifest as dilatation of the pupil, due to damage to the ipsilateral third nerve, and hemiparesis ipsilateral to the expanding mass lesion due to contralateral cerebral peduncle compression (Graham and Smith, 2001).

A common terminal event as a consequence of raised ICP is brainstem haemorrhage and infarction, due to axial displacement (Figure 1.3), found in up to 70% of brain injured patients who die. Such pathologies are seen at autopsy as small

foci of haemorrhage and /or infarction in the paramedian parenchyma of the pons and midbrain towards the ventral aspect (Graham et al., 1987).



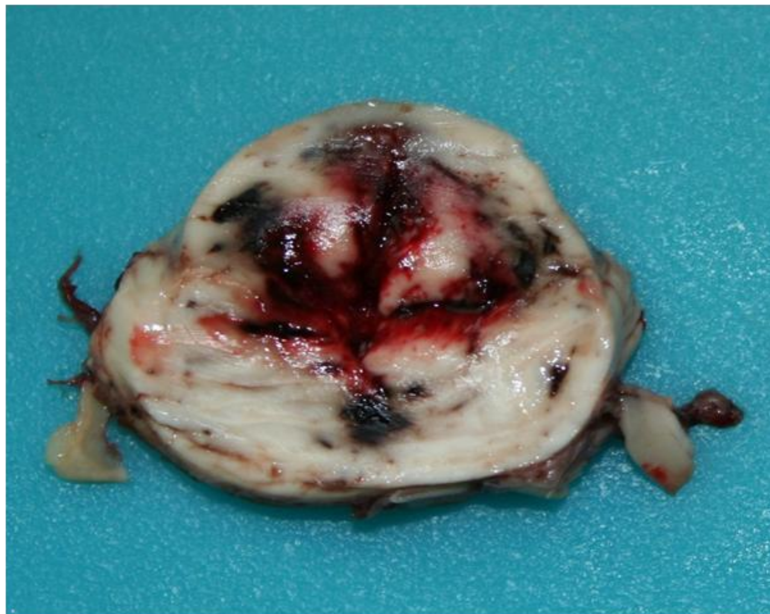


Figure 1.4: Axial displacement (upper) and haemorrhage (lower) of the brainstem. Images provided by Dr Colin Smith.

1.1.3.7 Ischaemia

Ischaemic brain injury is a common pathology in patients dying from TBI (Graham et al., 1978; Graham et al., 1989). In one study, it has been documented in 91% of cases (Graham et al., 1978). Ischaemia is often considered to be similar to hypoxia. However, while ischaemia is characterised by reduction or cessation of the systemic or regional blood flow, hypoxia is defined as a decrease of oxygen content in the blood (Oechmichen and Meissner, 2006). For instance, in cases of carbon monoxide (CO) poisoning, there is hypoxia but with no associated ischaemia.

Cerebral ischaemia can develop as a result of increasing ICP, often due to cerebral swelling secondary to cardiac arrest or due to an expanding intracranial lesion, or as a result of systemic hypotension (Smith and Whitwell, 2004). This, in turn, can cause oxygen and glucose deprivation as well as increased levels of potentially toxic substances, particularly lactic acid. These haemodynamic reductions can lead to the reduction in metabolites such as ATP, since nerve cells do not store alternative energy sources. This reduction may eventually result in metabolic stress, energy failure, ionic perturbations and ischaemic injury (Dirnagl et al., 1999; Siesjo et al., 1989). In hypoxia, there is only impairment of oxygen delivery. Metabolic products such as lactate and H^+ ions are still removed by the increased blood flow, and glucose and other substances continue to be supplied by the blood (Miyamoto and Auer, 2000). It has been described that there was no evidence of cellular necrosis or energy failure, or any other significant pathological changes in the brain at autopsy examination after severe hypoxia (Rie and Bernad, 1980).

Ischaemic insults can be global or focal and the cells that undergo severe ischaemia may die within minutes of the insult or display a delayed vulnerability

(Bramlett and Dietrich, 2004). In the brain, the cells most sensitive to ischaemia are neurons followed by other cell types such as oligodendrocytes, astrocytes and cells of blood vessels including endothelium and smooth muscle. Neuronal injury occurs rapidly after injury but not all neurons in the brain have the same vulnerability to ischaemia (Kubo et al., 1998). Neurons in the hippocampal subfield CA1, neocortical layers 3, 5, and 6, the outer segments of striatum, and the Purkinje cell layer of cerebellar cortex are most vulnerable and are referred to as selectively vulnerable (Bouma et al., 1992; Kotapka et al., 1992).

After the onset of ischaemia, the first sign of cellular injury to occur is neuronal swelling or shrinkage. If blood flow is restored prior to mitochondrial membranes beginning to rupture, these changes are potentially reversible. One to two hours later, neurons undergo irreversible necrotic changes which are recognized as ischaemic cell change. This phenomenon is characterised by condensed acidophilic cytoplasm, formation of triangular pyknotic nuclei and direct contact with swollen astrocytes. After 2-4 hours, ischemic cell change with incrustations becomes visible: this being an indication of irreversible neuronal damage. With ongoing ischaemia, the nuclei become smaller and the cytoplasm paler, a process described as homogenizing cell change (Stewart et al., 2004).

1.1.3.8 Brain swelling

Brain swelling is a commonly identified pathology in cases of fatal brain injury (Adams et al., 1980a). The swelling may be focal, usually in relation to contusions, or diffuse. The swelling may be congestive, secondary to an increase in the cerebral blood volume, or due to oedema, an increase in the water content of the brain tissue (Unterberg et al., 2004). Brain oedema is classified into vasogenic and cytotoxic

oedema, in relation to either extra- or intra-cellular accumulation of abnormal fluid (Klatzo, 1967; Klatzo, 1994). Vasogenic oedema results from functional impairment of the blood-brain barrier (BBB) leading to a high level of plasma proteins and water in the extracellular space. Abnormal water uptake by injured brain cells may cause cytotoxic oedema. Other types of oedema including hydrocephalic (interstitial) oedema and osmotic (hypostatic) oedema have also been described (Fishman, 1975; Klatzo, 1994). Hydrocephalic oedema is caused by an obstruction of cerebrospinal fluid outflow, while osmotic brain oedema is associated with osmotic imbalance between blood and tissue (Unterberg et al., 2004).

Depending on the time course of disease, different types of oedema can be found together in most clinical conditions (Nag et al., 2009). For instance, cellular swelling and cytotoxic oedema are associated with early cerebral ischaemia; however, when there is BBB breakdown due to the damage of the capillary endothelium vasogenic oedema will result. Cytotoxic, vasogenic, and interstitial oedema can be seen together in the case of meningitis, and both vasogenic and cytotoxic oedema are found in TBI (Marmarou, 2007). The pathogenesis underlying brain swelling is incompletely understood, although progress has been made from the study of molecules such as aquaporins; the matrix metalloproteinases; growth factors such as vascular endothelial growth factors A (VEGF-A) and B (VEGF-B); and angiopoietins (Nag et al., 2009).

In the setting of TBI, cytotoxic oedema appears to be more common than vasogenic oedema (Marmarou et al., 2000; Marmarou et al., 2006). Based on its extent and distribution, traumatic brain swelling may be categorised into focal, diffuse within one cerebral hemisphere or diffuse involving both cerebral

hemisphere. While focal swelling is related to contusion, global ischaemia is thought to be the cause of diffuse swelling of both cerebral hemispheres (Smith, 2005).

1.1.3.9 Diffuse traumatic axonal injury

Diffuse traumatic axonal injury (TAI) is an important pathological substrate of TBI due to the fact that it contributes to at least 35% of the mortality and morbidity of TBI cases without space occupying lesions and it is also related to the mortality and morbidity attributable to focal brain injuries (Gennarelli et al., 1982). The spectrum of TAI ranges clinically from mild diffuse injury being associated with mild concussion, in which consciousness is often preserved, to severe diffuse TAI resulting in a vegetative state (Gennarelli et al., 1993). Microscopic pathology of TAI in mild head injury has been described in only two published human studies and this may outline the basis of concussion (Blumbergs et al., 1994; Gorrie et al., 2002). Along with diffuse ischaemic injury, TAI is reported to be a main cause of severe disability and vegetative state in survivors of head injury (Graham et al., 2005).

The term diffuse axonal injury (DAI) is now reserved for the clinical syndrome with supporting neuroradiological changes. The term used for the pathological demonstration of damaged axons in a pattern supporting a traumatic aetiology is TAI (Geddes et al., 2000).

The typical pattern of TAI includes axonal injury in the corpus callosum, dorsolateral segments of the rostral brain stem adjoining the cerebellar peduncles, and in the internal capsule (Graham et al., 2002). In some cases, haemorrhagic lesions are also seen in the corpus callosum and dorsolateral quadrants. Three grades of TAI have been described; mild, moderate and severe (Adams et al., 1977; Adams et al., 1989). In mild TAI (Grade 1), there are microscopic changes in the white

matter of the cerebral cortex, corpus callosum, brain stem and the cerebellum. The moderate form of TAI (Grade 2) is distinguished by grossly obvious focal haemorrhagic lesions isolated to the corpus callosum. In severe TAI (Grade 3), additional focal haemorrhagic lesions are seen in the dorsolateral quadrants of the rostral brain stem.

In 1956, Strich was the first to describe the diffuse degeneration of white matter in patients with dementia after head injury (Strich et al., 1956). She then ascertained that the axonal damage can be created by mechanical forces shearing the fibres at the time of injury and the pathological correlate of this was retraction balls at the points of fibre disruption (Strich, 1961). Since that time research has investigated axonal injury pathways particularly through the development of animal models, which will be discussed below.

1.1.4 Animal models of TBI

Research using animal models of traumatic brain injury usually aims to understand the complex structural and functional events after traumatic brain injury (Wang and Ma, 2010). These models allow an opportunity to control the physiological parameters of the model to be able to replicate the sequence of changes occurring in the clinical condition of human brain injuries (Duhaime, 2006). A close correlation between the severity of injury and the post injury response and rate of recovery of brain-injured animals has been reported in a number of studies (Gennarelli, 1994; Povlishock et al., 1994; Mclntosh et al., 1998). Therefore, a classification for severity of traumatic brain injury in animal models has been developed, and models exist to focus specifically on focal and/or diffuse damage (Morales et al., 2005).

The most common experimental models for focal TBI are the fluid percussion injury and controlled cortical impact injury models (Morganti-Kossmann et al., 2010).

Optic nerve stretch injury: Much of the work relating to the basic science of axonal injury has been undertaken on the optic nerve stretch model, enabling axonal injury to be studied at different stages following injury (Gennarelli, 1994; Maxwell and Graham, 1997; Maxwell et al., 1997). This model has been developed in guinea pigs and allows a pure white matter injury to be produced. The process of axonal degeneration can be studied at different time-points post-stretch, and the process can be studied at great detail, down to the ultrastructural level.

Fluid percussion injury: There are two subsets of the fluid percussion injury model namely the midline and lateral models, based on their respective positions of craniotomy (Wang and Ma, 2010). The lateral fluid percussion model is the most commonly used experimental model for producing both focal and diffuse injury (Mclntosh et al., 1989). This method requires the delivery of a pressure pulse of a saline solution from a reservoir into a piston using a pendulum falling from different heights (Morganti-Kossmann et al., 2010). In this model the craniotomy is moved from the sagittal suture to a lateral position, generating a focal contusion lateral to the actual trephination and injury site with associated diffuse white matter damage remote from the injury site (Thompson et al., 2005). Focal TBI can be typically produced by midline fluid percussion whereby trephination is used to open the skull over the sagittal suture. A fluid bolus is accelerated onto the dural surface, the force being modified by variation of height from which the pendulum used to accelerate the bolus is released.

Cortical impact injury: The cortical impact device creates an injury by delivering a mechanical energy to the intact dura utilizing a pressurised rigid impactor (Cernak, 2005; Dixon et al., 1991; Thompson et al., 2005). Computerised software is used to regulate the damage in which varied parameters such as tissue depth, velocity, and duration of the impact can be selected.

Weight drop model: This model of closed head injury was established by Chen et al (1996). The weight drop model uses a specific mass falling on the closed skull which can lead to a unilateral skull fracture and a cortical cavity surrounded by a pericontusional lesion.

Although some of these rodent models have been modified for larger animals, these experiments have only been performed in the acute phase after injury and without behavioral testing (Duhaime, 2006).

Non-impact injuries (rotational injuries): Models to study diffuse traumatic axonal injury have been developed for rodents and higher species animals. Initially, a non-human primate model was established by Gennarelli et al (1982). This model uses a pneumatic shock tester to produce a non-impact injury. There is a controlled single rotation through a 60-degree arc in sagittal, oblique or lateral direction. They were able to generate prolonged post-injury coma associated with diffuse axonal damage, similar to that found in human brains after severe head injury. Marmarou et al (1994) subsequently developed a rodent model of diffuse TAI in the rat. Other large animal versions of diffuse injury have been developed in the pig using the adult and juvenile brain respectively (Smith et al., 2000; Duhaime, 2006). The acceleration-deceleration rat model has been shown to generate a widespread axonal injury, which extends up to the brainstem (Foda and Marmarou, 1994). Additionally,

the central fluid percussion model has been extensively used to characterize diffuse traumatic axonal injury (Singleton et al., 2002).

TAI in white matter tracts and in multiple regions of the brain has been demonstrated after inertial rotation of the piglet's head in the axial plane. Raghupathi and Margulies (2002) were the first to modify the non-impact inertial injury model for primates to immature piglets, and a number of reports have been published to describe responses at different ages with comparisons between ages. These injuries have not been scaled specifically to brain mass or other size parameters, although they have been graded as mild or more severe. In individuals subjected to higher deceleration forces, neuropathological findings of widespread axonal injury, scattered surface haemorrhages and sustained loss of consciousness have been demonstrated using this model. These observations were similar to the injuries attained when using this model in adult pigs and primates. Additionally, Ramesh et al (2004) have investigated the role of repeated mild injuries and found that the vulnerability to injury appeared to increase in the immature brain. Another study from the same group has developed neurobehavioral outcomes in neonatal piglets and suggested the possible application of these tests to other areas of animal modelling outcomes which use piglets, including cardiac resuscitation, stroke and global ischaemic brain injury (Friess et al., 2007).

Critical comparisons of the models: The vast majority of the published data surrounding animal models of TBI use rodent models, either fluid percussion or cortical impact. While these models have some value in assessing the biochemical cascades and behavioural outcomes of focal cortical injury, there are very serious limitations in the application of this data to the human setting. Rodents have very

little white matter and, as such, are unlikely to show a tissue response similar to the human. In addition, and of greatest concern, the lissencephalic morphology and small mass of the rodent brain poses unique challenges when assessing rotational injury; the gyral brain pattern significantly affects the movement of the brain within the cranial cavity (Cloots et al., 2008). Brain deformation is significantly greater in gyrencephalic animals, and biomechanical studies have demonstrated that axonal injury correlates with tissue deformation (strain) rather than the force applied per unit area (stress) (Margulies et al., 1990). As such to fully develop a model of TBI in any way comparable to the human a gyrencephalic animal with a large brain needs to be used. There are major ethical problems around the use of non-human primates, and the pig/piglet models are the best models currently available.

1.2 Mechanisms of axonal injury

1.2.1 Axonal transport system

The axon of a neuron is recognized to have two major functions; to electrically transmit information from the neuron cell body to the synaptic terminals and to carry proteins and organelles in both directions between the cell body and the synaptic terminals. This transportation process is called axoplasmic transport (Duncan and Goldstein, 2006).

Axonal transport has been classified into two major components, fast and slow. Fast axonal transport is responsible for the movement of membranous organelles, mitochondria, neurotransmitters, channel proteins, multivesicular bodies and endosomes at a rate of 0.5- 10 μm/ sec in the direction of the synapse (anterograde transport) or back to the cell body (retrograde transport). The slow axonal transport is

able to move cytoskeletal proteins and soluble enzymes at a rate of 0.1- 0.001 μm/ sec in the retrograde route (Miller and Heidemann, 2008).

The axonal transport system consists of motor proteins to move the cargo; cytoskeletal filaments that allow motors to produce force and movement; linker proteins to attach motor proteins to cargo; and other molecules which start and control transport. Disruptions to any of the mentioned components can lead to defective axonal transport and neurodegenerative diseases (Roy et al., 2005).

1.2.1.1 The cellular cytoskeleton

The process of axonal transport takes place along the cellular cytoskeleton. Although the cytoskeleton is dynamic allowing the cell to grow or change in size and shape over time, it also provides the neuron with structural support (Shah and Cleveland, 2002). Three main components of the neuronal cytoskeleton have been recognized, namely microtubules, actin mircofilaments and intermediate filaments (Nixon, 1998).

Microtubules: Microtubules are long filaments essential to all eukaryotic cells and are formed from the polymerization of α- and β-tubulin dimers. The α- and β-tubulins are proteins which show about 50% identity at the amino acid level, and are about 459 amino acids each (Burns et al., 1991). This polymerization is very dynamic in non-neuronal cells and in developing neurons. This dynamic process has been reported to be necessary for the normal outgrowth of the axon and growth cone. However, the microtubules become less dynamic as neurons mature (Valiron et al., 2001). This is essentially due to the stabilization of the polymer through interactions with microtubule-associated proteins (MAPs). In the axons of mature motor neurons, the high concentration of MAPs such as tau can significantly increase the stability of

theses microtubules (Brown, 2003). These microtubules have been found to be organized in a polar array along the axon. The polar structure is characterized by a microtubule 'minus' end, terminating in α-tubulin, directed toward the cell centre, and the microtubule 'plus' end of exposed β-tubulin directed outward (Salinas et al., 2008). Gamma-tubulin is a third member of the tubulin family, which may be involved in nucleation of microtubules and anchoring the minus end, and gamma-tubulin is found at microtubule organization centres (Joshi, 1993).

Actin microfilaments: Actin microfilaments are also known to be involved in both the dynamic function of the axon and stability. Assembly of actin monomers produces a flexible helical polymer with two distinct ends and actin microfilaments are frequently bundled into networks within the cell. Interacting proteins are thought to stabilize actin microfilaments, and actin microfilaments are most abundant in the sub-plasmalemmal area of the cell providing support at the cell periphery (Chevalier-Larsen and Holzbaur, 2006).

Intermediate filaments (neurofilaments): Intermediate filaments, primarily neurofilaments, are amongst the major components of the cellular cytoskeleton, and this will be discussed in detail later in this chapter.

1.2.1.2 Molecular motors

1.2.1.2.1 Kinesin and the kinesine superfamily

The first axonal transport motor to be recognized was kinesin; this was based on assays of vesicular motility along microtubules in extruded axoplasm (Vale et al., 1985). Kinesin, also known as kinesine-1, is a plus-end directed motor protein that conveys membranous organelles and synaptic vesicle precursors toward the direction of the synapse (Lawrence et al., 2004).

The hetero-tetramer structure of kinesin includes two heavy chains and two light chains. Each of the heavy chains comprises an an N-terminal motor domain, which fold forming a globular head that appears to be essential for motility in vitro. The α-helical coiled coil is formed by the central domain of the polypeptide and it mediates dimerization. The light chains of kinesin are related to the C-terminal domain, which may involve in cargo binding (Verhey et al., 1998; Stock et al., 1999). It is thought that the two heads of kinesin use the microtubule as a track for their movement (Yildiz et al., 2004). This movement appears to be highly processive which allows kinesin to transport cargo over the long distances of the axons prior to detaching (Vale et al., 1996). An extensive superfamily of kinesin-like proteins has been identified, based on homology with the kinesin motor domain. Many of the 45 members identified in humans also exhibit motor activity (Miki et al., 2005).

1.2.1.2.2 Cytoplasmic dynein and dynactin

Cytoplasmic dynein is the major motor driving retrograde transport which is involved in moving neurotrophic signals, endosomes and other organelles and vesicles toward the cell body (Muresan, 2000). Dynein was initially recognized as the motor protein driving ciliary and flagellar motility, but an intracellular dynein, which is particularly enriched in brain, was subsequently identified using biochemical approaches (Paschal et al., 1987). It has been reported to have important functions in both intracellular transport and cell division (Almenar-Queralt and Goldstein, 2001). The structure of cytoplasmic dynein includes two homodimerized heavy chains and multiple accessory proteins (King, 2000). The force for movement of dynein along microtubules is provided by the C-terminal portion, while the N-

terminal domain is required for binding of accessory proteins as well as homodimerization of the heavy chain (Roy et al., 2005).

Dynactin has been reported to interact with cytoplasmic dynein through dynein intermediate chains and controls its function. It may also act as an adaptor for binding to some of the membrane bound vesicles and organelles transported by dynein (El-Kadi et al., 2007). The dynactin molecule is large and complex and includes 11 various subunits. The actin-like filament is the most distinctive aspect of dynactin forming the base of the complex, consisting of the actin-related protein Arp1 (Schafer et al., 1994). A dimer of an extended coiled-coil protein, $p150^{Glued}$, is projected from this filament (Holzbaur et al., 1991), and then binds directly to cytoplasmic dynein (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995). The efficiency of dynein-mediated motility can be increased by binding $p150^{Glued}$ to microtubules forming an additional microtubule-binding site (Waterman-Storer et al., 1995; King and Schroer, 2000).

1.2.2 Theory of axonal injury

Initial observations in human brains suggested that the axons are disconnected at the time of the impact (primary axotomy) leading to axonal retraction and axoplasmic pooling (Strich et al., 1956). However, it has been shown through many studies that this hypothesis is inaccurate. More recent studies have illustrated that the forces applied to the axon modify focal axonal sections, causing local axonal transport disruption and local axonal swelling, followed by detachment over a period of time after injury (Povlishock and Jenkins, 1995; Maxwell et al., 1997). Although a small proportion of axons, particularly small unmyelinated axons, do undergo primary axotomy, the fact that theses axonal changes have been identified in

scattered axons associated with other normal axons and their vascular elements is strongly supportive of secondary axotomy. The molecular changes that can contribute to the pathogenesis of the axonal injury are being elucidated and are discussed below (Farkas and Povlishok, 2007). Many intra-axonal changes evolve during TAI, which then progress into axonal transport dysfunction, disconnection, and ultimately axonal bulb formation (Figure 1.4) (Serbest et al., 2007).

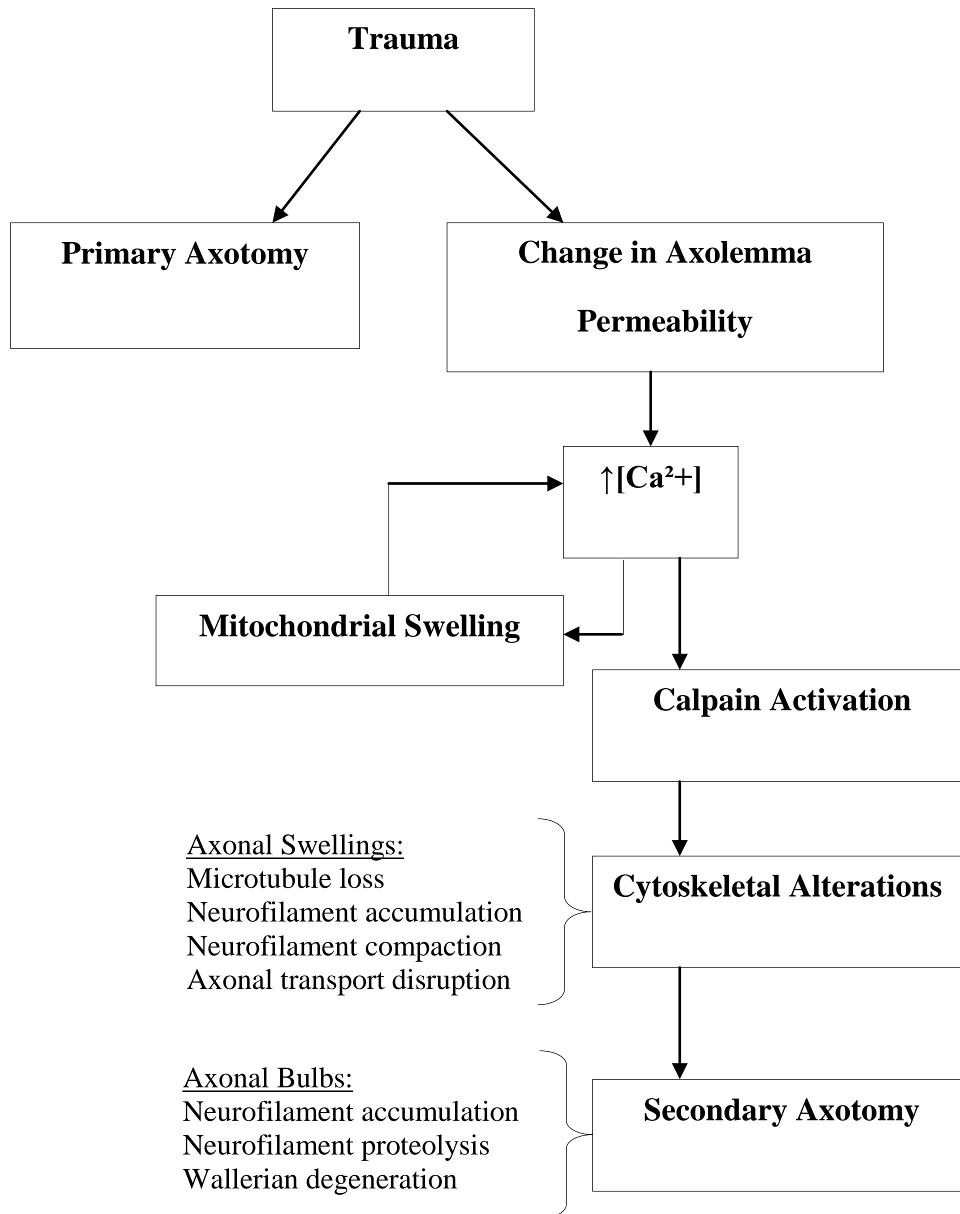


Figure 1.5: Flow chart representing the consequences of TAI. TAI often leads to secondary axotomy, which is initiated by calcium influx, calpain activation and cytoskeletal damage. Axonal swelling is caused by early cytoskeletal changes, while secondary axotomy contributes to axonal bulbs which then lead to Wallerian degeneration and associated proteolysis. Adapted from (Serbest et al., 2007).

1.2.2.1 Altered axolemmal permeability

It has been thought for some time that altered axolemmal permeability is a consequence of the shear and tensile forces caused by the mechanical injury (Medana and Esiri, 2003). Using the fluid percussion injury model, Pettus et al (1994) have employed extracellular horseradish peroxidase (HRP) and the neurofilament 68 kD subunit to determine if altered axolemmal permeability occurs after the traumatic injury. They have demonstrated alterations in axolemmal permeability associated with rapid local compaction of axonal neurofilaments following moderate TBI. Similar events in the axolemma and the cytoskeleton have also been observed within 5 minutes and up to 6 hours post-TBI (Pettus and Povlishock, 1996), suggesting that this initial mechanism occurs over a relatively prolonged period of time.

The importance of mechanoporation is linked to the fact that altered axolemmal permeability can allow the entry of normally excluded ions such as calcium (Ca^{2+}) into the internal environment of the axon (Siesjo et al., 1999). Mechanoporation of the axolemma has been demonstrated to involve the mechanical formation of transient membrane pores during axonal injury. It acts as a major factor in the Ca^{2+} -induced pathophysiology in animal models of brain trauma (Maxwell et al., 1993; Buki et al., 2000). The dysregulation of axonal calcium stimulates different Ca^{2+}-dependent deleterious cascades, including calpain, caspase and calcineurin activation, which contribute to the cytoskeletal disruption leading to impaired axonal transport (Buki et al., 1999; Hall et al., 2005; Kurz et al., 2005; Okonkwo et al., 1999; Okonkwo and Povlishok, 1999; Singleton et al., 2001; Stone et al., 2002; Sattman et al., 2003).

Although it is thought that all injured axons follow the same mechanism of Ca^{2+} mediated damage, a number of studies have suggested that after TBI, the response of axons are more complex than previously considered (Buki et al., 1999; Maxwell et al., 1997). Stone and colleagues, using rodent models, have demonstrated that neurofilament compaction can take place independent of axonal transport disruption (Stone et al., 2001). They were able to illustrate two different anatomical substrates of TAI by employing different antibodies targeting different functions; β-APP to detect impaired axonal transport and RM014 to identify neurofilament compaction. Further studies in 2005, again using rodents, have supported this premise by concluding that there is no correlation between neurofilament compaction and impaired axonal transport in the majority of damage axons (Marmarou et al., 2005). However, to date this observation has not been described in any model outside of the rodent fluid percussion model.

In more severe forms of axonal injury, Marmarou et al (2005) have also reported that the huge Ca^{2+} influx alters local transport kinetics by converting anterograde to retrograde transport and also to influence the formation of reactive axonal swellings. Moreover, the further complexity of the mechanisms of TAI has emerged with studies showing no evidence of changed axonal permeability in unmyelinated axons subjected to in vitro mechanical loading (Wolf et al., 2001). Their study surmised that the activation of sodium (Na^{+}) channels can be a source of local Ca^{2+} dysregulation and the activation of the voltage sensitive Ca^{2+} channel was due to the subsequent depolarization. In addition, they discussed other events as well as the activation of the Na^{+}/ Ca^{2+} exchangers, which resulted in a significant increase in intra-axonal calcium.

As has been shown, previous studies have demonstrated that there are several sources of Ca^{2+} dysregulation, particularly the direct mechanical membrane poration, the activation of Na^{+} channels and / or a direct channelopathy. Furthermore, whatever the origin of the intra-axonal accumulation of Ca^{2+}, Ca^{2+}-induced proteolytic pathways seem to play important roles in the pathogenesis of the axonal injury (He et al., 1999; Maxwell et al., 1995; Wolf et al., 2001).

Maxwell and colleagues have reported that the activation of these proteolytic mechanisms can be the reason for instantaneous disruption of the axonal cytoskeleton (Maxwell et al., 1993). By contrast, ultrastructural investigations have suggested that there is no alteration in the general structural integrity of the local axonal cytoskeleton for several hours after injury, although morphological changes such as neurofilament compaction and loss of microtubules can be detected rapidly (Povlishock and Pettus, 1996).

1.2.2.2 Induction of calpain-mediated structural proteolysis

The impact acceleration rat brain injury model was used to investigate the subject of Ca^{2+}-induced proteolytic alteration (Marmarou et al., 1994). This model is uncomplicated and it has the ability to generate focally injured axonal segments that can be identified within the brain stem tracts. It has been demonstrated that calpain can help to identify Ca^{2+}-induced proteolytic activation in injured axonal segments and this has been achieved using a set of antibodies (Ab38) against breakdown products of the structural protein spectrin (Robert-Lewis et al., 1994; Siman et al., 1984). Immunohistochemical studies utilizing single and double labelling light and electron microscopy have demonstrated the calpain-mediated spectrin proteolysis (CMSP) indicative of early focal intra-axonal activation of the Ca^{2+}-induced protease

calpain (Buki and Povlishock, 2006). Calpain activation co-localised with markers of traumatic axonal injury such as RMO-14 antibody, enabling cytoskeletal alterations such as neurofilament compaction associated with traumatic axonal injury to be shown (Lee et al., 1987; Maxwell et al., 1993; Okonkwo et al., 1998).

In experimental animal models of TAI there was a significant increase in the number of CMSP-immunorective axonal profiles over time after injury. Buki and colleagues have noted that following an injury, the calpain-mediated proteolysis of the cytoskeletal structural protein spectrin seemed to have different spatial compartmentalization (Buki et al., 1999). Within 15 minutes after injury, predominant subaxolemmal and perimitochondrial CMSP-localization was found by utilizing ultrastructural analysis of injured axonal segments, which was then followed by widespread degradation of the intra-axonal protein 1-2 hours post injury. Buki et al (1999) have also described a direct co-localisation of neurofilament compaction (RMO-14)-imunoreactive and CMSP (Ab38). When taken together the data supports the view that CMSP has an important role in the pathobiology of traumatic axonal injury (Buki et al., 1999).

The early Ca^{2+} influx seems to occur secondary to the mechanoporation of the axolemma. This is followed by the initial induction of CMSP in the subaxolemmal compartment, changing the subaxolemmal network. With time, this could influence the structural integrity of the axolemma causing permanent permeability change which can lead to more adverse downstream consequences such as an excessive stimulation of calpain and CMSP in injured axons. Proteolytic side-arm modification occurs after the calcium entry and results in neurofilament compaction (NFC). Calcineurin can be stimulated by Ca^{2+} and alters the repelling forces of the side-arms

by modification of the neurofilament phosphorylation state leading to NFC (Medana and Esiri, 2003; Marmarou et al., 2005).

1.2.2.3 Mitochondrial damage

It has been noted that local morphological changes of mitochondria, for example swelling and degeneration of mitochondrial crisate and membranes can be associated with TAI. Such changes are reminiscent of those caused by Ca^{2+} induced opening of the mitochondrial membrane permeability transition (MPT)-pore (Lifshitz et al., 2004).

Several studies have reported that the opening of the MPT-pore is linked to the pathogenesis of different diseases and is responsible for apoptotic neuronal death (Cai et al., 1998; Lee et al., 1997; Nunez et al., 1998; Saikumar et al., 1998). The excessive sequestration of Ca^{2+} post injury has been thought to be a moderator of these mitochondrial alterations. Therefore, the calcium overloading would lead to the dissipation of the mitochondrial transmembrane potential and opening of the MPT-pore, which permealizes the mitochondrial membrane for molecules less than 1.5 kDa (Medana and Esiri, 2003). This course of action leads to the uptake of water, mitochondrial swelling, (Hirsch et al., 1998; Lemasters et al., 1998; Trost and Lemasters, 1996; Zoratti and Szabo, 1995) and eventually mitochondrial disruption (Buki et al., 2000).

One important consequence of the abnormal mitochondria and MPT is the release of pro-apoptotic substances such as cytochrome-c, the apoptosis activating factor and various members of the caspase enzyme family (Krajewski et al., 1999; Mancini et al., 1998; Susin et al., 1999a). These substances, via the development of the apoptosome (caspase-9, apoptosis protease activating factor-1 and cytochrome-

c), are capable of enhancing the caspase death cascade especially caspase-3 (Cai et al., 1998; Montal, 1998; Susin et al., 1999b; Susin et al., 1998).

1.2.2.4 Activation of the caspase death cascade

The stimulation of two members of the cystein protease group, the calpains and caspases, would initiate the molecular pathways of traumatic axonal injury (Kilinc et al., 2009). It has been reported that the calpains are concerned with limited structural proteolysis, neural plasticity and synaptic transmission (Bartus, 1997; Pasqualin, 1998; Wang, 2000). It is acknowledged, however, that the caspases seem to implement the end of apoptotic processes through signals from the environment, the genome or abnormal mitochondria (Banki et al., 1999; Kruman and Mattsen, 1999; Pettus and Povlishock, 1996; Saikumar et al., 1998; Sun et al., 1999; Susin et al., 1999b; Wolf and Eastman, 1999; Zhao et al., 1999).

Using a rodent model Buki et al (2000) have established that the injured mitochondria are able to release cytochrome-c within traumatically damaged axons. Using double labeling immunofluorecent staining methods they described that the foci of cytochrome-c release were also labeled with CMSP-immunoreactivity. Hence, calpain-mediated protelytic alterations that are induced by Ca^{2+} play a fundamental function in axonal pathology and co-localise with cytochrome-c release (Kilinc et al., 2009). It has also been demonstrated, using other approaches, that spectrin breakdown protein 120KDa-immunoreactive, a protein indicative of caspase-3 activation, has been consistently co-localised with both CMSP- and cytochrome-c-immunoreactivity in traumatic axonal injury (Buki et al., 2000).

It has been suggested that the caspases have no direct apoptotic effect in the neuronal soma but produce a localized effect within the injured axon, the final

degeneration of the damaged axonal parts, particularly the cytoskeletal network, being the result of localized activation of these caspases (Buki and Povlishock, 2006).

The alpha chain of the brain-spectrin tetramer is the initial target of CMSP, whereas the beta chain, which is essential for the membrane-binding of subaxolemmal spectrin, is processed by caspase-3 (Wang et al., 1998). The cleavage of the beta chain is thought to result in collapse of the subaxolemmal membrane cytoskeleton. This then leads to destructive changes in axonal morphology and axolemmal permeability. This is believed to be the most important step in neuronal degradation in different CNS diseases (Bartus, 1997; Wang et al., 1998). It has been suggested that, as the caspases are able to cleave the primary intracellular inhibitor of calpains, namely calpastatin, the caspases are involved in the over stimulation of calpains (Wang, 2000; Wood and Newcomb, 1999). On the basis of the above, in traumatically damaged axonal segments, the activation of the caspase death cascade has been considered to ultimately result in axonal detachment (Raghupathi, 2004).

1.3 Detection of axonal injury

1.3.1 Beta-amyloid precursor protein

Beta-amyloid precursor protein (β -APP) is a member of the homologous type-I transmembrane proteins (King and Turner, 2004). Aberrant proteolytic processing of β –APP produces the amyloid ß-protein (Aß), a 38-43 amino acid residue peptide which is the core of the amyloid cascade hypothesis of AD. It has been reported that two proteases, ß- and γ-secretase, are necessary to release Aß form the precursor molecule (Thinakaran and Koo, 2008). β-APP is processed by Golgi apparatus and conveyed along the axon by fast anterograde transport (Koo et al., 1990; Sisodia et

al., 1993). When there is axonal transport interruption ß-APP has been shown to accumulate, indicative of dysfunction or possibly ultimate detachment of axon (Yam et al., 1997).

Structurally β –APP consists of a large extracellular domain, a single transmembrane region, and a small cytoplasmic tail (Gralle and Ferreira, 2007). On the basis of its structure, β –APP has been hypothesised to function as a receptor in intracellular axonal transport. It is thought that β –APP may interact with kinesin and the microtubule cytoskeleton enabling the transport of cargo to the synaptic terminal (Kamal et al., 2001). Several studies have also shown the role of this protein in adhesion to other cellular or to extracellular matrix components (Kibbey et al., 1993; Qiu et al., 1995; Sabo et al., 2001; Soba et al., 2005). β –APP has been hypothesised to be involved in tissue maintenance (Breen et al., 1991), brain development (Trapp and Hauer, 1994) and response to injury (Mattson, 1997).

Gentleman and colleagues were the first group who used the ß-APP as a marker for injured axons (Gentleman et al., 1993).

1.3.2 Neurofilament subunits

Neurofilaments (NFs) are the intermediate filaments of neurons that provide strength and stability to the axon, and are involved in regulating intracellular transport to axons and dendrites (Serbest et al., 2007). They are members of the cytoskeleton proteins and are thought to have roles in forming and preserving cell shape, as well as facilitating the cytoplasmic transport of particles and organelles (Liu et al., 2004). On the basis of the differences in molecular weights, NFs have been considered to consist of three subunits, namely the light (NF68), the medium (NF160) and the heavy (NF200) neurofilament subunits (Shea and Chan, 2008).

Like other members of the intermediate filament family, NFs are composed structurally of a central α- helical rod domain (~ 310 amino acids) which is flanked by a globular N-terminal region and non- α- helical carboxy-terminal side-arm domains (Figure 1.5). The central rod domains including regions 1a, 1b, and 2 are characterized by their highly conserved motifs which are essential for their proper assembly (Barry et al., 2007). Every seventh residue of the central rod is thought to be hydrophobic which helps to form the α- helical coiled-coil parallel homodimers or heterodimers containing NFL and either NFM or NFH (Al-Chalabi and Miller, 2003). There is also a linker region aligned to the hydrophobic residue (Liu et al., 2004).

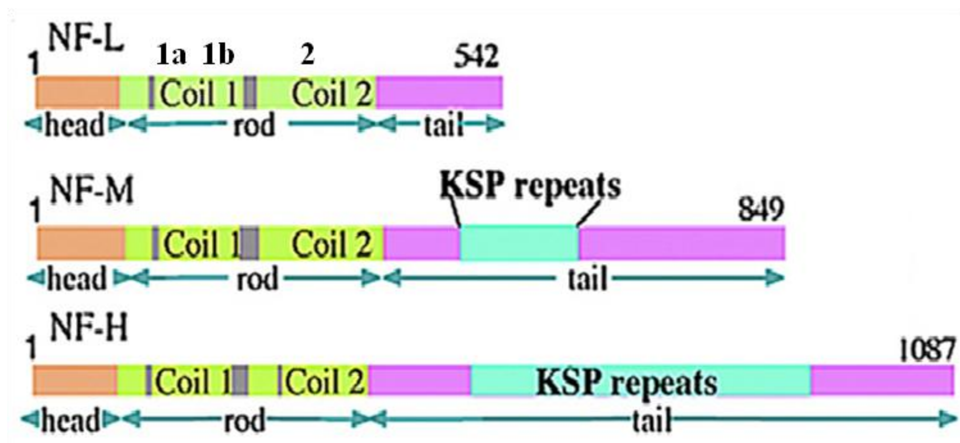


Figure 1.6: Structure of the neurofilament subunit proteins. The size of the NF fibre is 10 nm and its core made of the head and rod domains. The 'side-arms' are formed by the tail domains of NFM and NFH. Adapted from Barry et al., 2007.

The assembly of NF is believed to occur in a multistep process (Petzold, 2005). The first step of NF formation is thought to be the dimerization of NFL with either NFM or NFH subunits. This is followed by forming a dimer when the highly conserved rod domains of the NFs are coiled together in a head- to- tail shape. The next stage is the overlapping of two coiled dimers with each other in an anti-parallel, half-staggered manner to form the tetramers. Eight of formed tetramers bind to each other laterally and longitudinally to form ultimately a rope-like 10 nm filament.

Phosphorylation has been considered one of the major post-translational modifications involved in the formation and functions of NFs (Liu et al., 2004). Lasek and colleagues have stated that the vast majority of axonal NFs (80%) are highly phosphorylated representing the “static pool” (Lasek et al., 1985). The “dynamic pool” constitutes the remaining 20% of less extensively phosphorylated NFs (Lasek et al., 1985; Nixon, 1993). It is clear that the most extensively phosphorylated protein of the human brain, and possibly the entire human body, is NFH (Petzold, 2005).

Neurofilament phosphorylation and dephosphorylation are predominately regulated within the axonal compartment with the targets being different phosphate acceptor sidearms in the NFs (Nguyen et al., 2001; Dewaegh et al., 1992). The phosphorylation of NFs in vivo seems to occur in a slow manner and is orchestrated by two types of kinase enzymes, cyclin-kinases and mitogen-activated protein (MAP) kinases. The region of the phosphorylation of the C-terminal tail domain is thought to be the sidearm (Grant and Pant, 2000). In humans, there are 42 KSP (Lys-Ser-Pro) repeats forming the C-terminal tail domain (Miller et al., 2002; Strong et al., 2001; Betts et al., 1997). Serine within the KSP repeats couples with high affinity to

phosphate (Strong et al., 2001; Betts et al., 1997). Threonine is known to be an additional phosphate acceptor site with high binding affinity (Grant and Pant, 2000). Phosphorylation of the N-terminal domain is regulated by glycogen synthetase kinase-3 (Guan et al., 1991; Guidato et al., 1996), extracellular signal-regulated kinases (Roder and Ingram, 1991; Roder et al., 1995) and cyclin-dependent kinase-5 (Nguyen et al., 2001; Lew et al., 1992; Shetty et al., 1993), while the C-terminal domain is phosphorylated by a range of protein kinases (Sihag and Nixon, 1989; Sihag and Nixon, 1990; Sihag and Nixon, 1991).

With regard to axonal transport, it has been demonstrated that there is an inverse correlation between the degree of phosphorylation and the velocity of axonal transport (Watson et al., 1989; Watson et al., 1991; Nixon et al., 1994). Phosphorylation dependent dissociation of NFs from the kinesin motor has been suggested by Yabe and co-workers to be the reason for this observation (Yabe et al., 1999).

Axonal injury has been reported, through several animal studies, to be associated with an increase in the immunoreactivity or an accumulation of NFs at the sites of axonal swelling or bulbs (Saatman et al., 1998; Yaghmai and Povlishock, 1992; Chen et al., 1999). Equally, it has been demonstrated that the accumulation of NF protein at the axonal swelling site can be accompanied by a general decrease of NF immunostaining or low level of NF protein within white matter post injury (Saatman et al., 1998; Saatman et al., 2003; McCracken et al., 1999). Whereas axonal NFs showed a decrease after experimental traumatic axonal injury owing to sidearm modification, axonal injury can lead to misalignment or compaction of NFs (Yaghmai and Povlishock, 1992; Okonkwo et al., 1998; Maxwell et al., 2003). When

this data is considered together, it is not obvious then whether trauma primarily results in the pathological reorganization of NF proteins, or whether there is a secondary decrease in the level of NF protein because of proteolysis (Serbest et al., 2007).

1.3.3 Calpain

Calpains are non-lysosomal, calcium activated neutral cysteine proteases (Saatman et al., 2010). The two best characterized isoforms of calpains that are expressed in the brain are the ubiquitous calcium-regulated isoforms μ-calpain and m-calpain (Bevers and Neumar, 2008). They both exist as heterodimers, each with a common small (28 kDa) regulatory subunit and a unique large (80 kDa) catalytic subunit, and each of the large and small subunits encloses multiple calcium binding sites (Saatman et al., 2010). When intracellular free calcium levels reach a certain threshold, this can lead to the pathological activation of calpain (Pineda et al., 2004).

Physiologically, the function of calpains has been extensively reviewed (Goll et al., 2003). It has been shown that calpains are implicated in cell cycle regulation, differentiation, cell migration, adhesion, signal transduction, and synaptic plasticity (Goll et al., 2003; Wu and Lynch, 2006). Within the brain, the activated calpains have been involved in neurodegeneration associated with both Parkinson's disease and Alzheimer's disease, and acute damage due to traumatic brain injury, stroke and hypoxia (Vosler et al., 2008; Kampl et al., 1996; Saatman et al., 1996; Bevers and Neumar, 2008; Arai et al., 1991; Hiramatsu et al., 1993).

The major cytoskeletal protein component of the cell membrane is spectrin which has a molecular weight of 280 kDa (Czogalla and Sikorski, 2005). Calpain cleavage produces 150 and 145 kDa frogments of the sub-membrane cytoskeleton

protein in a sequential manner (Saatman et al., 2010). Antibodies specific to calpain-medaited spectrin breakdown products (SBDPs) can be used to recognize calpain activity through immunohistochemistry or western blotting (Bevers and Neumar, 2008).

Calpain-mediated SBDPs have been seen in the cortex and hippocampus following trauma (Saatman et al., 1996; Newcomb et al., 1997). Three models of injury, specifically lateral fluid percussion brain trauma in the rat, optic nerve stretch in the mouse, and impact acceleration injury in the rat have been used to assess the activity of calpain in injured axons: SBDPs appear within minutes after injury (Saatman et al., 1996; Saatman et al., 2003; Buki et al., 1999). The detection of calpain-mediated SBDPs in the corpus callosum after human traumatic injury has also been reported (McCracken et al., 1999). This suggests the possible role of calpain activation in axonal damage after human head trauma (Huh et al., 2006).

1.3.4 Caspase-3

Caspase-3 is part of the caspase family of cysteine proteases (Chowdhury et al., 2008). Caspase-3 activity leads to the appearance of the morphological criteria of apoptosis (Lamkanfi et al., 2007). Apoptosis is a well-ordered process that allows eukaryotic cells to degrade their DNA and nuclei. The morphological criteria of apoptosis are cell shrinkage, chromatin condensation DNA fragmentation and membrane blebbing. The final stage of this process is reformation of apoptotic bodies, the membrane-enclosed vesicles, which are then engulfed by nearby phagocytes (Shi, 2004). Two distinct pathways of caspase activation are recognized, an intrinsic, or mitochondrial, pathway and an extrinsic pathway (Gashequ et al., 2007). Apoptotic caspases are classified into two groups, the initiator caspases such

as caspase-8 and -9 and the effector caspases such as caspase-3, -6, and -7 (Chowdhury et al., 2008). The activation of executioner caspases, mainly caspase-3, appears to be involved in both apoptotic pathways (Gashequ et al., 2007).

In vitro studies using an axonal stretch injury model have shown that caspase-3 will cleave α-spectrin into the apoptotic-linked 120-kDa fragment 24 hours after moderate injury, but not following mild or severe damage (Beer et al., 2000). *In vivo* studies have shown that caspase-3 has been activated after TBI (Pineda et al., 2004). The activity of caspase-3 has been reported to increase as a result of TBI, but no effect has been observed for caspase-1 activity (Yakovlev et al., 1997). Pike and colleagues have reported the absences of caspase-3-mediated BDPs to αII-spectrin in cortex, while increasing significantly in hippocampus and striatum immediately after TBI (Pike et al., 1998). From 6 to 72 hours after controlled cortical impact injury, immunohisotochemical investigations have demonstrated that the active proteolytic subunit of caspase-3, p18, has been highly expressed in neurons, astrocytes and oligodendrocytes (Pineda et al., 2004). After lateral fluid percussion TBI in rats, caspase-3 appeared to be activated in injured cortex and hippocampus (Knoblach et al., 2002). The specific formation of caspase-3-cleaved amyloid ß-peptide fragment has been observed in injured axons, which suggest the association between the active caspase-3 and the pathogenesis of traumatic axonal injury (Stone et al., 2002) and may be linked to subsequent neurodegeneration (Johnson et al., 2010).

1.4 Wallerian degeneration

Wallerian degeneration is now considered to be a process of axonal self-destruction, similar to the process of apoptosis involving the cell body, although the mechanisms appear very different. It has been proposed that there are at least three

distinct phases in axonal degeneration: competence to degenerate; commitment to degenerate; and execution phase (Saxena and Caroni, 2007). Different insults to the axon may initiate axonal damage through different mechanisms, such as disruption of anterograde axonal flow, energy failure (mitochondrial dysfunction) and calcium influx, but it is likely that these initial mechanisms of axonal damage converge on a single terminal molecular pathway involving calpain activation with subsequent axonal protein degradation (Coleman, 2005).

Much of the understanding of the molecular mechanisms involved in Wallerian degeneration has come from studying the *slow Wallerian degeneration* mutant mouse (Wld^S) (Lunn et al., 1989). In this mouse model axon stumps distal to a point of crush injury survive up to 10 times longer than wild-type mice. The Wld^S gene is the result of a genomic re-arrangement which includes the complete protein sequence of nicotinamide mononucleotide adenyltransferase 1 (Nmnat1), a short fragment of the gene for an E4-type ubiquitin ligase, which lacks ligase activity, and a unique 18 amino acid sequence (Coleman and Freeman, 2010). It is thought this novel gene results in a gain of function of Nmnat1, probably by taking over the function of an existing protein. Nmnat2 is an endogenous protein which is rapidly down-regulated after axonal injury and recent studies suggest the novel protein generated in the Wld^S mutant is not down-regulated after injury and can take over the normal function of Nmnat2 (Gilley and Coleman, 2010). In wild-type mice axonal injury results in a decrease of nicotinamide deaminase (NAD) by unknown NAD depleting factors, which then leads to mitochondrial dysfunction, calcium influx and calpain activation, with subsequent degeneration of the axonal cytoskeleton. The Wld^S gene product may function by greatly reducing the rate of NAD depletion resulting in axonal

protection (Yan et al., 2010). This greater understanding of the active mechanisms underlying Wallerian degeneration raises the possibility of pharmacological intervention to limit the rate of axonal degeneration after trauma, although to date the precise point at which trauma activates the axonal degenerative mechanism has not been characterised.

1.5 Immunohistochemistry in forensic neuropathology practice

Several techniques have been used to identify damaged axons with variable degrees of sensitivity, dependent on survival time after head trauma (Hortobágyi and Al-Sarraj, 2008). Haematoxylin and eosin (H&E) stains can visualize damaged axons as eosinphilic swelling 24 hours after injury. They can also be detected using silver impregnation in cases with survival times of 15-18 hours (Adams et al., 1989).

Immunoreactivity of Glial Fibrillary Acidic Protein (GFAP) and CD68 were shown to identify reactive changes days after damage (Hortobágyi et al., 2007). Other proteins including ubiquitin, cathepsin D, chromogranin A and synaptosomal associated protein have been employed immunohistochemically to highlight axonal damage several hours after injury, but they have been shown to be less reliable markers (Sherriff et al., 1994a). In early stages of axonal injury, it was thought the Neuron-Specific Enolase (NSE) immunostaining could be used to detect damaged axons (Ogata.M. and Tsuganezawa.O., 1999), but this limited study looked only at sections of the corpus callosum.

It has been widely recognized that immunostaining for ß-APP is the most sensitive method for detecting TAI and most publications in human tissue use this marker (Graham et al., 2004; Sherriff et al., 1994a; Sherriff et al., 1994b; Gentleman

et al., 1995; Blumbergs et al., 1995; McKenzie et al., 1996; Geddes et al., 2000; Oehmichen et al., 1998). In 2007, Hortobagyi and co-workers demonstrated that ß-APP immunoreactivity could be used to detect TAI in the brain with survival times as short as 35 minutes after head injury (Hortobagyi et al., 2007). Previous studies have shown that the expression of ß-APP remained detectable as long as 99 days after head injury (Blumbergs et al., 1994). A number of studies have also exposed the useful application of ß-APP immunoreactivity as a diagnostic tool in medico-legal practice (Sherriff et al., 1994b; Geddes et al., 1997; Oehmichen et al., 1998; Geddes et al., 2000; Graham et al., 2004; Reichard et al., 2005; Hortobágyi et al., 2007).

Although this marker appears to be sensitive to traumatic axonal injury it is not specific, since other causes such as brain injury such as ischaemia without head injury can result in the accumulation of ß-APP, and in the setting of trauma this is often seen in cases with raised intracranial pressure (Graham et al., 2004; Geddes et al., 2000; Dolinak et al., 2000b). Increased immunoreactivity of ß-APP may also be seen in ischaemia (Dolinak et al., 2000a), hypoglycaemia (Dolinak et al., 2000b), multiple sclerosis (Ferguson et al., 1997), human immunodeficiency virus (HIV) encephalitis (Giometto et al., 1997), infarction (Nukina et al., 1992) and abscess formation (Ohgami et al., 1992).

With regard to the different patterns of ß-APP immunoreactivity, it is suggested that mechanical forces create a wave-like shape, but the ischaemic insult causes an aggregated pattern (Oehmichen et al., 2003). Graham et al have shown that an irregular or 'Z' shaped pattern can be seen in cases of infarction or haematoma (Graham et al., 2004), and ß-APP staining was stated to be linear or geographical not outlined to individual white matter bundles in ischaemia (Reichard et al., 2003a;

Reichard et al., 2003b; Dolinak et al., 2000b). Nevertheless, it has been difficult to differentiate between these types, particularly in cases that mimic the pattern of traumatic axonal injury leading to incorrect diagnosis (Reichard et al., 2005). Hence, in order to determine the cause of axonal injury in most cases, it is recommended that there should be sufficient sampling, adequate time of sample fixation, and standardization of ß-APP immunohistochemistry technique (Graham et al., 2002; Graham et al., 2004; Geddes et al., 2000; Reichard et al., 2005). However, it must be recognised that the patterns of TAI can be subtle and can be obscured by extensive ischaemic injury, and in some cases it is not possible to conclusively comment on the presence or absence of TAI.

1.6 Summary

The current opinion in relation to TAI is that the forces of injury modify focal axonal sections resulting in mechanoporation with calcium influx and microtubule disruption causing local axonal transport impairment and axonal swelling, followed by detachment over a period of time after injury (secondary axotomy). Factors against a significant mechanical effect include the fact that theses axonal changes have been identified in scattered axons associated with other normal axons and their vascular elements. Hence, it has been suggested that there are more molecular changes that can contribute to the pathogenesis of the axonal injury.

Different pathological mechanisms have been described in association with axonal damage, including Wallerian degeneration and complete energy failure. In trauma there appears to be transport failure followed by Wallerian degeneration. In ischaemia there is complete energy failure with immediate localised axonal degeneration. Axonal transport is mediated by microtubules and neurofilaments, and traditionally neurofilaments have been used as a marker of axonal swelling in experimental animals.

The first aim of the current study was to document the neuropathological features of TBI amongst Edinburgh population, although previous studies have described cohorts from the West of Scotland. Based on ethnicity there is an expectation that the current studies would be very similar to those seen in the West of Scotland, and there is unlikely to be any significant genetic or cultural influence to cause significant differences. The second aim was to investigate the presence of the different TAI subpopulations in piglet and human brains.

1.7 Hypotheses

This initial hypothesis is that range of neuropathology associated with TBI cases in Edinburgh cohort are similar to those seen in the West of Scotland cohort. The second hypothesis is that the published descriptions of the different populations of damaged axons found in rodent models are detectable in both an animal model of TBI and in human autopsy material from the Edinburgh cohort.

To address these hypotheses, there are a number of components to this study:

1. Development of a database of autopsy human traumatic brain injury cases within the archive of Department of Pathology (Neuropathology), University of Edinburgh.
2. Critically evaluate the published data relating to human traumatic axonal injury.
3. Immunohistochemical analysis of a range of antibodies investigating defined injury pathways, in an experimental model of non-impact head injury (piglet model).
4. Immunohistochemical analysis of these same pathways in human autopsy material.

2. Materials and Methods

2.1 Generation of pathological data

A retrospective cohort study was conducted using cases from the archive of the Academic Department of Pathology (Neuropathology), University of Edinburgh. The Lothian and Borders data was obtained from 406 consecutive cases between 1982 and 2005 and logged onto a database, each case being assessed according to a standardized study proforma. A proforma was developed which listed 15 sections for which entries could be coded (Appendix 1). The current proforma is based on previously used proformas and was modified after the discussion with Dr Colin Smith. Inclusion data were age, sex and the mechanism of injury. The data also included the length of survival time between injury and death, and the description of the tissue available for histological study. Pathological data included; focal pathologies namely skull fracture, contusions, intracranial haemorrhage and raised intracranial pressure: and diffuse pathologies, namely diffuse hypoxic brain damage, diffuse traumatic axonal injury (TAI) and brain swelling. Injury severity was not coded since it was not available for every case. All data was extracted from the neuropath logical reports, police reports and from the autopsy report of cases where available. All the details of the documented cases are shown in Appendix 2.

2.1.1 Skull fractures and contusions

Skull fractures were documented as being either present or absent, and contusions were initially graded using the total contusion index (TCI) developed by Adams et al (1980b) and subsequently modified (Adams et al., 1985). The TCI has been developed to assess the extent (0-3) and depth (0-4) of contusions in various

parts of the brain, producing a numerical score for each hemisphere which is then combined and interpreted as absent, mild, moderate or severe. The frontal, temporal, parietal and occipital lobes, the cortex above and below the Sylvian fissure and the cerebellum are among the different anatomical locators. The maximum score for an anatomical locator is 12 (4x3=12), and the TCI has a maximum value of 144 (each side 6x12=72, 2x72=144). Contusional injury is considered mild if the TCI is less than 20, moderate if the TCI is between 20 and 37 and if the TCI is more than 37 the contusional injury is considered to be severe (Graham et al., 1988). However, a decision was ultimately made to record the contusions as either present or absent, since no information about the extent and depth of contusions was documented in a significant number of neuropathological reports used in this study.

2.1.2 Brain swelling and intracranial haemorrhages

Brain swelling was recorded as being secondary to contusion, intracranial haematoma, ischaemic brain damage or related to a combination of different causes. Intracranial haemorrhages were documented in relation to the anatomical compartment involved (extradural, subdural or intracerebral). The volume of the intracranial haematomas and any history of surgical evacuation of the haematomas were recorded after reviewing all reports available.

2.1.3 Raised intracranial pressure

Raised intracranial pressure was documented as being either present or absent. It was considered to be present if there were tentorial herniae (either macroscopic or microscopic). This is often observed with associated vascular complications within the distributions of the anterior choroidal artery, the pericallosal artery, the posterior

cerebral artery and the arterial supply to the cerebellum and brainstem (Adams and Graham, 1976).

2.1.4 Ischaemic brain injury

Ischaemic brain injury was documented as being present or absent. Graham et al (1989) developed a grading system to assess ischaemic brain damage; severe when the lesions are diffuse, multifocal, and large within arterial territories: moderate, in which the lesions are limited to the arterial boundary zones, singly or in combination with subtotal infarction in the distribution of the cerebral arteries or if there are 6-10 subcortical lesions: and, mild if there are five or less subcortical lesions in the brain.

2.1.5 Traumatic axonal injury

Traumatic axonal injury (TAI) was recorded as being present or absent, and if present was assessed as grade 1, 2, or 3 according to the established grading system (Adams et al., 1989). In grade 1, there was widespread axonal damage in the corpus callosum and the cerebral hemisphere. In grade 2 there was, in addition, focal haemorrhagic lesion in the corpus callosum. Grade 3 lesions, in addition, had a haemorrhagic lesion in the rostral brain stem.

Diffuse vascular injury (DVI) was documented as being either present or absent. DVI is a diffuse injury in which small multiple haemorrhages are seen throughout the brain, although predominantly in the frontal white matter. The condition is usually fatal and is the result of shear stress and traction of parenchymal blood vessels (Hortobágyi and Al-Sarraj, 2008).

2.2 Human study

2.2.1 Fixation and processing of human brains

Human tissue was fixed in 10% formalin for approximately 3 weeks and then processed using a Leica ASP 300S processor (Leica, UK) with a 41 hour protocol. Tissue was embedded into paraffin wax and 5 µm sections were cut to be evaluated in the current study.

2.2.2 Identification of human cases

Forty seven cases from the completed database for the human study were found to have TAI. Haematoxylin and eosin staining and β-APP immunostaining were carried out on these cases for diagnostic purposes. Eight cases were then selected with a total of 34 sections from different regions including corpus callosum, internal capsule and pons (Figure 2.1). 4 cases were identified to have ischaemic damage and 4 cases were found to have TAI as the predominant injury. One case had an associated tentorial hernia and a second had focal cortical ischaemia. None of the TAI cases had global ischaemic injury or incidence of ischaemic axonal injury based on morphological distribution (Reichard et al., 2005). All pathological findings in these cases are summarized in Table 2.1.

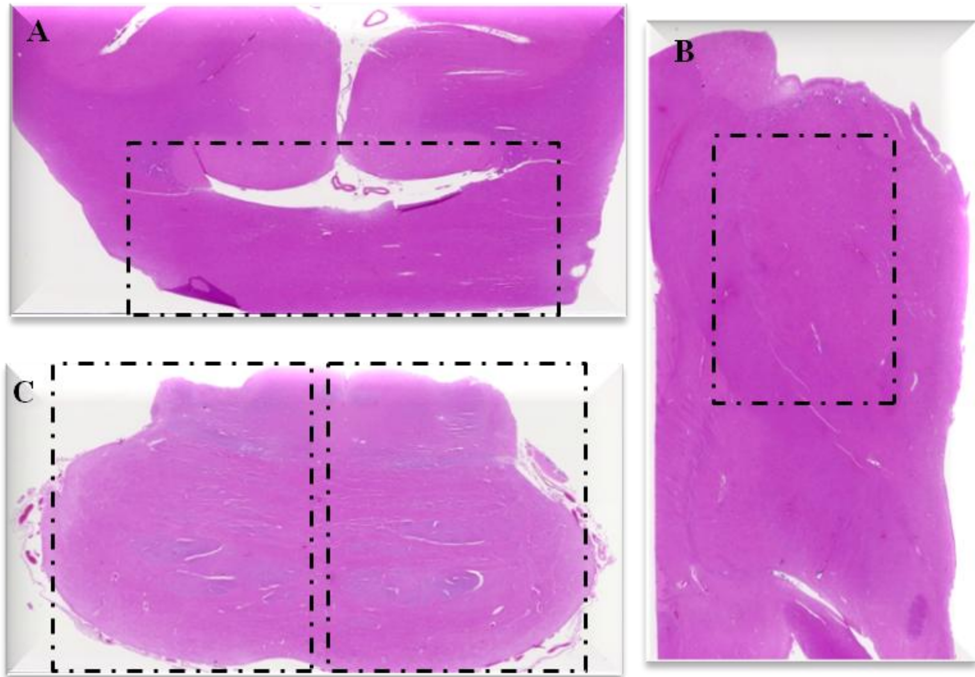


Figure 2.1: Representative images showing the examined regions in the human study. A: corpus callosum; B: internal capsule; C: pons.

Table 2.1: Details of human cases used in the immunohistochemistry study.

Case no.	Age and sex	Survival Time	Type of trauma	Fracture of skull	Contusions	ICH	Swelling	Ischaemia	No of available sections
Traumatic cases									
1	40 Male	2 days	RTA	-	-	-	-	-	4
2	41 Male	Instant	Assault	+	+	H in Pons	-	-	4
3	26 Male	12 days	Fall	-	+	ICH	+	+	5
4	32 Male	1 day	Assault	-	+	-	+	+	5
Ischaemic cases									
1	66 Male	1 day	Fall	+	+	BSDH,SAH,ICH,IVH	+	+	4
2	9 Female	7 days	RTA	-	+	SDH,SAH	+	+	4
3	4 Male	1 hour	RTA	+	+	SAH	+	+	4
4	52 Male	NA	NA	-	-	SDH	-	-	4

Legend: RTA= Road traffic accident
ICH= Intracranial haemorrhage
H= Haemorrhage
BSDH= Bilateral subdural haemorrhage
SAH= Subarachnoid haemorrhage
IVH= Intraventricular haemorrhage
SDH= Subdural haemorrhage
NA= Not available

2.2.3 Issues related to organ retention

The retention of organs at post-mortem examination and the use of retained tissue in medical research has been the focus of much public and political attention over the past ten years. The practise of removing and retaining organs at autopsy examination had been widespread and long-standing in the United Kingdom. While this was considered to be part of the normal autopsy examination by many health care professionals it became clear that many relatives who consented to autopsy examination after a death were unaware of the possibility of organ retention for diagnostic purposes and, in some cases, use of the retained tissues in research. The issue came to light during an inquiry into paediatric heart surgery at Bristol Royal Infirmary (Kennedy, 2001). At this inquiry it was disclosed that organs (malformed hearts) had been retained as part of the diagnostic process. Subsequent investigations focussed on Liverpool (Alder Hey Children's Hospital) where the practises of an individual paediatric pathologist were scrutinised (Redfern, 2001). It became apparent that there was inadequate information being provided to relatives and that the whole process of consent for autopsy examination had to be addressed. In Scotland Professor Shelia McLean headed an inquiry, the findings being published in 2001 (McLean, 2001). The medical profession responded to the recommendations made by these various reports and have improved the process of seeking and documenting informed consent such that relatives are fully aware of any retained tissues and give specific consent allowing the use of any retained tissues in medical research and education. Much of the discussion relating to retained organs focussed on autopsies that had been performed in cases outwith the authority of the Procurator Fiscal (or Coroner in England, Wales and Northern Ireland). There was, however,

considerable concern relating to retention of organs in cases which had been instructed by the Procurator Fiscal (or Coroner) and the subsequent use of tissues for medical research and education. A particularly high profile case resulted in a review of the practises by Her Majesty's Inspector of Anatomy (Metters, 2003) which coincided with a review of the Coroners system in England and Wales (Luce, 2003). There was some uncertainty in Scotland with regard to the use in medical research of tissue retained for diagnostic purposes under the authority of the Procurator Fiscal, and archived material which was retained in good faith using the appropriate mechanisms available at the time. There was a clear willingness that research which will benefit the general public should continue and as such, following a 12 month moratorium on research using human tissues during which time relatives could reclaim retained tissue if they so wished, research has been able to continue. The result of these investigations and discussions was the publication of the Human Tissue Act (2004) and the Human Tissue (Scotland) Act (2006), with activity relating to human tissues in England and Wales being overseen by the Human Tissue Authority. In Scotland all autopsy - derived tissues prior to 2006 are deemed as existing holdings, and they can be used for research with appropriate research ethical approval. All tissues derived from autopsies after this time require explicit consent from relatives for their use in research. All work undertaken in this study has full ethical approval. The local research ethics committee (LREC) number for the current study is 2002/4/36 (use of post mortem tissues for research studies).

2.3 Animal study

2.3.1 Piglet injury

Studies using a piglet animal model were performed with head rotational acceleration in the axial plane using the HYGE pneumatic actuator (Figure 2.1 and 2.2) at University of Pennsylvania with our collaborator, Professor Susan Margulies (Raghupathi and Margulies, 2002). All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Neonatal (3-5 day old) and 4-week old (corresponding in brain development and myelination to a 2-4 year old human), farm piglets were studied. All littermates were acquired from the University of Pennsylvania swine facility to ensure uniformity in birthing, handling and physical and social environment prior to arrival. Animals were subjected to rapid, purely impulsive, non-impact rotations. Brain injury was induced using a well-characterized head rotational acceleration device to impart a rapid, single 110° axial rotation with its center in the cervical spine. To achieve this motion, the animals' heads were secured to a padded snout clamp, which, in turn, was mounted to the linkage assembly of a HYGE pneumatic actuator (Bendix Corp) that converts the impulsive linear motion to an angular (rotational) motion. Axial angular velocities of 214–286 rad/sec have been used previously in the adult pig producing unconscious periods from 2 hours to over 8 hours. Applying a relationship proposed by Ommaya and colleagues (Ommaya et al., 1967) with a scaling factor defined as the inverse ratio of the brain masses raised to the one-third power, and using average brain mass in the piglet and adult, target angular velocities were developed for the 3-5 day and 4 week piglets with their smaller brains. In the 3-5 day animal angular velocities ranged from 170 rad/s (low) to 205 rad/s (high), and in the 4 week animal

from 130 rad/s (low) to 177 rad/s (high). Immediately prior to inducing brain injury the animals were taken off anesthesia. Controls (sham) were anesthetized, but were not surgically prepared and did not receive injury. Immediately following the rotational load, the snout of the animal was removed from the bite plate, and the animal was placed on heating blankets to maintain core body temperatures between 36°C and 38°C. Upon return of the pinch reflex, anesthesia was re-administered (3% isofluorane for induction and 1.5% for maintenance). When the diastolic BP fell below 25 mm Hg, animals received an intravenous fluid bolus of normal saline (10 mL/kg). Oxygen saturation was maintained at 95–99% at all times.

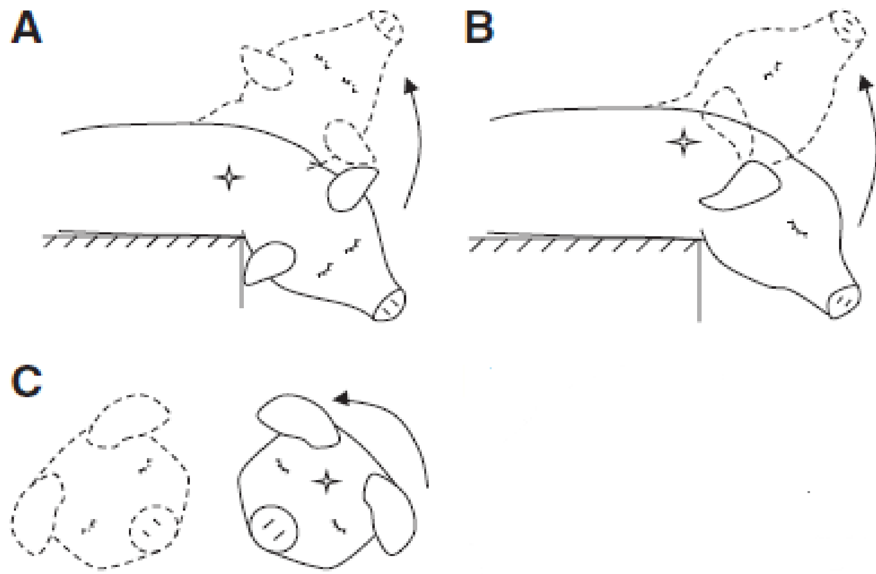


Figure 2.2: Directions of injury produced in the piglets. A= Sagittal, B= Axial, and C= Coronal. Adapted from (Raghupathi and Margulies, 2002).

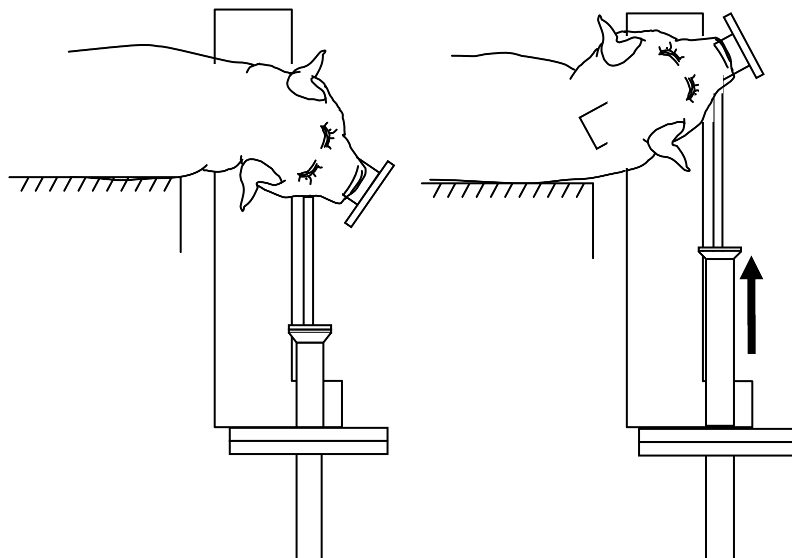


Figure 2.3: HYGE mechanism producing a non-impact controlled injury. Adapted from (Raghupathi and Margulies, 2002).

2.3.2 Piglet brain removal, fixation, and processing

The piglet was euthanized by sodium pentobarbital overdose (150mg/kg IV + small amount of heparin) and a transcardiac perfusion was performed, first with ~2L normal saline followed by 2.5-3L unbuffered formalin. The head was separated from the body and the scalp was reflected from the skull. The cranium was removed in pieces with bone rongeurs until the whole brain was exposed. The dura was then reflected and the brain including olfactory bulbs was gently teased away from the base of the skull, severing cranial nerves along the way. Once the brain was removed, photos were taken; the brain was weighed, and after that placed in unbuffered formalin for one week. After one week, the formalin was replaced with PBS until the brain was cut for pathology.

Prior to cutting, the brain was rinsed with water and photographed. It was then placed in a brain matrix and serial slices (~3mm section thickness) were cut through the length of the brain. Photographs of the slices were taken, and the slices were placed in labelled histology cassettes for processing. The tissue was processed using Shandon Excelsior ES processor (Thermo Electron Corporation, UK) with 21 hour protocol and embedded into paraffin wax. 5 µm sections were cut from the embedded blocks to be used in this study.

2.3.3 Identification of piglet cases

Sixteen cases of TAI and 11 control cases were selected from two different age groups (3-5 day old and 4 weeks old) for this study. The group of 3–5 day old piglets consisted of 6 injured cases and 4 control cases with 6 hours survival and 3 injured cases and 3 shams cases with a survival time of 6 days. A second group of 4 week old piglets consisted of 4 cases with traumatic injury and 2 control cases with a

survival time of 6 days as well as 3 injured cases and 2 sham cases with 6 hours survival. From each case, two regions were chosen to be stained and evaluated in this part of the project (Figure 2.4); the regions selected being based on previous studies which demonstrated areas of maximal traumatic axonal injury. All piglet cases used in the immunohistochemical study are listed in table 2.2.

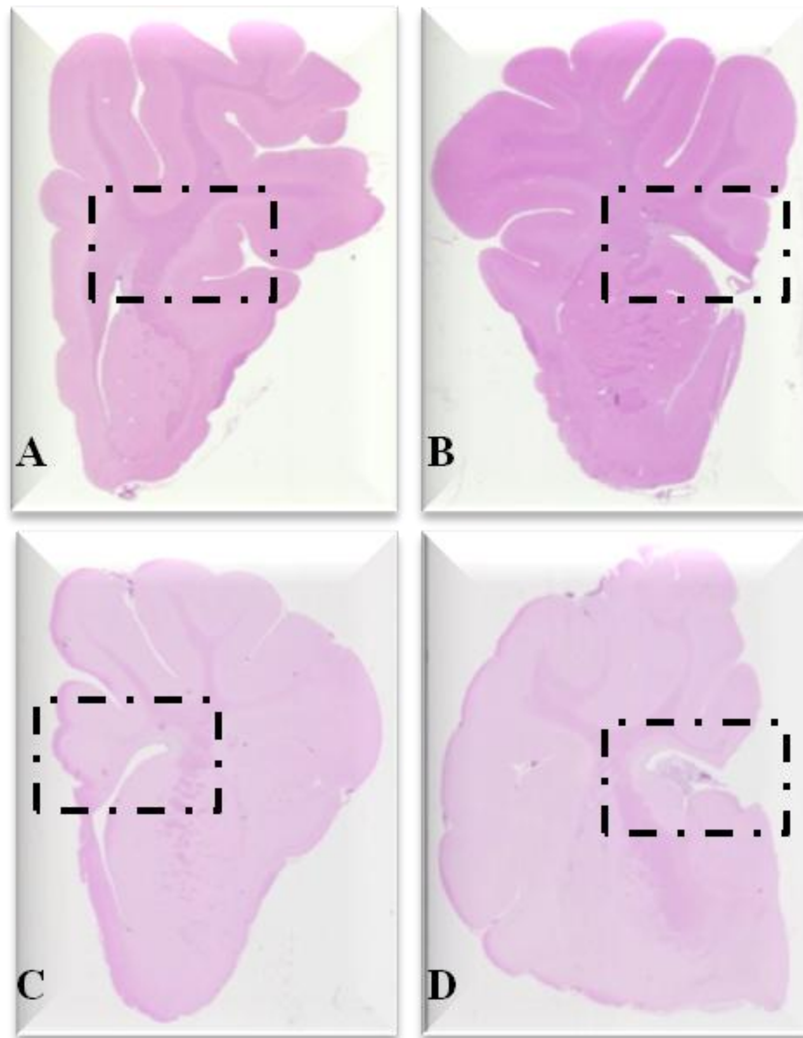


Figure 2.4: Representative images showing the regions examined in the piglet study. Coronal sections from 4-week old animal (level 6 and level 8) [A+B] and from 3-5 day animal (level 6 and level 8) [C+D]. A and C correspond to level 17.5 mm whereas B and D correspond to level 14.5 mm (Felix et al., 1999).

Table 2.2: Details of piglet cases used in the immunohistochemistry study.

	Shams		**Injured**		
	6 hrs survival	**6 day survival**	**6 hrs survival**		**6 day survival**
			Low	**High**	
3-5 day old	051220 060816	041001-1 061212-1 041123-2	070925 070912 070920	070815 051108 050119-1	050718-1 051003-1 051003-2
4 weeks old	040729 041215	060719-3 060705-2	050504 050913	050106 050111	060705-1 060719-1 060719-2

Legend: Low= Low velocity injury
High= high velocity injury

2.4 Immunohistochemistry (IHC)

2.4.1 General principles of immunohistochemistry

The concept behind IHC is the localization of antigens by means of specific antibodies that can then be demonstrated through a coloured histochemical reaction (Ramos-Vara, 2005). Immunohistochemical staining has become widely used in morphological analysis in histopathology diagnosis (Dodson, 2002). Immunostaining may be affected by factors involved in the different steps of tissue processing such as the fixation procedure, and the dehydration and embedding processes, leading to loss or variable expression of antigens. Specificity and sensitivity of the antibody, buffer solutions, detection system, chromogen and antigen retrieval system are important variables that need to be considered when optimising immunohistochemical methods (Bussolati and Leonardo, 2008).

2.4.1.1 Antigen-antibody interaction

The antibodies are immunoglobulins that consist of two heavy and two light polypeptide chains forming a Y-shaped structure (Montero, 2003). They have been identified as bifunctional molecules (Ritter, 1986), in that they have two reactive antigen-binding sites at one end (Fab region) and allow linking to other antibody, complement, or inflammatory cells through the other end (Fc region).

Monoclonal and polyclonal antibodies are the two major types of antibodies used in immunocytochemical studies. Monoclonal antibodies are mostly developed in mice whereas polyclonal antibodies are produced by antigenic stimulation in multiple animal species, particularly rabbit, horse, goat, chicken and pig (Bunea and Zarnescu, 2001). It has been reported that monoclonal antibodies have higher specificity, but lower affinity and narrower reactivity when compared with

polyclonal antibodies (Hayat, 2002). In immunohistochemical studies, high specificity and affinity for the antigen of interest and the production in high titre are considered to be the major criteria of a useful antibody. A number of features, which influence the antibody-antigen interaction, have been described including time of incubation with the primary antibody, the temperature, tissue antigens accessibility and buffer pH and ionic strength (Mao et al., 1994).

2.4.1.2 Fixation

Fixation of tissue is essential to preserve cellular morphology and antigenicity (Hayat, 2002). Two types of fixatives have been used in histopathology; cross-linking (non-coagulating) fixatives such as formalin, and coagulating fixatives such as alcoholic solutions (Bussolati and Leonardo, 2008).

The current gold standard of fixative for routine histology and immunohistochemistry is formaldehyde which maintains the general structure of cellular organelles and peptides (Montero, 2003). It has a little effect on carbohydrates and interacts with nucleic acids (Eltoum et al., 2001). Lipids are well preserved by formaldehyde if the fixative contains calcium (Jones, 2002). Formaldehyde has the ability to bind to amino acids such as lysine, tyrosine, asparagine, histidine, arginine, cystein, and glutamine if they found in solution (Shi et al., 2000). The formation of additional products between the formalin and uncharged reactive amino groups (-NH or NH_2), forming cross-links is the basic mechanism of fixation with formaldehyde (Dapson, 1993).

A profound change in the conformation of macromolecules is the ultimate result of formaldehyde fixation. This can make the detection of proteins by antibodies impossible or at best difficult due to conformational changes “hiding” the

epitope of interest (Arnold et al., 2006). These changes have an effect on the primary and secondary structures, and the three dimensional (tertiary and quaternary) structures of proteins are modified (Mason and O'Leary, 1991). The process of formalin fixation is known to be progressive, and to be temperature and time-dependent. The results of the immunostaining process can be affected by the length of fixation, by both under-fixation and over-fixation (Dapson, 1993).

2.4.1.3 Antigen Retrieval

The purpose of antigen retrieval is to recover the antigenicity of tissue sections that has been masked by the process of fixation. Enzymatic and heat-based retrieval are the two most common antigen retrieval procedures used in immunohistochemistry (Shi et al., 2001).

Antigen retrieval with enzymes: Protease-induced epitope retrieval (PIER) was the most commonly used antigen retrieval method before the advent of heat – based method's (Huang, 1975; Huang et al., 1976). Subsequent studies have used different enzymes for the same purposes including trypsin, proteinase K, pronase, ficin, and pepsin (Battifora and Kopinski, 1986; Jacobsen et al., 1980; Miettinen, 1989; Ordonez et al., 1988; Pinkus et al., 1985). Although the mechanism of PIER is still unclear, it is thought that it might be related to the non-specific cleavage of the protein (Van Hecke, 2002). There are factors that influence the effect of PIER including the duration of fixation, the concentration and type of enzymes and incubation parameters specifically, time, temperature and pH (Battifora and Kopinski, 1986; Miettinen, 1989; Pinkus et al., 1985). The drawbacks of PIER are possible alteration of tissue morphology, possible destruction of epitope, and the low

number of antigens optimally detected by this method (Ordonez et al., 1988; Van Hecke, 2002).

Heat-induced epitope retrieval: The concept of antigen retrieval by means of heating was initially introduced by Shi et al (1991). The final effect of heat-induced epitope retrieval (HIER) is the reversal of conformational modifications produced during fixation, but the mechanism is unknown (Ramos-Vara, 2005). Epitopes might be unmasked with heating via hydrolysis of methylene cross-links (Van Hecke, 2002). Other molecular mechanisms have been suggested including precipitation of proteins, extraction of diffusible blocking proteins, heat mobilization of trace paraffin and rehydration of tissue sections allowing better penetration of antibody (Sompuran et al., 2004).

Heat and antigen retrieval buffer are the two major elements of HIER (Pileri et al., 1997; Taylor et al., 1996; Shi et al., 2001). As a source of heating, microwave ovens (Shi et al., 1991) or pressure cookers (Norton et al., 1994) have been widely utilized producing the sufficiently long heating time required to achieve maximal results (Balaton, 1999). The most commonly used antigen retrieval buffers are citrate and ethylenediamine-tetraacetic acid (EDTA) (Dodson, 2002). It has been reported that the use of 0.01 M sodium citrate buffer (ph 6.0) can give acceptable results and good cellular morphology for most antigens when compared with buffer with high pH or solution containing EDTA (Battifora, 1999; Ehara et al., 1996). The pH of the solution is recognized to influence the antigen retrieval process (Shi et al., 1995).

Other antigen retrieval methods: Another antigen retrieval method reported is incubation of slides in concentrated formic acid, which seems to improve the signal in some immunohistochemical tests (Kitamoto et al., 1987). Pre-treatment

with strong alkaline solution, urea, acid solutions, borohydride and a solution of sucrose has also been used (Shi et al., 1997; Shi et al, 2000).

2.4.1.4 Detection systems

Labelling the antigen-antibody reaction is necessary to allow the reaction to be seen with light microscope. Therefore, the antigen-antibody reaction can be visualized by attaching labels (reporter molecules) to the primary, secondary or tertiary antibodies of a detection system. Fluorescent compounds, enzymes, and metals are illustrations of the variety of labels that have been used (Taylor et al., 2002). Enzymes are the most commonly used labels including peroxidise, alkaline phosphatase and glucose oxidase. In the presence of a specific substrate and a chromogen, enzymes are able to produce a coloured precipitation at the site of the antigen- antibody reaction (Ramos-Vara, 2005). There are two major types of detection systems; direct and indirect methods (Bunea and Zarnescu, 2001).

Direct methods: This is the simplest process to visualise the antigen-antibody interaction. A primary antibody conjugated with a reporter molecule (label) forming a one-step reaction (Coons and Kaplan, 1950). Fluorochromes, enzymes, colloidal gold and biotin have been used as different labels for detection via this process. Although this method is quick, its sensitivity for the detection of most antigens in routinely processed tissue is low (Haines et al., 1991).

Indirect methods: Coons et al were able to develop a two step method for more sensitive antigen detection (Coons et al., 1955). The second layer raised against the primary antibody is labelled but the first layer of antibodies is unlabelled. This method has a higher sensitivity when compared with a direct method because it has high numbers of labels per molecule of primary antibody leading to an increase in

the intensity of reaction. Further, it can produce a strong signal by retaining the activity of unlabelled primary antibody. With this process, different primary antibodies raised in the same species can also be detected with the same secondary antibody (Polak and Van Noorden, 2003).

There are different types of indirect methods employed including the avidin-biotin methods, the peroxidise-antiperoxidase (PAP) method, the polymeric labelling two-step method and the tyramine amplification method. The avidin-biotin complex (ABC) method is one of the most widely used detection systems. Avidin is a large glycoprotein extracted from egg white which has been shown to bind a low-molecular mass vitamin called biotin with high affinity. Biotin has been shown to have one binding site for avidin and can attach to antibody through other sites (biotinylated antibody) or any other macromolecules such as an enzyme, fluorochromes or other labels (Chen et al, 2010). The large number of biotin molecules that can be attached to a primary antigen can increase the sensitivity of avidin-biotin methods (Guesdon et al., 1979; Hsu and Raine, 1981; Hus et al., 1981). In the process of the ABC method, the second antibody is biotinylated and the mixture of avidin and biotin forms the third layer which links with appropriate label (Haines et al., 1991).

2.4.2 β-APP immunohistochemistry

The antibodies used in the immunohistochemistry studies are listed in table 2.3. These antibodies are known to recognise epitopes in both human and porcine tissues. Initially, optomisation of each antibody was performed using a range of antibody concentrations and different pretreatments to be able to determine an optimal condition where the antibody reaction is proper and the results are correct.

Immunohistochemistry was carried out for the marker of axonal transport dysfunction beta-amyloid precursor protein (ß-APP) (mouse monoclonal antibody; clone 22C11, cat # MAB348, Millipore, 1:12000 and 1:1000 diluted in TBS, for human and animal sections respectively). The high dilution of ß-APP antibody with human tissue is a reflection of how the NovoLink Polymer Detection System Kit is very sensitive to such antibody. Human sections were pre-treated with citric acid (1.05 g citric acid, 500 ml distilled water, pH 6) using a Menarini pressure cooker (Menarini Diagnostic Limited, Berkshire, UK) and they were stained using NovoLink Polymer Detection System Kit (cat # RE7150-K, Novocastra laboratories Ltd, Newcastle, UK). Animal sections were pre-treated with citric acid (1.05 g citric acid, 500 ml distilled water, pH 6) using a Menarini pressure cooker (Menarini Diagnostic Limited, Berkshire, UK), and then 96% formic acid (Fisher Scientific) for 5 minutes. Avidin and biotin blocking kit (Vector Laboratories, Peterborough, UK) was required to block non-specific binding of avidin and biotin for both animal and human sections before the primary antibody was applied for 30 minutes. The biotinylated antibody (polyclonal rabbit anti-mouse immunoglobulin/biotinylated antibody; Dako, 1:200 diluted in TBS) was labelled using the ABC kit (Vecta stain, Vector Laboratories, Peterborough, UK) and visualized with 3,3'-diaminobenzidine (DAB) (Vector Laboratories, Peterborough, UK).

2.4.3 NF-160 immunohistochemistry

Immunohistochemistry was undertaken for NF-160 (mouse monoclonal antibody; clone NN18, cat # N 5264, Sigma, 1:40 diluted in TBS). Animal and human sections were pre-treated with trypsin (0.1 g trypsin, 0.1 gm calcium chloride, 100 ml TBS, pH 7.8) for 20 minutes in a 37°C water bath before applying the

antibody for 30 minutes at room temperature. The biotinylated antibody (polyclonal rabbit anti-mouse immunoglobulin/biotinylated antibody; Dako, 1:200 diluted in TBS) was labelled using the ABC kit (Vecta stain, Vector Laboratories, Peterborough, UK) and visualized with 3,3'-diaminobenzidine (DAB) (Vector Laboratories, Peterborough, UK).

Table 2.3: Primary antibodies used for the immunohistochemical staining of human and piglet brain sections.

Antibody	Source	Tissue tested	Primary antibody concentration and incubation	Pre-treatment	Secondary antibody
β-APP **Mouse monoclonal**	Millipore cat# MAB348	Piglet	1:1000 30 minutes incubation	Citric acid and formic acid	Secondary biotinylated antibody (Dako)
		Human	1:12000 30 minutes incubation	Citric acid	
NF-160 **Mouse monoclonal**	Sigma cat# N 5264	Piglet	1:40 30 minutes incubation	Trypsin	secondary biotinylated antibody (Dako)
		Human	1:40 30 minutes incubation	Trypsin	
NF-200 **Mouse monoclonal**	Sigma cat# N 0142	Piglet	1:250 30 minutes incubation	Citric acid	Secondary biotinylated antibody (Dako)
		Human	1:250 30 minutes incubation	Citric acid	
SMI-34 **Mouse monoclonal**	Abcam cat# ab24571	Piglet	1:500 Overnight incubation	Citric acid	Secondary biotinylated antibody (Dako)
		Human	1:1000 Overnight incubation	Citric acid	

2.4.4 NF-200 immunohistochemistry

Immunohistochemistry was carried out for NF-200 (mouse monoclonal antibody; cat # N 0142, Sigma, 1:250 diluted in TBS). This antibody reacts with both phosphorylated and non-phosphorylated forms of NF-200, and labels the neurofilaments of molecular weight 200 kDa. It was applied for 30 minutes at room temperature after pre-treating animal and human section with citric acid (1.05 g citric acid, 500 ml distilled water, pH 6) using a Menarini pressure cooker (Menarini Diagnostic Limited, Berkshire, UK). The biotinylated antibody (polyclonal rabbit anti-mouse immunoglobulin/biotinylated antibody; Dako, 1:200 diluted in TBS) was labelled using the ABC kit (Vecta stain, Vector Laboratories, Peterborough, UK) and visualized with 3,3'-diaminobenzidine (DAB) (Vector Laboratories, Peterborough, UK).

2.4.5 SMI-34 immunohistochemistry

Immunohistochemistry was undertaken for a marker of phosphorylated neurofilaments (mouse monoclonal antibody; clone SMI 34, cat # ab24571, abcam, 1:500 and 1:1000 diluted in TBS, for animal and human respectively). This antibody recognises a phosphorylated epitope in extensively phosphorylated 200 kDa and to a lesser extent in 160 kDa neurofilament. Animal and human sections were pre-treated with citric acid (1.05 g citric acid, 500 ml distilled water, pH 6) using a Menarini pressure cooker (Menarini Diagnostic Limited, Berkshire, UK). Avidin and biotin blocking kit (Vector Laboratories, Peterborough, UK) was used with human sections and the primary antibody was applied overnight (18 hours) at 4°C for both animal and human sections. The biotinylated antibody (polyclonal rabbit anti-mouse immunoglobulin/biotinylated antibody; Dako, 1:200 diluted in TBS) was labelled

using the ABC kit (Vecta stain, Vector Laboratories, Peterborough, UK) and visualized with 3,3'-diaminobenzidine (DAB) (Vector Laboratories, Peterborough, UK).

2.4.6 Optimisation of caspase-3 and calpain antibodies

Immunohistochemistry was performed for markers of active caspase-3 (Rabbit polyclonal antibody; cat # ab2302, abcam) and calpain activation (Ab37 and Ab38, Rabbit polyclonal antibodies, both antibodies were gifts of Dr. Robert Siman, University of Pennsylvania). While the caspase antibody preferentially detects the p17 fragment of the active caspase-3, Ab37 and Ab38 recognise a proteolytic fragment of α-spectrin cleaved by activated calpain. For the purpose of optimising the antibodies, animal and human sections were pre-treated with different treatments including citric acid (1.05 g citric acid, 500 ml distilled water, pH 6) using a menarini pressure cooker (Menarini Diagnostic Limited, Berkshire, UK); EDTA (0.37g EDTA per litre distilled water, pH 8) using a Menarini pressure cooker, and trypsin (0.1 g trypsin, 0.1 gm calcium chloride, 100 ml TBS, pH 7.8) for 20 minutes in a 37°C water bath. Avidin and biotin blocking kit (Vector Laboratories, Peterborough, UK) was used with human and animal sections, whereas the primary antibodies were applied for a range of time interval including 30 minutes at room temperature, 1 hour at room temperature, and overnight (18 hours) at 4°C. The biotinylated antibody (polyclonal swine anti-rabbit immunoglobulin/biotinylated antibody; Dako, 1:200 diluted in TBS) was labelled using the ABC kit (Vecta stain, Vector Laboratories, Peterborough, UK) and visualized with 3,3'-diaminobenzidine (DAB) (Vector Laboratories, Peterborough, UK).

2.4.7 Immunofluorescence

2.4.7.1 Identification of cases for immunofluorescent study

Eighteen cases (9 piglet cases and 9 human cases) with TAI were selected for this study in which the immunoreactivities of β-APP and each marker of NFs were observed in the same anatomical region.

2.4.7.2 Double immunofluorescent staining protocol

The double immunofluorescent staining was carried out using a vector Mouse-On-Mouse (MOM) immunodetection kit (Cat # FMK-2201, Vector Laboratories Inc.) where both primary antibodies came from mice. The sections were pre-treated with citric acid using a Menarini pressure cooker and subsequently incubated with formic acid for 5 minutes. Endogenous avidin/biotin was blocked using Avidin and Biotin blocking kit (Vector Laboratories, Peterborough, UK).

Slides were incubated with the first primary antibody (β-APP, clone 22C11, cat # MAB348, Millipore, 1:100 for human and piglet sections) for 30 minutes and subsequently incubated with working solution of MOM biotinylated anti-mouse IgG as a secondary antibody for 10 minutes. The immunoreaction was visualized by Fluorescein Avidin DCS for 5 minutes.

For staining the second antigen, the human and piglet sections were incubated with the second primary antibodies (NF-160, clone NN18, cat # N 5264, Sigma, 1:40, for human and piglet sections; NF-200, cat # N 0142, Sigma, 1:250 for human and piglet sections; SMI 34, cat # ab24571, abcam, 1:500 and 1:1000 for human and piglet sections respectively) overnight at 4ºC except for NF-200 which was applied for 30 minutes at room temperature, then incubating with the same secondary antibody for 10 minutes. Texas Red Avidin DCS (Cat # A-2016, Vector Laboratories

Inc.) was applied for 30 minutes to visualize the second reaction. Slides were viewed with a fluorescence microscope and subsequently processed to adjust the black level, and image intensity was performed using Microsoft Office Picture Manager. The sections were kept at 4 ºC in the dark if not viewed immediately.

2.5 Image analysis

Image analysis is a technique that allows capture and manipulation of digital images. Image analysis was carried out to assess the immunostaining load within defined anatomical region in cases of TAI and to compare these with control cases. In human cases the regions of interest were the corpus callosum, internal capsule and pons.

2.5.1 Image capture and generation of tiled images

The morphometric study used an image analysis system consisting of a digital CCD-Camera (CoolSnap-Pro®) linking an Olympus BX 40 Light Microscope to a PC with the image analysis software (Image-Pro® Plus 6.3, Media Cybernetics).

Immunostained sections were placed on an automated stage (Prior®) which could move in both X- and Y- axes. Multiple non-overlapping colour images of the area of interest were captured using x 4 stage objective lens and the images were tiled together automatically to give a large composite image. Images were stored using the RGB 24 (red, green, blue) colour model. This system allows the production of colour images as every colour can be produced by varying levels of red, green, and blue. The brightness values range from 0 to 225 for each colour and allow colour images to be created digitally.

The composite image was stored as a data file in jpeg format. Lamp intensity, digital camera set-up, and calibration were kept constant throughout the capture of images.

2.5.2 Area of interest function

Using the stored image an ‘‘area of interest’’ (AOI) could be defined for data generation. The AOI too allowed a freehand or geometric area to be defined; the area defined was dependent on the anatomical area being studied. Each AOI was defined in terms of pixels such that the number of pixels forming the X- and Y- axes of the shape was known. The system was calibrated such that this figure could be converted to microns (μm) or millimetres (mm).

2.5.3 Assessment of immunostaining load

The image analysis software (Image-Pro® Plus 6.3, Media Cybernetics) used in this study allowed the definition of immunoreactive profiles based on a defined threshold (segmentation). Segmentation is a process that allows the isolation of certain colours from an image as a whole. In this study, the immunoreaction was developed by DAB which produces a brown precipitate. A manual segmentation technique was used to isolate the brown immunostaining in the captured image. The sections were all weakly counterstained with haematoxylin to allow greater differentiation between the brown immunostaining reaction and the bluish background. The stored images were magnified to allow greater sensitivity during the segmentation process. The colour cube-based model was used for these images. This allows a square measuring 3x3 pixels to be assessed for segmentation; this produces greater sensitivity in differentiating between an area of brown immunostaining and adjacent haematoxylin counterstaining.

While this programme allowed the threshold setting to be applied as a constant to all images this was not done due to immunostaining intensity variation between batches of immunostaining. Therefore, each slide had unique parameters set for segmentation based on the intensity of immunostaining. While this was more labour intensive it allowed greater sensitivity in the segmentation process. All results were generated by one analyst to remove an inter-observer variation. To assess intra-observer variation, the same field of the same slide was analysed at the start of each session and the load scores checked to see if they were similar. This showed less than 5% variation over a ten day period.

The ‘‘Per Area’’ function found in the measurements tool was used to determine immunostaining load within a given AOI. This gave information regarding the ratio between the area of the counted object (the immunostained area) to that of the entire area of the pre-defined AOI.

2.6 Statistical analysis

2.6.1 Analysis of the Lothian and Borders data

This study consisted of 406 autopsy cases with a diagnosis of TBI. In order to assess the common associated neuropathologies amongst cases, a pairwise comparison between groups was made using a Mann Whitney test with a Bonferroni correction.

2.6.2 Analysis of the immunohistochemistry results

Injured and sham animal cases were included in this study and each animal was assigned a specific group defined by age, injury and survival time. The initial step was to see if there was any consistency within each group. The effect of age, velocity and survival time was then evaluated. Taken together, for each antibody one-way

Analysis Of Variance (ANOVA) was used to try to answer the following questions in which a p value of less than 0.05 was considered significant:

Question 1- sham vs injured

(6 hour survival)

3-5 day old sham *vs* 3-5 day old injured (low velocity)

3-5 day old sham *vs* 3-5 day old injured (high velocity)

4 week old sham *vs* 4 week old injured (low velocity)

4 week old sham *vs* 4 week old injured (high velocity)

(6 day survival)

3-5 day old sham *vs* 3-5 day old injured

4 week old sham *vs* 4 week old injured

Question 2- effect of age

(6 hour survival)

3-5 day old injured (low velocity) *vs* 4 week old injured (low velocity)

3-5 day old injured (high velocity) *vs* 4 week old injured (high velocity)

(6 day survival)

3-5 day old injured *vs* 4 week old injured

Question 3- effect of velocity

(3-5 day old, 6 hour survival)

low velocity *vs* high velocity

(4 week old, 6 hour survival)

low velocity *vs* high velocity

Question 4- effect of survival time

(3-5 day old)

6 hour survival low velocity *vs* 6 day survival

6 hour survival high velocity *vs* 6 day survival

(4 week old)

6 hour survival low velocity *vs* 6 day survival

6 hour survival high velocity *vs* 6 day survival

For the human cases, statistical analysis was carried out to see whether there was any difference for each antibody for each region between TAI and ischaemia. This was done using parametric analysis namely the Two-Sample T-test with significance defined at $p<0.05$. Then, the two-way ANOVA test was used to look if there was difference in the staining of each antibody in three anatomical regions including corpus callosum, internal capsule and brainstem. A p value of less than 0.05 was considered significant.

3. Results

3.1 The Lothian and Borders database

This was a retrospective study based on existing data from the Academic Department of Pathology (Neuropathology), University of Edinburgh. 406 consecutive cases were selected and reviewed from 1982 to 2005. The data recorded included the case number, sex, age, survival time, type of trauma, skull fracture, contusions, intracranial haematoma, brain swelling, ischaemia and TAI. The data was gathered from the neuropathological report of each case and from the police and forensic autopsy reports if available. The intention of this study was to build a pathological database of TBI by reviewing cases with TBI, to build a picture of the common pathological findings amongst Edinburgh population.

3.1.1 Histological study

Of the 406 cases, a histological study was not done in 12 cases (3%) while limited histology was carried out in 27 cases (7%). However, there were 364 cases (90%) that had a comprehensive histological examination. The histology was unknown in 3 cases.

3.1.2 Gender and age

280 cases (69%) were male and 126 (31%) were female. Of the study cases, there were 4 cases (1%) below 12 months of age and 74 cases (7.7%) aged 80 years or older. The most common age group affected was 20-39 years (26.7%), followed by 60-79 years (25.7%), 40-59 years (20.5%), and 1-19 years (18.3%) (Figure 3.1).

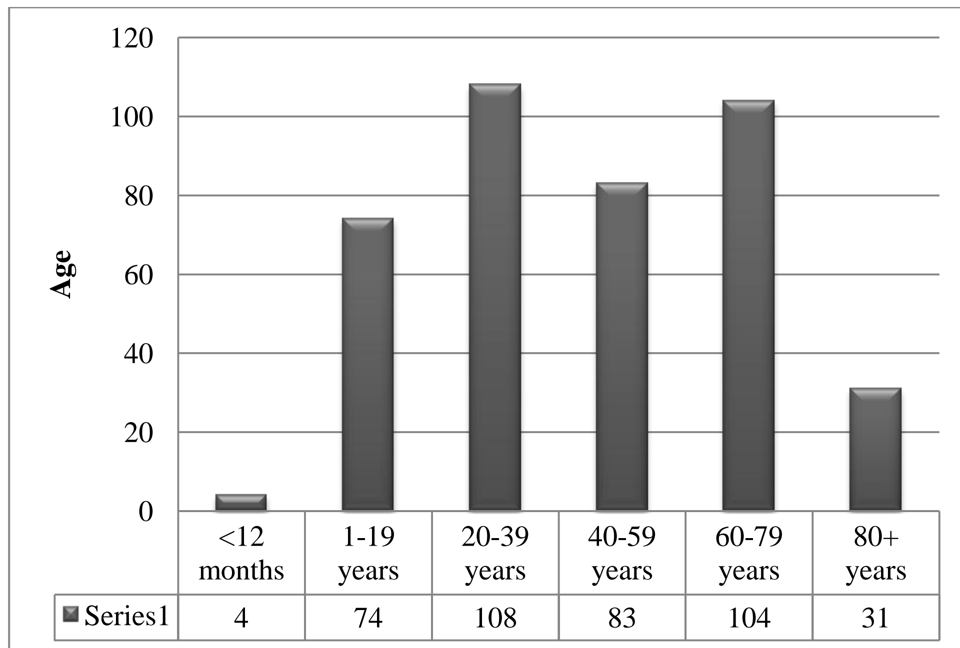


Figure 3.1: Column chart representing case distribution by age.

3.1.3 Type of injury

From a total number of 406 cases, road traffic accidents (RTA) were the leading mode of injury accounting for 48.5% (196 cases) (Figure 3.2). Other modes of injury consisted of fall from height in 99 cases (24.5%), and assault in 24 injuries (5.9%). 15 cases had different types of injury including work or home accident and gunshot. In 72 cases (17.8%), the mechanism of injury was unknown, due to limited documentation being available.

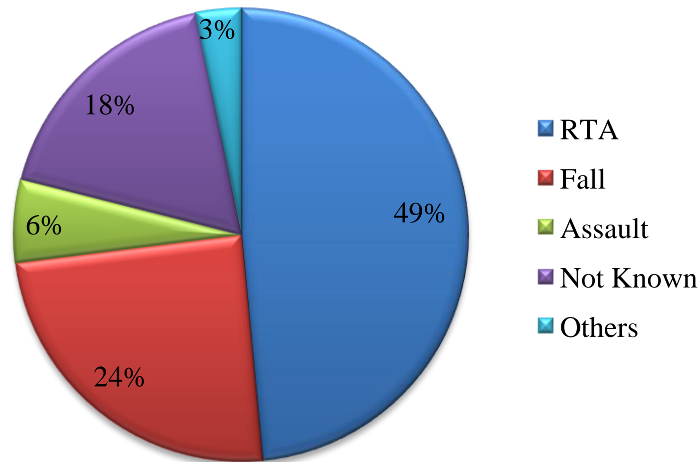


Figure 3.2: Pie chart representing the frequency of types of injury. RTA= Road traffic accidents.

3.1.4 Survival time

208 (51.4%) out of 406 cases had a survival time of less than 24 hours, amongst which 84 cases were said to have died instantaneously, 79 cases had a survival time between > 0 and ≤ 3 hours, 17 cases survived from > 3 hours up to 6 hours, 12 cases had a survival time ranging from > 6 hours up to 12 hours, 12 cases with survival time between > 12 hours and 18 hours, and in 4 cases the survival time was from > 18 hours up to 24 hours (Table 3.1).

Review of the survival times of other cases revealed that 21 cases (5.2%) were ≤ 48 hours survival, 6 cases (1.5%) were between > 48- ≤ 72 hours survival, 16 cases (4%) were between > 3- ≤ 7 days survival, 12 cases (3%) were between > 7- ≤ 14 days survival, 10 cases (2.5%) were between >14- ≤ 28 days survival, 4 cases (1%) were between > 28 days- ≤ 3 months survival, and 3 cases (0.7%) were between > 3- ≤ 6 months survival. For survivors more than one year (4.2%), 3 survived between 1and 5 years, 4 survived from 5 up to 10 years, and 10 survived over 10 years. Out of the 406 cases reviewed, the survival times of one hundred and nine cases (26.7%) were unknown due to limited documentation.

Table 3.1: Distribution by survival.

Duration of survival	Number of cases
≤ 24 hours	208
> 24- ≤ 48 hours	21
> 48- ≤ 72 hours	6
3- ≤ 7 days	16
>7- ≤ 14 days	12
>14- ≤ 28 days	10
> 28 days- ≤ 3 months	4
> 3- ≤ 6 months	3
> 6- ≤ 12 months	0
> 12 months	17
Not known	109

3.1.5 Skull fracture and contusion

281 cases (69.2%) had no skull fractures. However, a total of 123 cases (30.3%) had fractures of skull. There was no documented information about skull fracture in 2 cases. Of the 406 cases reviewed in the current study, contusions were seen in 219 cases (53.9%).

3.1.6 Diffuse ischaemic brain damage

136 cases (33.5%) were observed to have evidence of diffuse ischaemic brain injury (global ischaemia), whereas this was absent in 269 cases (66.3%). The evidence of ischaemic injury was unknown in only one case.

3.1.7 Intracranial haematomas

There were 255 individuals with a supratentorial haematoma. Of them, 5 cases (2%) had haematomas with size of ≤ 2 cm diameter, 9 cases (3.5%) had haematomas of > 2 cm diameter in size, and the vast majority of cases (89.4%) had haematomas of unknown size. There were also 13 individuals (5.1%) who had haematomas removed at operation. Amongst the cases with supratentorial haematomas, 18 cases had left sided EDH, 11 cases had right sided EDH, 78 cases had left sided SDH, and 76 cases had right sided SDH. However, in 187 cases supratentorial haematomas were localized to the subarachnoid space and were considered to be clinically insignificant.

There were 55 cases with ICH in which 33 cases (60%) had ICH of unknown size, 16 cases (29.1%) had ICH of ≤ 2 cm diameter in size, and in 6 cases (10.9%) there were ICH with size of > 2 cm diameter. The review of the areas where ICH was found revealed that such haematomas were localized to the left frontal lobe in 16 cases, the right frontal lobe in 13 cases, the left temporal lobe in 10 cases, and the

right temporal lobe in 13 cases. ICH was also seen in the left parietal lobe in 5 cases, in the right parietal lobe in 4 cases, in the left occipital lobe in 3 cases, and in 4 cases ICH was seen in the right occipital lobe. In 10 cases ICH was localized to the basal ganglia. Out of the total cases in the current study, only four individuals were identified with a burst lobe. Haemorrhage in the infratentorial region was seen in 5 cases.

Taken together, the current review revealed that 270 out of 406 cases had some form of haematoma wherein the majority of cases (88.2%) developed haematomas of unknown size, 11 cases (4.1%) had haematoma of more than 2 cm diameter in size, and haematoma with size of ≤ 2 cm diameter was seen in 8 cases (3%).

3.1.8 Raised intracranial pressure

This study showed that 186 cases had some pathological indication of raised ICP, amongst which 180 cases (96.8%) were diagnosed macroscopically, 3 cases (1.6%) were diagnosed microscopically, and in 3 cases there were microscopic and macroscopic descriptions of raised ICP. Out of 180 cases, 34 had supracallosal hernia (18 left only, 11 right only and 5 bilateral), 162 had tentorial hernia (24 left only, 13 right only and 125 bilateral), and tonsillar hernia was seen in the right side in only 65 individuals.

Raised intracranial pressure pathology was associated with secondary brainstem haemorrhage in 34 cases. Secondary brainstem haemorrhage was identified grossly in 22 cases (64.7%), 8 cases (23.5%) were diagnosed microscopically and in 4 cases (11.8%) there were macroscopic and microscopic evidences of such haemorrhage. In 217 cases, no pathological evidence of increased ICP was observed. Three case had no available information about raised ICP.

3.1.9 Diffuse traumatic axonal injury and acute vascular injury

The records of all 406 cases revealed that 360 cases (88.6%) had no evidence of TAI. However, TAI was seen in 46 cases of which 41 cases (10.1%) had grade 1, only one case (0.2%) was considered to have grade 2, and grade 3 was seen in 4 cases (1%).

Diffuse vascular injury was seen in 30 cases which accounted for 7.4%, and absent in 376 cases (92.3%).

3.1.10 Brain swelling

Of the 406 cases examined, 129 cases (31.8%) had no brain swelling. There were 274 cases with bilateral brain swelling and 3 cases with unilateral brain swelling. In cases with brain swelling, the causes of the brain swelling were described as being secondary to contusions in 21 cases (5.2%), intracranial haematoma in 92 cases (22.7%), ischaemic brain damage in 11 cases (2.7%), and a combination of pathologies in 98 cases (24.1%). The cause of the brain swelling could not be ascertained for a number of cases.

3.2 Critical evaluation of TAI literature

TAI remains an evolving concept, and as such the historical literature needs to be assessed critically. The older literature, prior to the introduction of immunohistochemistry, was based on the identification of axonal spheroids using silver stains and H&E stains. It is well established that such changes require many hours to develop and are much less sensitive than immunohistochemistry, which can identify axonal flow dysfunction within 35 minutes (Hortobagyi et al., 2007). We reviewed several large cohorts published by the Glasgow group, recognised experts in the field of TAI diagnosis, and compared these with the Edinburgh cohort. The data is presented in table 3.2.

Table 3.2: Data regarding the incidence of TAI across 4 separate cohorts. As discussed in the text, different methods of identification of TAI were used across the cohorts.

TAI grade	1968-1972 (Adams et al 1977)	1981-1982 (Graham et al 1989)	1968-1982 (Adams et al 1989)	1987-1999 (Smith 2001)	1982-2005 Edinburgh cohort
Absent	130 (86%)	75 (67%)	312 (71%)	134 (59%)	359 (88.75%)
Grade 1			10 (2%)	38 (17%)	41 (10%)
Grade 2			29 (7%)	16 (7%)	1 (0.25%)
Grade 3			83 (20%)	38 (17%)	4 (1%)
Present but not graded	21 (14%)	37 (33%)	(total 29%)	(total 41%)	(total 11.25%)

This table clearly demonstrates how the sensitivity of identification of axonal dysfunction has improved since the introduction of immunohistochemistry. The cohorts reported between 1968 and 1982 were based on histological examination of both celloidin and paraffin blocks, and used palmgren staining on the paraffin sections, a silver stain technique which requires survival of 12-18 hours before damaged axons can be seen. Identification of axonal injury in celloidin sections is difficult and no specific stains were developed. This technique is now no longer used.

Damaged axons are seen in greater numbers of cases when immunohistochemistry is employed (1987-1999 Glasgow cohort and the 1982-2005 Edinburgh cohort). However, a significant difference is seen between the most recent Glasgow cohort (1987-1999) and the Edinburgh cohort. All cases in the Edinburgh cohort used histological criteria to differentiate between ischaemic and traumatic axonal injury. This data suggests that older immunohistochemistry studies were incorrectly identifying ischaemic axonal inujury as TAI, and this has been recognised in other published studies (Smith et al., 2002; Smith et al., 2003). However, previous published data in Glasgow should be reviewed immunohistochemically to confirm such suggested misinterpretations. This review clearly highlights how important it is to critically evaluate historical literature, particularly as new techniques are developed and new concepts evolve. It is likely that the development of new protocols for the immunohistochemical analysis of TAI will further modify our understanding and diagnosis of this entity. The presence of an uneven accrual rate of cases over the timeframe is also noticed which might be explained by the different regulations of the procurator fiscal practice throughout the years.

3.3 Immunohistochemistry Results

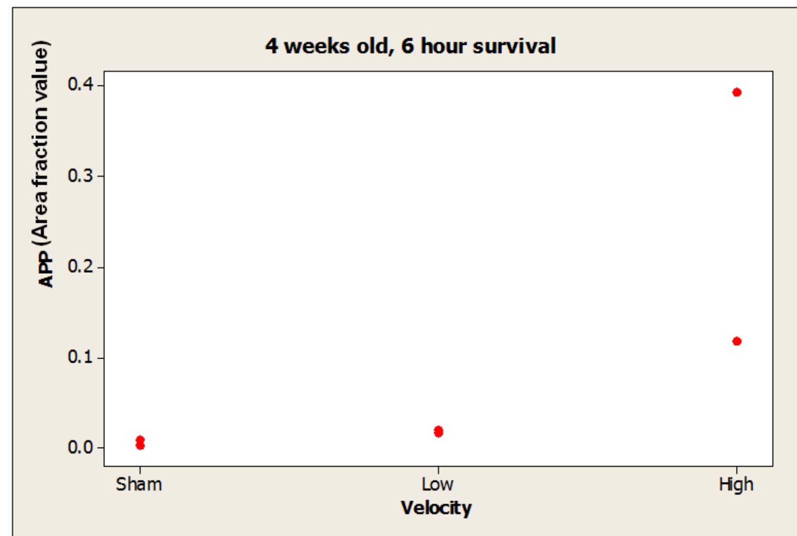
3.3.1 Immunoreactivity profile of β-APP and neurofilament markers in piglet

Immunohistochemistry for β-APP, NF-160, NF-200 and SMI-34 was undertaken on selected sections for the piglet study looking at specific groups defined by age, injury, and survival time. The first part of the study looked to see if there were differences in immunoreactivity between the sham and injured groups. The effects of age, velocity, and survival time among injured piglets were then investigated based on the immunostaining of the listed antibodies. The last part of the animal study aimed to assess the association between the presence of axonal swellings and /or axonal bulbs with the immunoloading for each antibody, comparing the control and trauma cases.

3.3.1.1 Sham v injured piglets

A comparison of sham cases and injured cases immunostained for β-APP revealed significantly increased β-APP immunoreactivity in the 4 week old injured piglets ($p=0.01$) when compared to sham (Figure 3.3), but no significant difference was noted between other groups.

NF-160, showed decreased immunostaining in the 3-5 day old injured animals when compared to control (sham) animals ($p=0.02$) (Figure 3.4). SMI-34 immunoreactivity was increased in the 3-5 day old piglets with a low velocity impact (6 hour survival) when compared to control cases ($p=0.03$) (Figure 3.5). There was no significant difference between groups when the NF-200 was used.



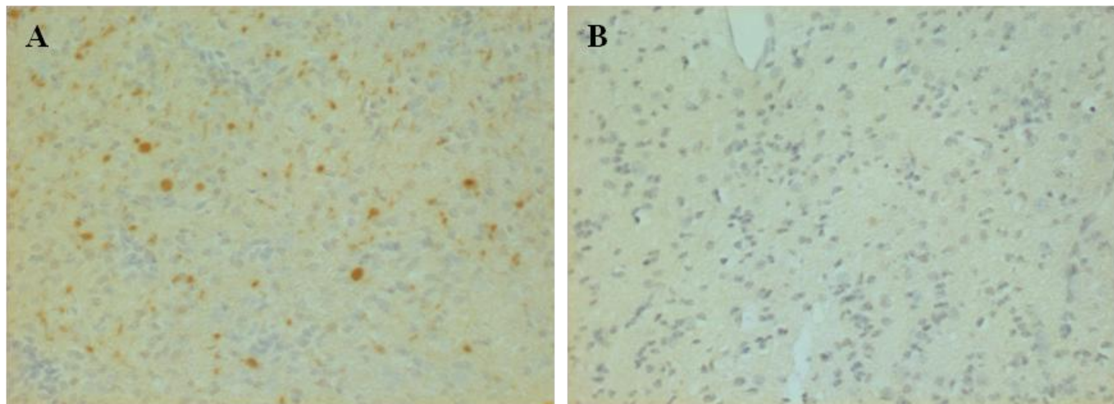
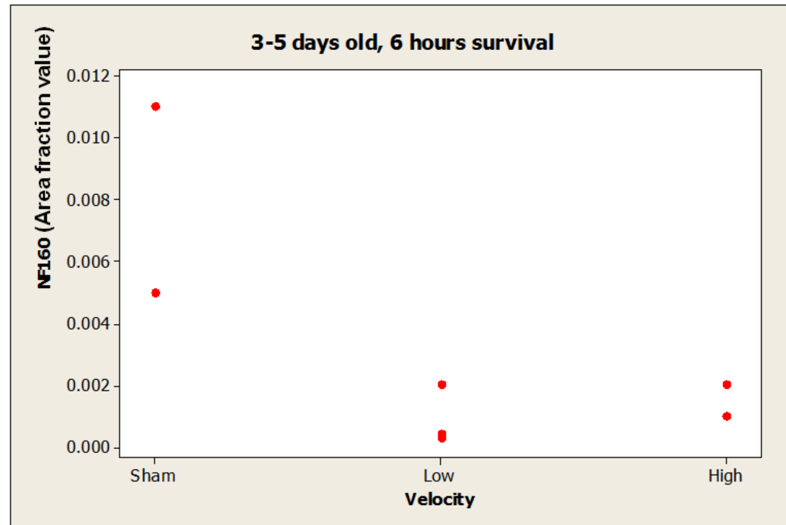
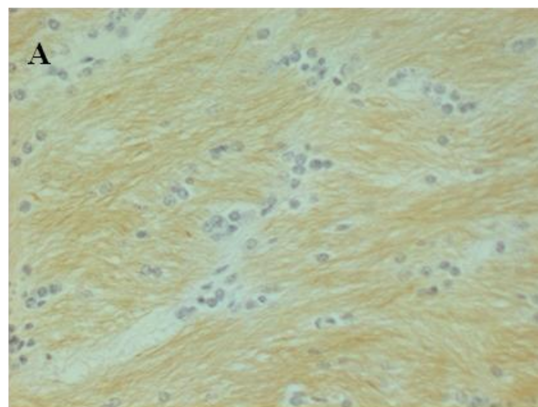
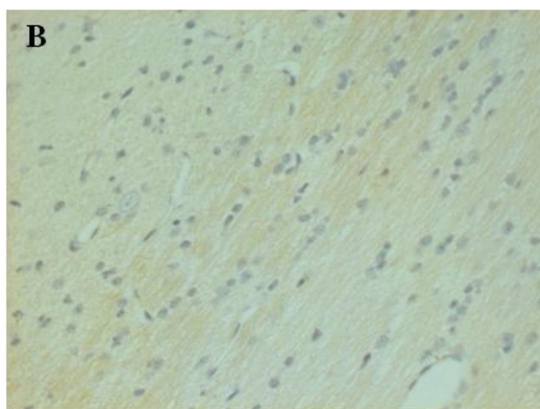


Figure 3.3: Accumulation of β-APP in injured and sham piglets. Plot with representative images demonstrating increase β-APP immunoreactivity in the 4 week old piglets with TAI (A) compared to the sham piglets (B). β-APP immunostaining x20.







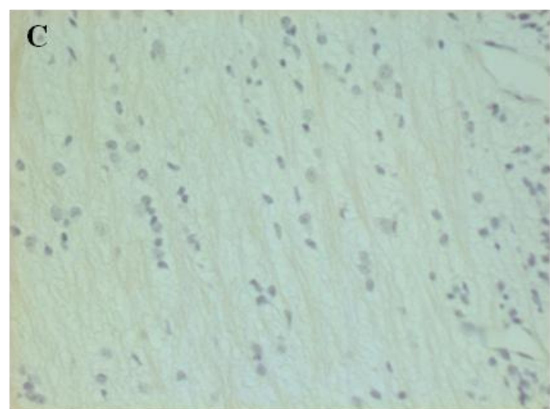


Figure 3.4: Accumulation of NF-160 in injured and sham piglets. Plot with representative images demonstrating increase NF-160 immunoreactivity in the 3-5 day old sham piglets with 6 hours survival (A) compared to the low (B) and high (C) velocity injured piglets. NF-160 immunostaining x20.

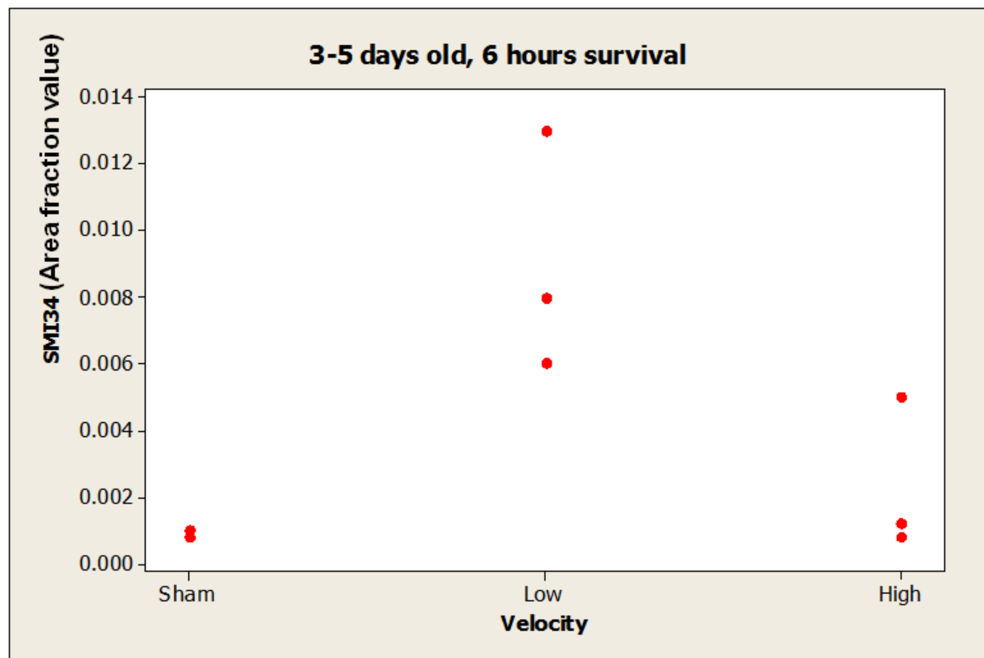


Figure 3.5: Accumulation of SMI-34 in injured and sham piglets. Plot represents increased SMI-34 immunostaining after low velocity injury in the 3-5 day old piglets with 6 hours survival compared to the sham piglets.

3.3.1.2 Effect of age

One-way ANOVA did not show any significant differences in β-APP, NF-160, NF-200 or SMI-34 immunoreactivity between 3-5 day and 4 week old injured animals. The effect of age on the immunostaining for each antibody is illustrated on figure 3.6.

3.3.1.3 Effect of velocity

None of the antibodies gave a significant result for the one-way ANOVA when comparing low and high velocity injury in the 3-5 day old group. In the older injured group (4 week old), there was no significant difference between low and high velocity injury according to the immunoload of NF-160, NF-200 and SMI-34 (Figure 3.7). Iincreased immunoreactivity of β-APP was observed in piglets sustaining high velocity injury but it did not reach significance (Figure 3.8).

3.3.1.4 Effect of survival time

One-way ANOVA with Dunnett's test was used to test the effect of survival time comparing 6 hours survival against 6 day survival. The current study revealed that there were no significant differences in the immunostaining for any of the antibodies in either the 3-5 day old cases or 4 week old cases (Figures 3.9 and 3.10).

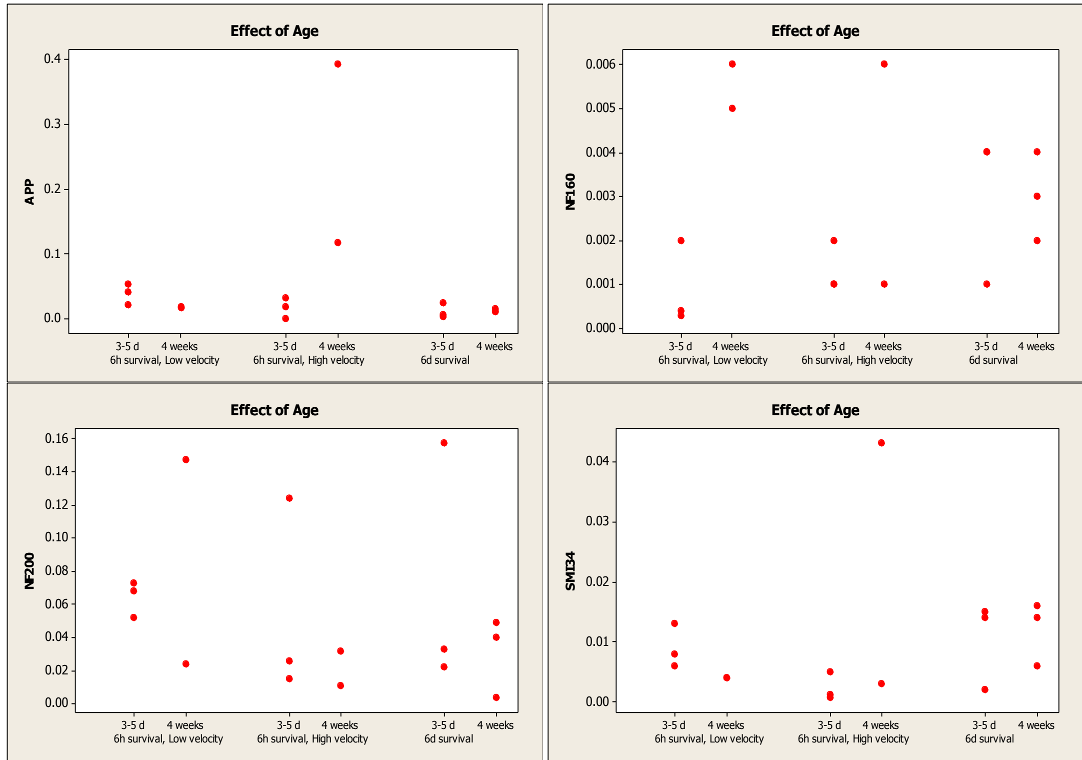
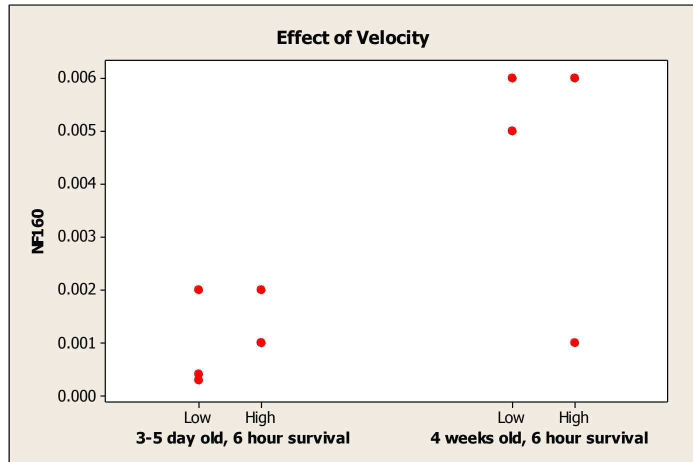
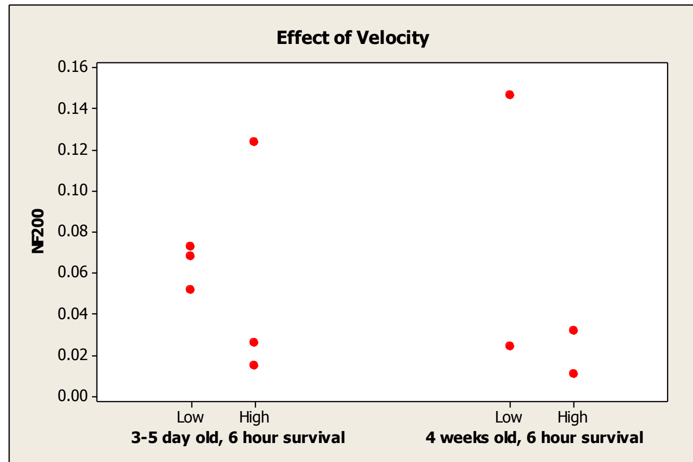


Figure 3.6: Effect of age on β-APP, NF-160, NF-200 and SMI-34. The y-axis demonstrates the area fraction value.





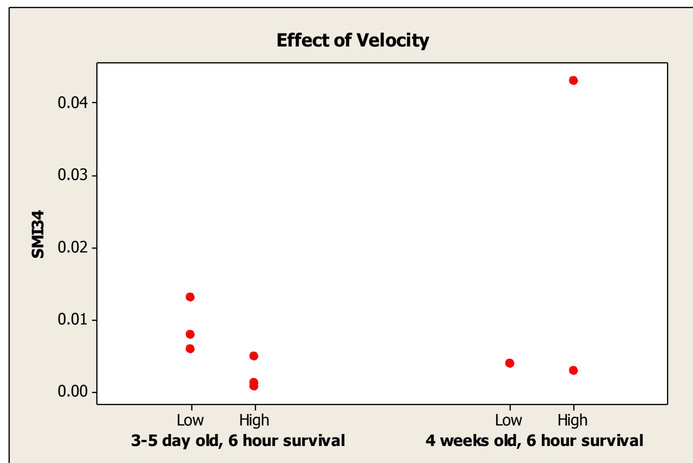
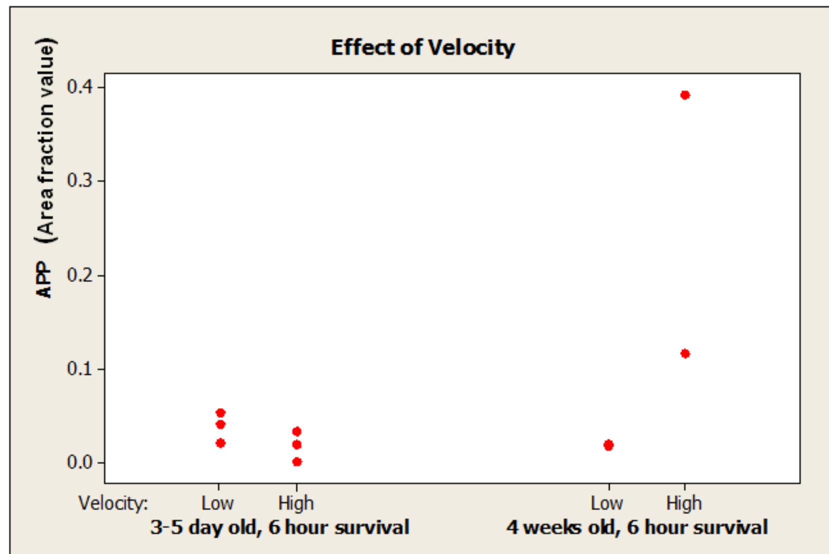
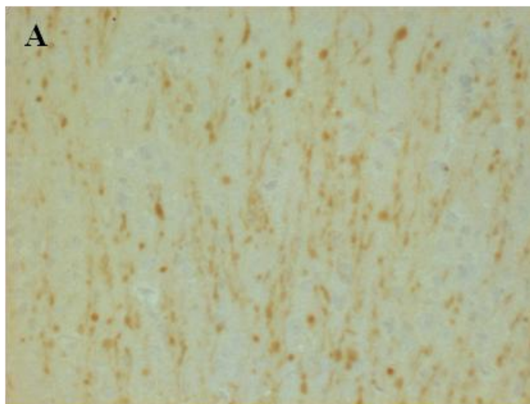


Figure 3.7: Effect of the velocity of injury on NF-160, NF-200 and SMI-34.

The y-axis demonstrates the area fraction value.





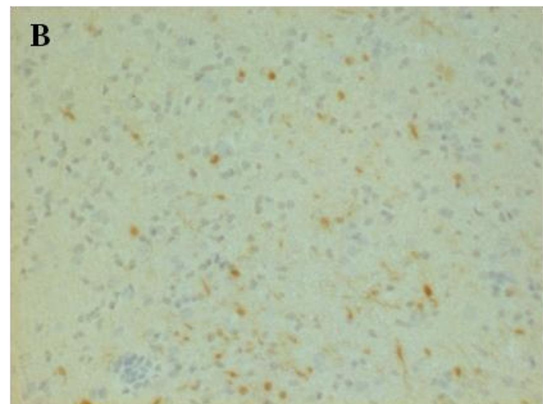


Figure 3.8: Effect of velocity on β-APP immunoreactivity. Plot with representative images demonstrating the accumulation of β-APP in the brains of piglets after high (A) and low (B) velocity injury. β-APP immunostaining x20.

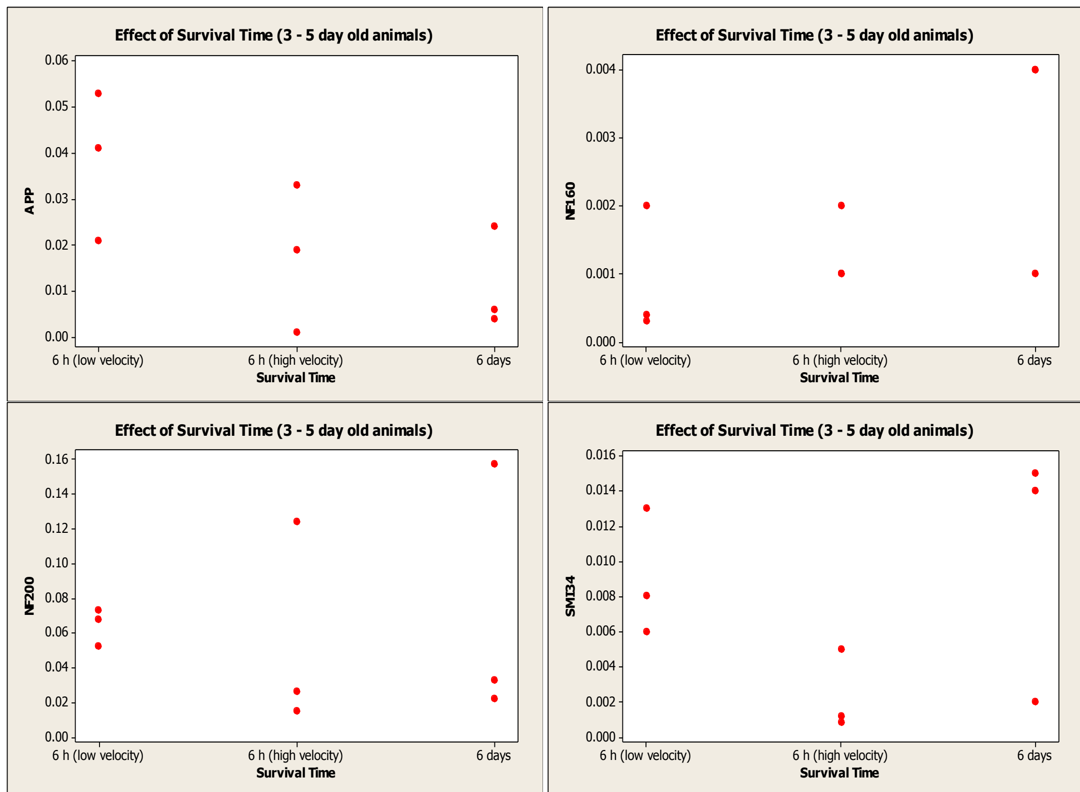


Figure 3.9: Effect of survival time on β-APP, NF-160, NF-200 and SMI-34 in the 3-5 day old piglets. The y-axis demonstrates the area fraction value.

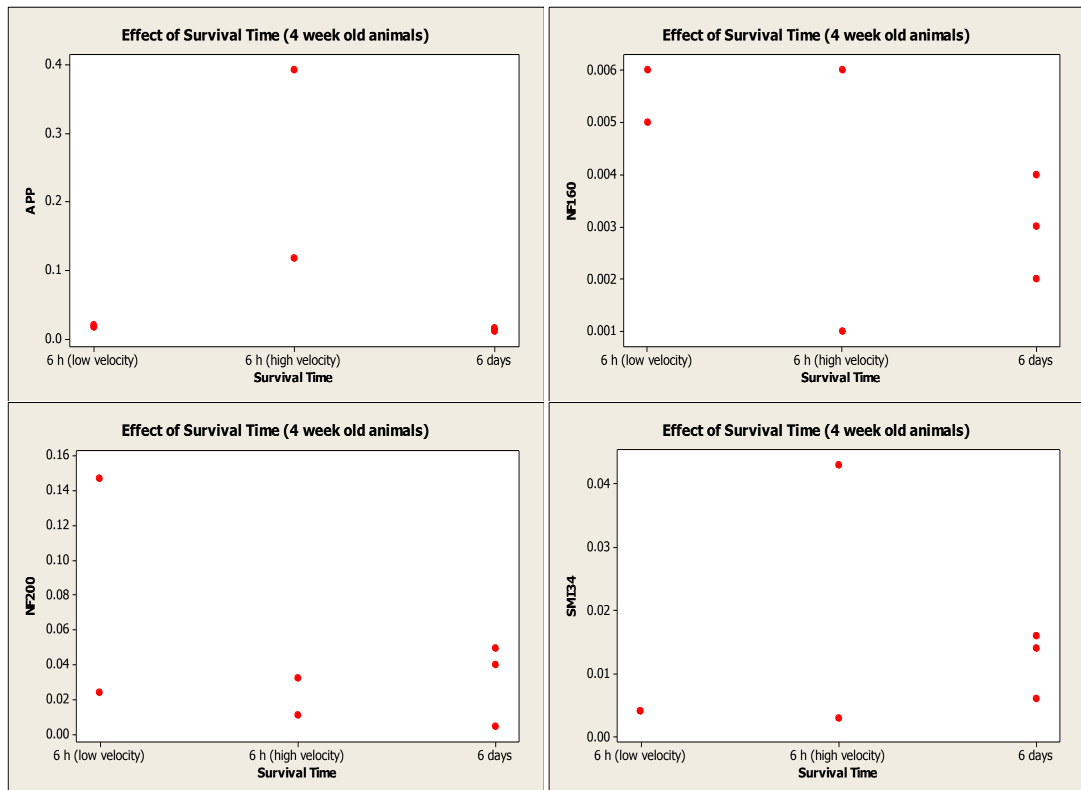


Figure 3.10: Effect of survival time on β-APP, NF-166, NF-200 and SMI-34 in the 4 week old piglets. The y-axis demonstrates the area fraction value.

3.3.1.5 Correlation of the antibodies with the presence of axonal swellings and terminal bulbs

β-APP positive axonal swellings or terminal bulbs were not seen in any sham cases. However, a majority of injured piglets showed evidence of β-APP positive axonal swelling and/or axonal bulb formation, this pathology being seen in 10 out of 16 cases. Control piglet brains exhibited normal staining for NF-160 and NF-200 proteins. Axonal swelling and retraction bulbs, indicative of axonal injury were observed less commonly (5 out of 16 animals) in the brains of injured piglets when immunohistochemistry for NF-160 and NF-200 was employed. Normal patterns of immunoreactivity for the SMI-34 antibody were demonstrated in control brains, indicated by the absence of axonal swellings and terminal bulbs. Interestingly, axonal swellings were highlighted by SMI-34 immunohistochemistry in 2 out of 16 injured piglets. The various immunoreactivities of the different antibodies within the axonal swellings and axonal bulbs are shown in figure 3.11.

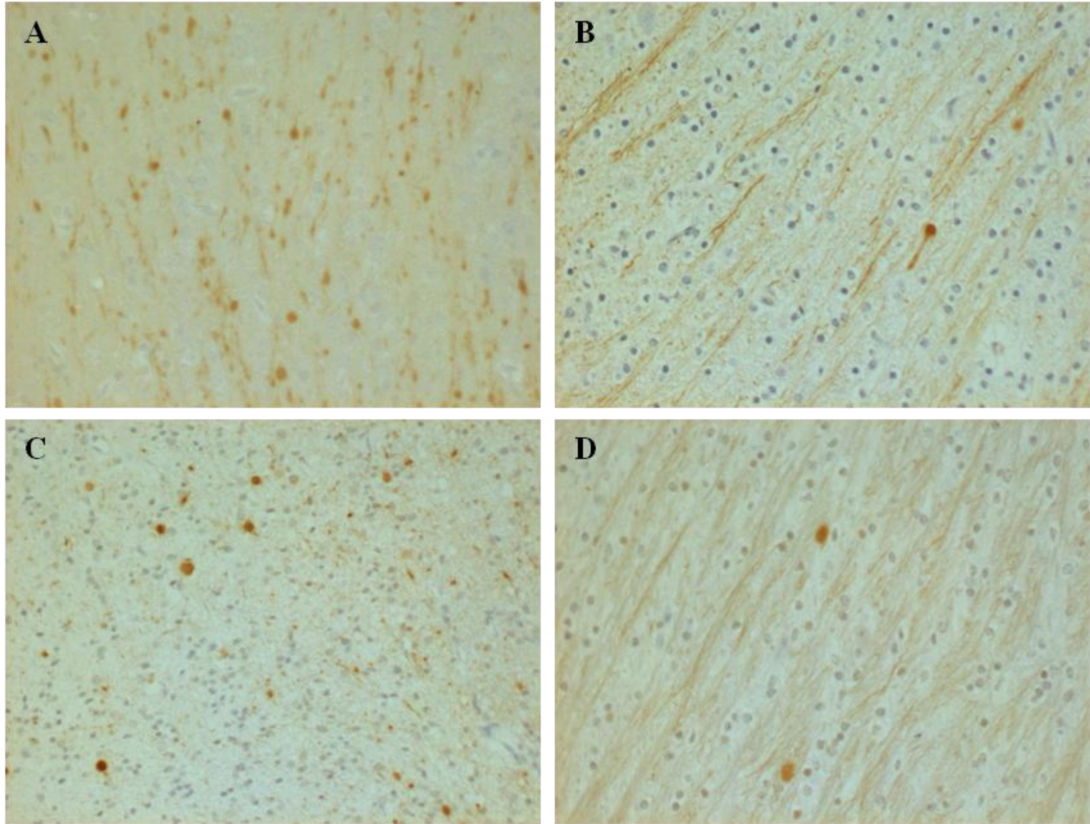


Figure 3.11: Traumatic evidences of axonal injury. Representative images demonstrating the range of TAI, based on the presence of axonal swelling and terminal bulbs. TAI is detected using β-APP (A), NF-160 (B), NF-200 (C) and SMI-34 (D). Immunostaining of all markers x20.

3.3.2 Immunoreactivities of β-APP and neurofilament markers in human

3.3.2.1 Trauma vs ischaemia

To assess the differences between ischaemic cases and cases with TAI, an antibody panel including β-APP, NF-160, NF-200 and SMI-34, was applied to trauma cases and to ischaemic cases. Boxplots for each of the antibodies in each region (corpus callosum, internal capsule and brain stem) for traumatic and ischaemic cases are shown (Figures 3.12 and 3.13). Statistical analysis of the immunohistochemistry results revealed no significant differences when comparing TAI cases with ischaemia cases.

3.3.2.2 The anatomical distribution of immunoloading in the brain

The human study assessed the distribution of the immunoreactivity of the different markers in each tested region (corpus callosum, internal capsule and brain stem) and comparisons were made between traumatic and control cases. The level of β-APP immunostaining was significantly increased ($p=0.004$) in the brain stem compared with corpus callosum or internal capsule (Figure 3.14). The brain stem displayed elevated NF-160 immunostaining when compared to other regions, but did not reach statistical significance (Figure 3.15). There were no significant regional effects when NF-200 and SMI-34 antibodies were analyzed (Figure 3.16).

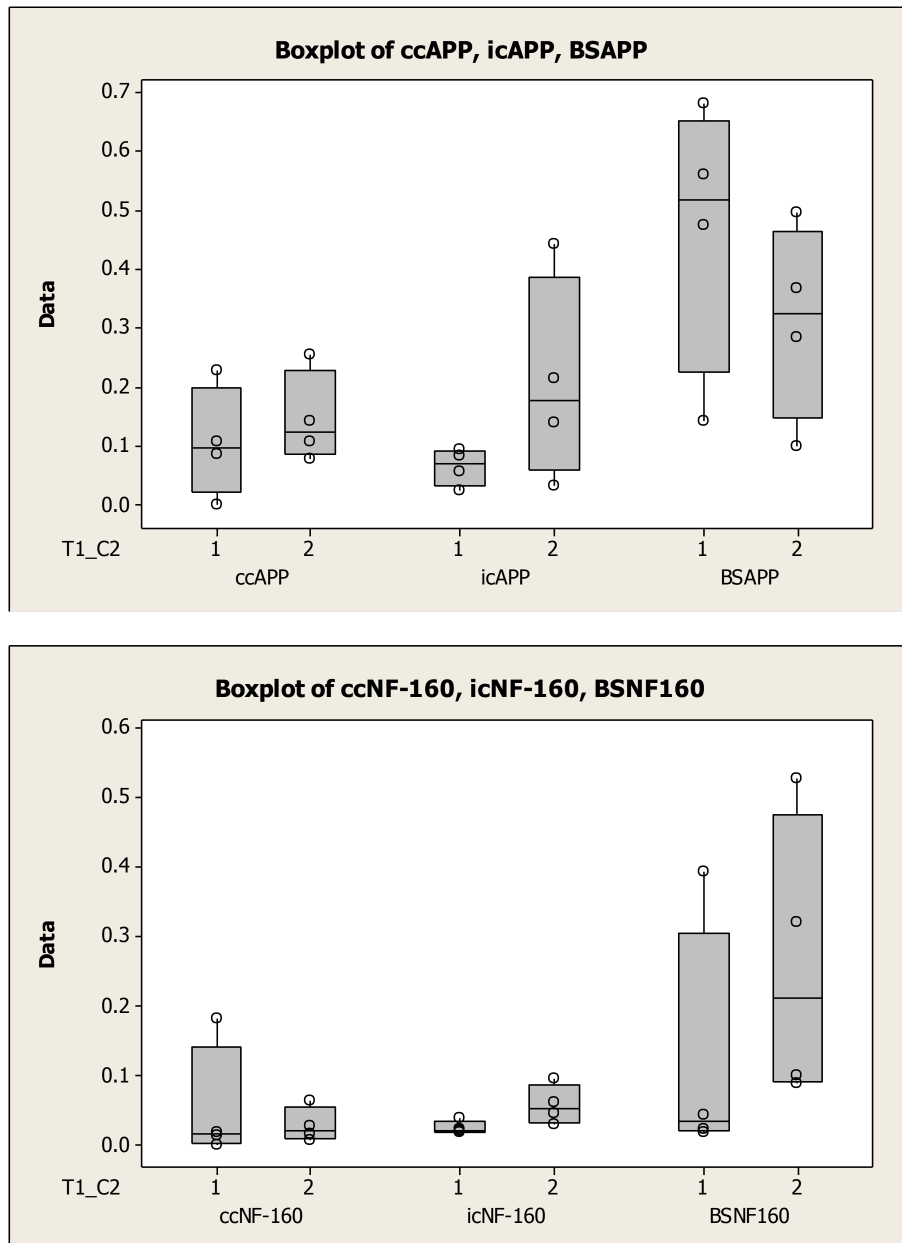


Figure 3.12: Accumulation of β-APP and NF-160 after TAI and ischaemia. Boxplots for β-APP and NF-160 antibodies in different regions of traumatic and control cases. 1= T1 (TAI), 2= C2 (Control), cc= corpus callosum, ic= internal capsule, BS= brainstem.

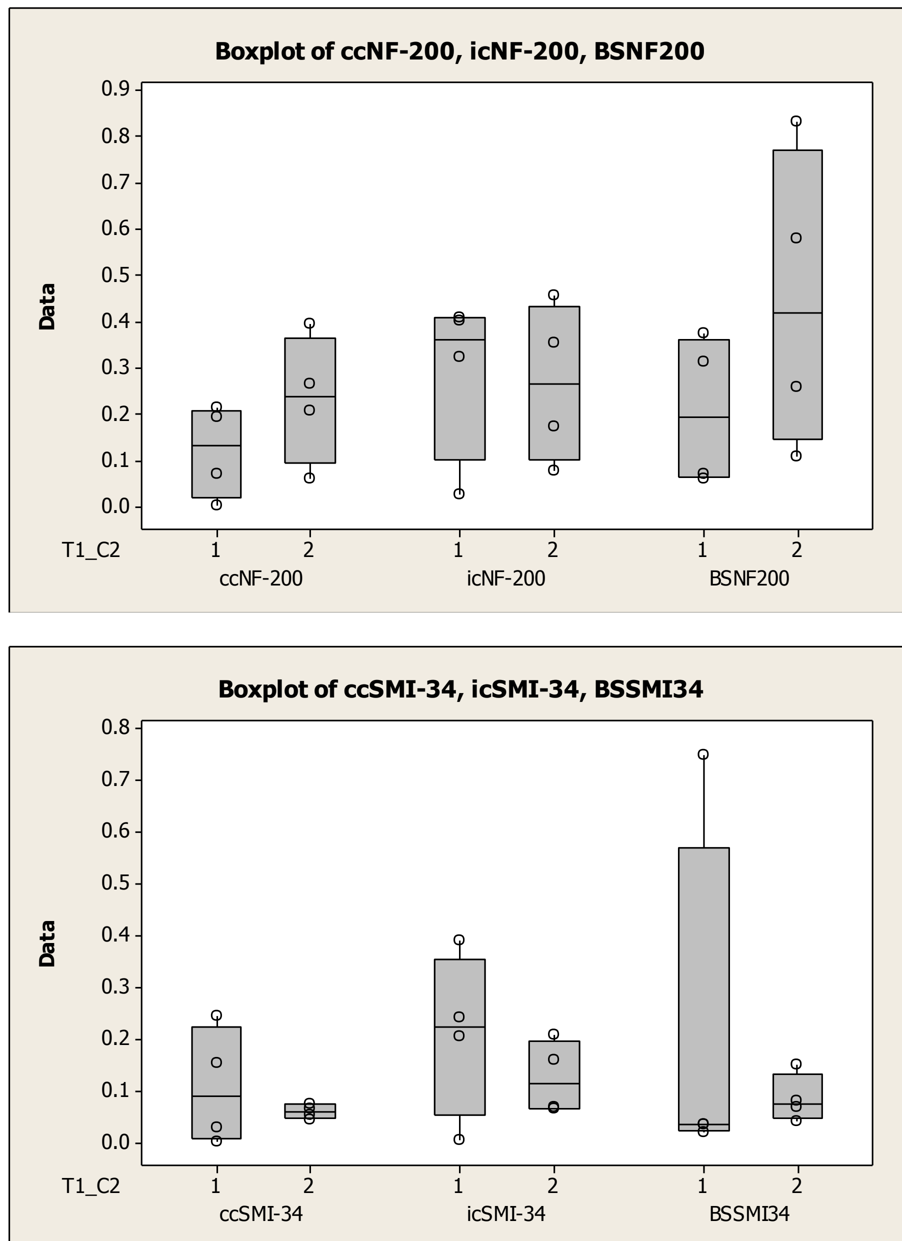
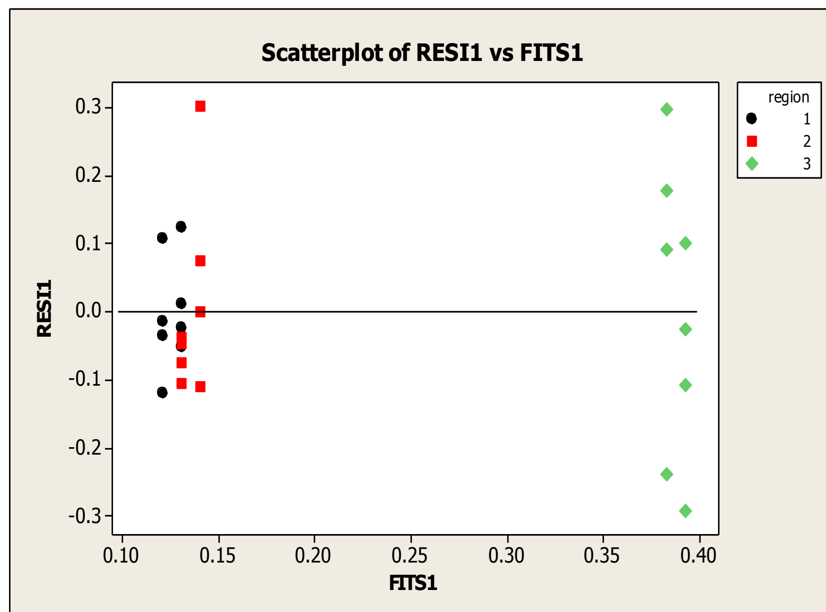
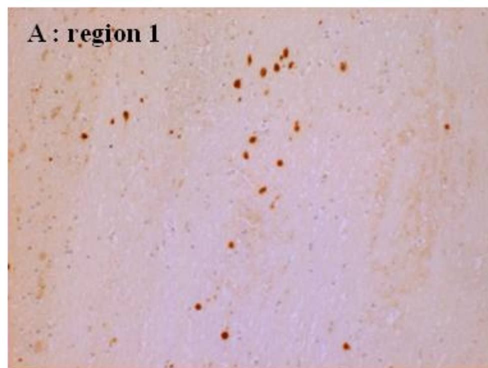
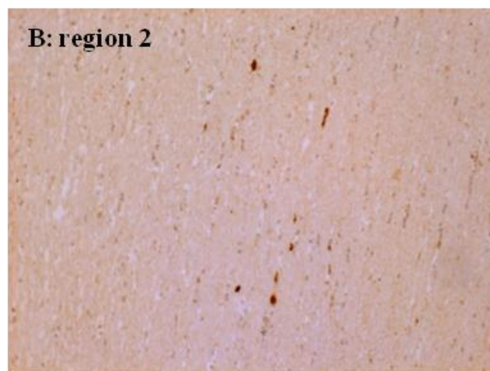


Figure 3.13: Accumulation of β-APP and NF-160 after TAI and ischaemia. Boxplots for NF-200 and SMI-34 antibodies in different regions of traumatic and control cases. 1= T1 (TAI), 2= C2 (Control), cc= corpus callosum, ic= internal capsule, BS= brainstem.







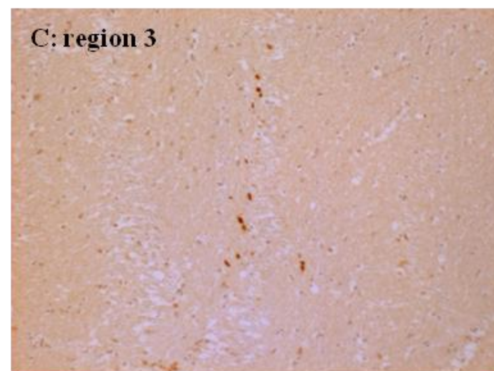


Figure 3.14: Anatomical distribution of β-APP immunostaining.

Scatterplot of β-APP immunoreactivity in three regions for each case, with representative images showing higher accumulation of β-APP in pons (A) when compared to corpus callosum (B) or internal capsule (C) of the same case. 1= corpus callosum, 2= internal capsule, 3= brainstem, FITS1=fitted value, RESI1=residual error. β-APP immunostaining x10.

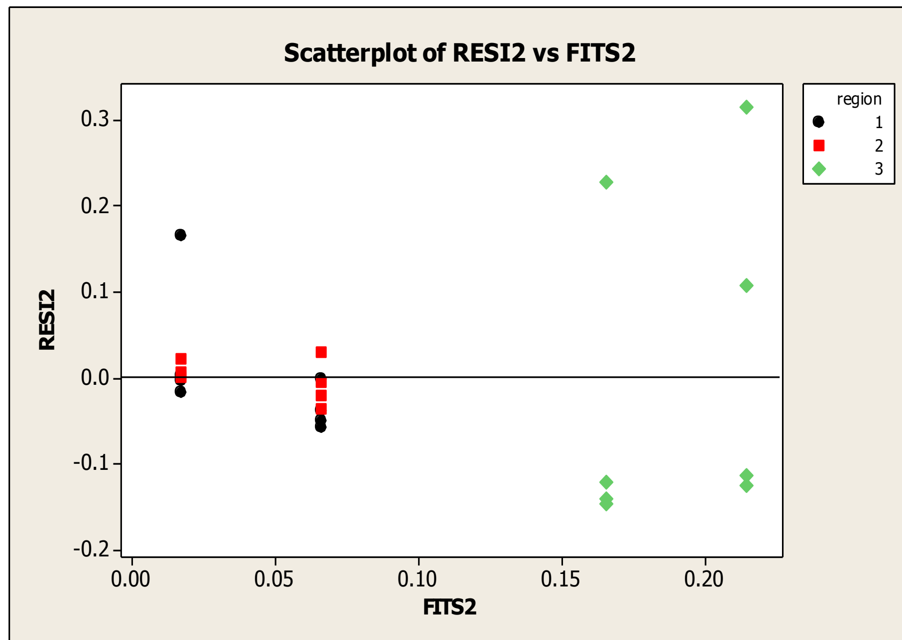


Figure 3.15: Anatomical distribution of NF-160 immunostaining.

Scatterplot of NF-160 immunoreactivity in three regions for each case. 1= corpus callosum, 2= internal capsule, 3= brainstem, FITS2=fitted value, RESI2=residual error.

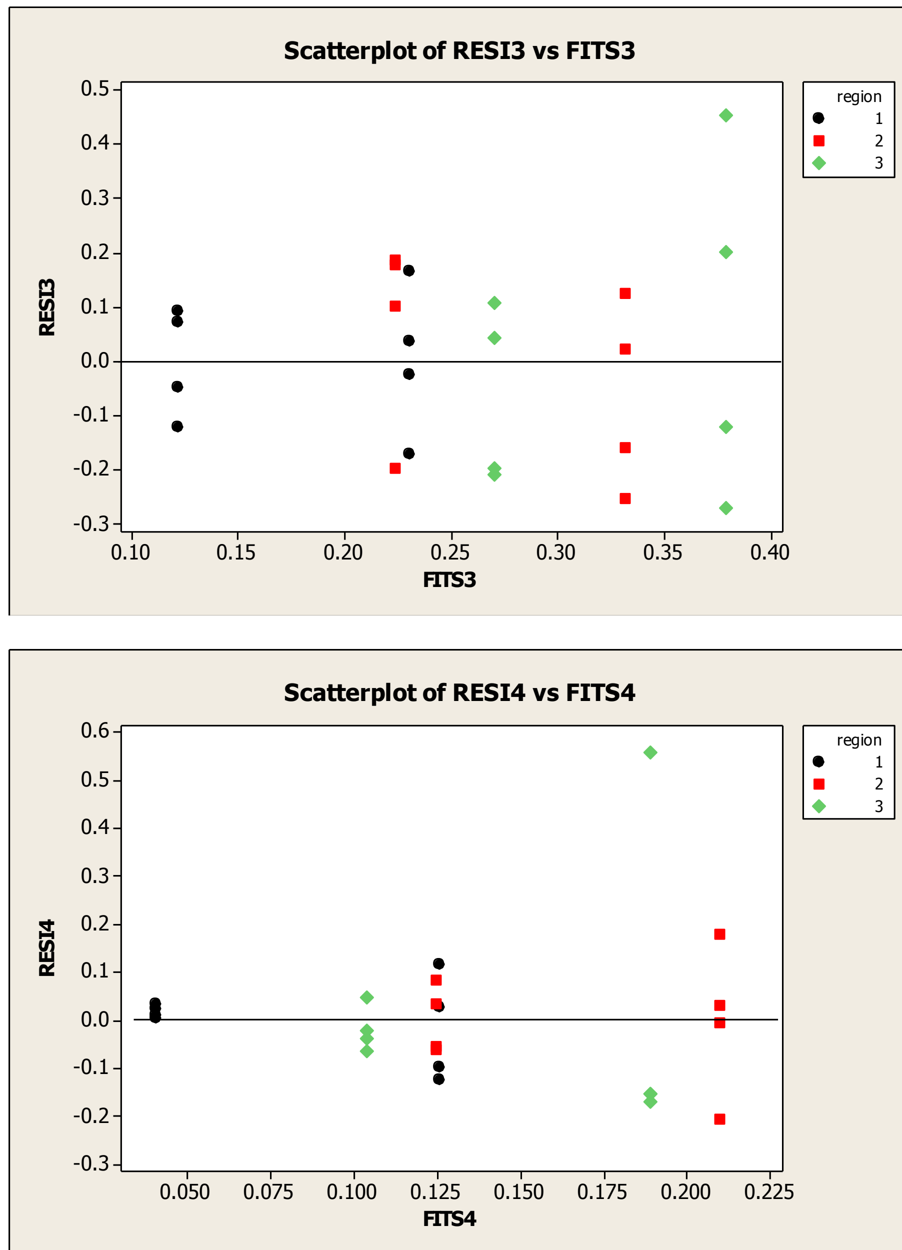


Figure 3.16: Anatomical distribution of NF-200 and SMI-34 immunostainings. Scatterplots of NF-200 and SMI-34 immunoreactivities in three regions for each case. 1= corpus callosum, 2= internal capsule, 3= brainstem, FITS3 and FITS4=fitted values, RESI3 and RESI4=residual errors.

3.3.3 Active caspase-3 and calpain in piglet and human brains

The purpose of this section of the study was to determine the activity of caspase-3 and calpain in human and piglet brains with TAI. To assess this, three antibodies were employed: active caspase-3 antibody as a marker of activated caspase-3, and two antibodies (Ab37 and Ab38) to detect the activation of calpain by recognizing a proteolytic fragment of α-spectrin cleaved by activated calpain. These antibodies were a gift from Dr R Siman and have been well characterised and used for western blotting studies. However, the current study failed to show any significant staining by any of these antibodies in the injured brains of animal or human, when citric acid and different dilutions of the primary antibodies were used. To investigate the possibility that the antibodies were not responsive to such pre-treatment, the immunohistochemical procedure was repeated under conditions where EDTA and trypsin were employed. However, despite multiple antigen pre-treatments and differing staining protocols there was no detectable immunopositive staining of caspase-3 and calpain markers in piglet and human cases with TAI. In addition, a formalin-fixed paraffin embedded human lymph node with high levels of apoptosis was used as a control tissue and this also failed to show any significant immunostaining. There was also no alternative antibody available to active caspase-3 to be tested in the present study.

3.3.4 Double labelling studies

In this part of the study, double immunofluorescence labelling was carried out to examine whether β-APP and each of the neurofilament markers co-localised, highlighting the same population of axons. In all images acquired, β-APP staining was indicated by the green chromogen, while the red chromogen was the signal for

the neurofilament (NF-160, NF-200 or SMI-34) staining. The co-localisation was observed as a yellow colour, the combination of green and red signals.

3.3.4.1 Co-localisation of β-APP and NF-160 in piglet and human

Two cases of injured piglets with TAI were selected for double labelling. They revealed evidence of co-localisation of stained axons with β-APP and NF-160 antibodies in both case, one case showing strong co-localisation (Figure 3.17: A, B, and C) and the second showing less consistent co-localisation. All human cases of TAI showed β-APP immunoreactivity co-localising with NF-160 immunostaining (Figure 3.17: D, E, and F), but to a lesser degree in one case. In addition, there was a significant population of NF-160 immunopositive axonal swellings which did not co-localise with β-APP, although the vast majority of the β-APP immunopositive axons did co-localise with NF-160 stained axons. In summary, while β-APP did co-localise with NF-160 in all piglet and human cases, there was an obvious second population of axonal swellings that were NF-160 positive, β-APP negative. There was a much smaller population of β-APP positive, NF-160 negative axons.

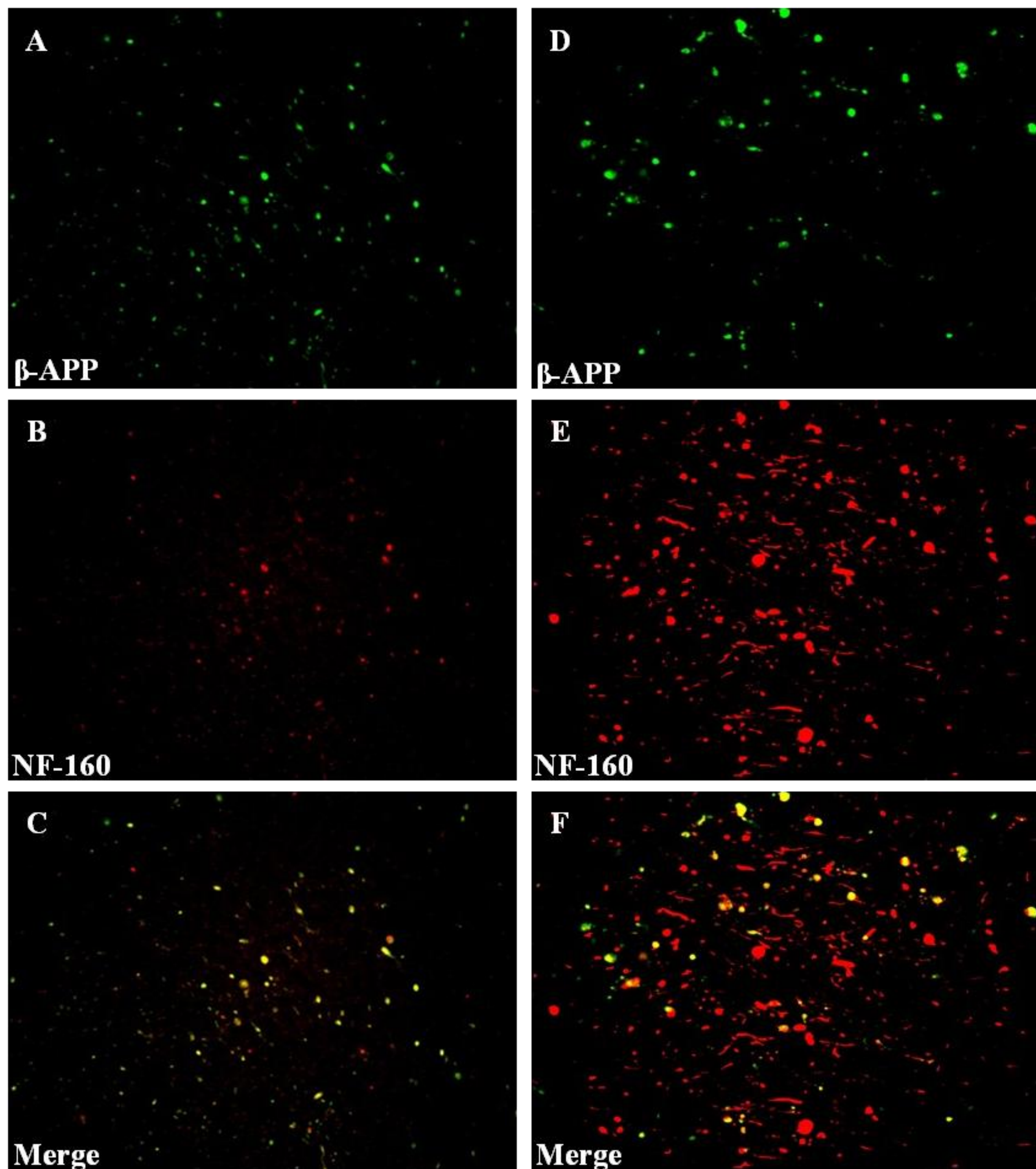


Figure 3.17: Co-localisation of β-APP and NF-160. Representative images of double-label immunofluorescence of traumatically injured axons in corpus callosum showing intra-axonal β-APP accumulation (green) in piglet (A) and in human (D), NF-160 immunoreactivity (red) in piglet (D) and in human (E), and co-localisation (yellow) of both markers in piglet (C) and human (F). Immunostaining x20.

3.3.4.2 Co-localisation of β-APP and NF-200 in piglet and human

In injured piglets with TAI, there was strong co-localisation between β-APP and NF-200 positive axons, although there was also a prominent second population of damaged axons which were β-APP immunoreactive only. Only a very small NF-200 positive β-APP negative population of axons was seen (Figure 3.18: A, B, and C). However, as can be seen from figure 3.18 (D, E, and F), the results obtained from the human cases of TAI show that antibodies to β-APP and NF-200 appear to stain predominantly separate populations of damaged axons, with only focal co-localisation.

3.3.4.3 Co-localisation of β-APP and SMI-34 in piglet and human

In piglets, co-localisation of β-APP and SMI-34 markers was observed in both cases albeit to a lesser degree in one case (Figure 3.19: A, B, and C). Virtually all β-APP positive axons co-localised with SMI-34, although there was a small population of SMI-34 positive β-APP negative axons. In human cases with TAI, there was a similar pattern of co-localisation of β-APP and SMI-34 (Figure 3.19: D, E, and F), although SMI-34 positive β-APP negative axons were numerous. Only very occasional β-APP positive SMI-34 negative axons were seen.

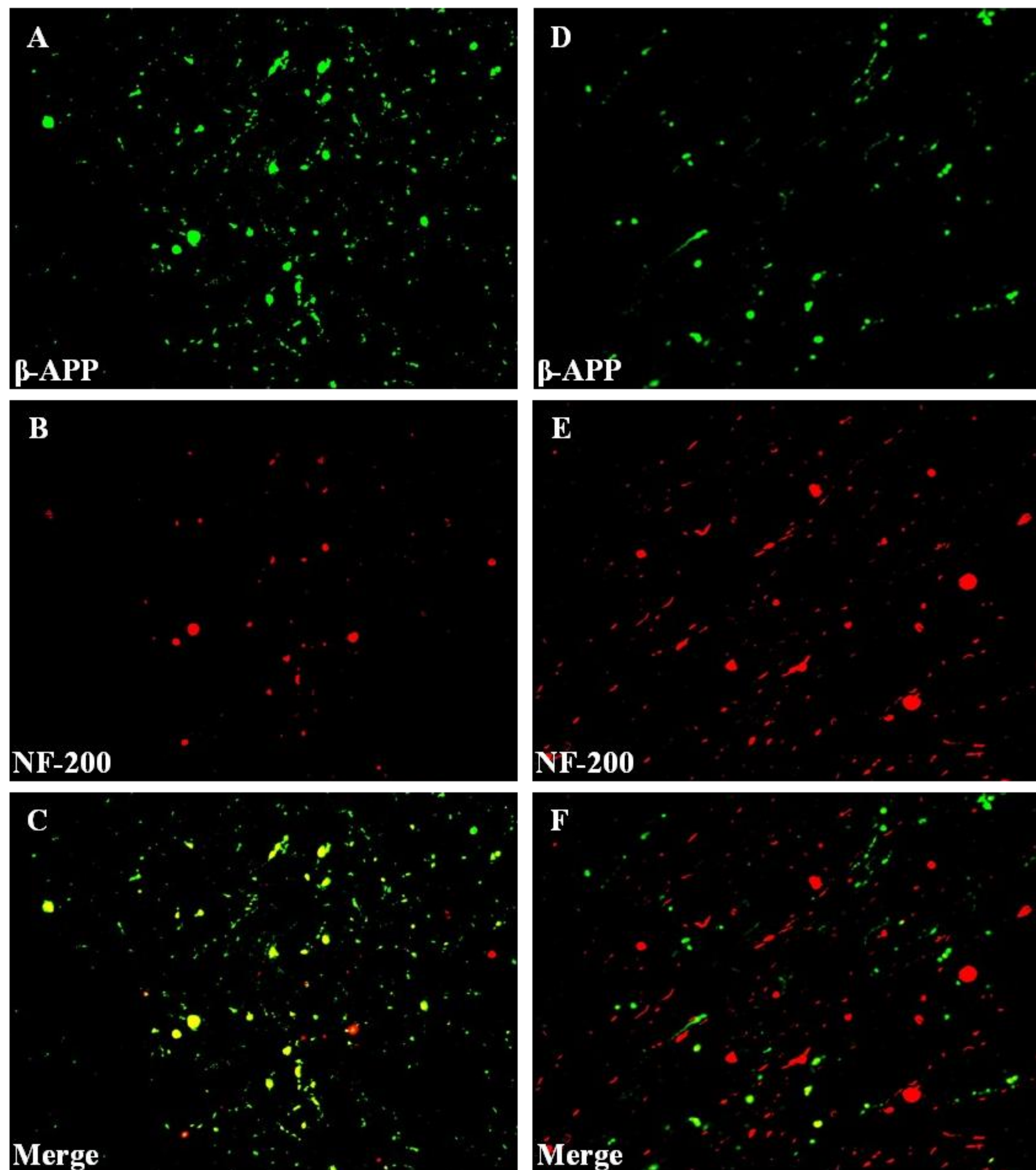


Figure 3.18: Co-localisation of β-APP and NF-200. Representative images of double-label immunofluorescence of traumatically injured axons in corpus callosum showing intra-axonal β-APP accumulation (green) in piglet (A) and in human (D), NF-200 immunoreactivity (red) in piglet (D) and in human (E), and co-localisation (yellow) of both markers in piglet (C) and human (F). Immunostaining x20.

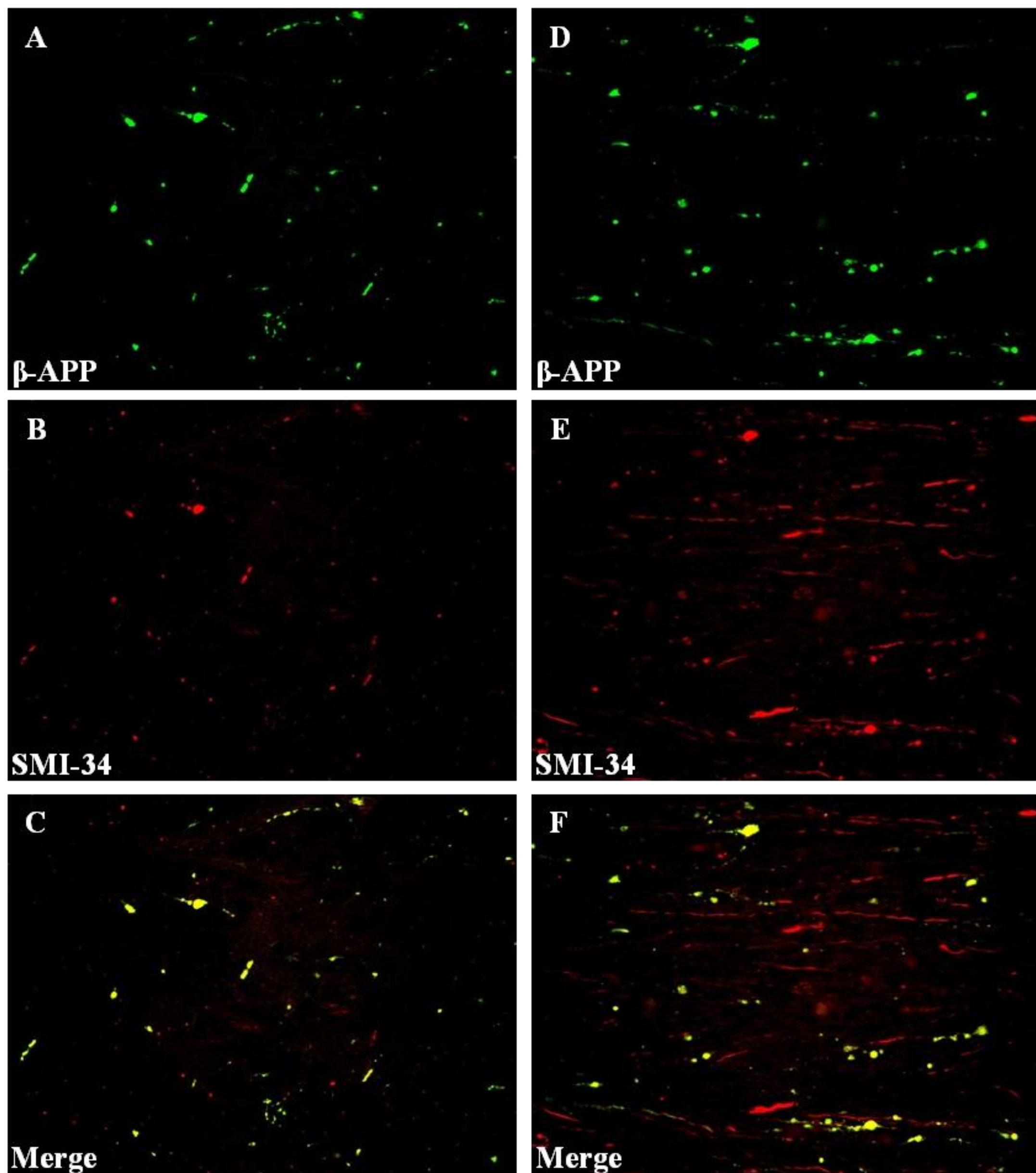


Figure 3.19: Co-localisation of β-APP and SMI-34. Representative images of double-label immunofluorescence of traumatically injured axons in corpus callosum showing intra-axonal β-APP accumulation (green) in piglet (A) and in human (D), SMI-34 immunoreactivity (red) in piglet (D) and in human (E), and co-localisation (yellow) of both markers in piglet (C) and human (F). Immunostaining x20.

4. Discussion

4.1 Pathological database of the Lothian and Borders cohort

The current study described retrospectively the neuropathological findings associated with TBI among people living in the Lothian and Borders region (Scotland, United Kingdom) over the period 1982 to 2005. This population-based cohort study reviewed the data of 406 cases from the archive of the Academic Department of Pathology (Neuropathology), University of Edinburgh. This is the first study of its kind to document the neuropathological features of TBI amongst this population, although previous studies have described cohorts from the West of Scotland (Adams et al., 1977; Graham et al., 1989; Adams et al., 1989; Smith et al., 2006). Based on ethnicity there is an expectation that the findings would be very similar to those seen in the West of Scotland, and there is unlikely to be any significant genetic or cultural influence to cause significant differences.

4.1.1 Sampling of the brain

A comprehensive histological examination had been carried out in almost 90% of the cases, whereas 27 cases (7%) had a limited histological study. No histology had been done in only 12 cases (3%). When sampling the brain for histology a routine protocol should be applied, modified to specific focal pathology or clinicopathological question (Kalimo et al., 2004). It has been reported that a minimum of at least 12 different brain regions require to be sampled in trauma cases, particularly for the assessment of TAI (Geddes et al., 2000). In cases of ischaemic injury, selected areas should include the neocortical border-zone region, hippocampi with subiculum, basal ganglia, thalamus and cerebellum (Graham, 1977). For

verification of axonal injury, sampling of the corpus callosum just rostral to the splenium with parasagittal white matter, the body of the corpus callosum with parasagittal white matter, posterior limb of the internal capsule, midbrain, pons and medulla is required (Geddes et al., 1997). Bilateral sampling is encouraged especially if TAI is being considered (Dolinak and Reichard, 2006), as it has been demonstrated that a significant number of cases of TAI can be missed without bilateral sampling (Geddes et al., 1997). As for many other neurological disorders, it is recommended to follow internationally accepted sampling protocols for the neuropathological investigation of various conditions since the accuracy of diagnosis is dependent on the representative adequacy of the retained tissue and the quality of the histology (Love, 2004). In addition, retrospective assessment of cases is entirely dependent on appropriate sampling and description of pathology at the time of examination. In this cohort it was not possible to provide a detailed assessment of contusional injury due to the limited description available from the archived reports.

4.1.2 Gender differences

The incidence of TBI was shown to be higher in males than females, with 69% of TBI cases within the cohort being males (Summers et al., 2009). This is consistent with other studies. Chau et al (2007) reported that the risk of TBI in males is 3 to 4 times higher than in females. In the UK, an epidemiological study of individuals with a head injury attending an emergency department revealed that males were more at risk than females (Yates et al., 2006). In the USA, national emergency department studies have uniformly found that males are at higher risk of TBI than females where the male-to-female (M/F) ratios were 1.5:1 and 1.7:1(Guerrero et al., 2000; Jager et al., 2000). The M/F ratios were also reported to be > 2:1 and 2:1in Olmsted Country

and San Diego respectively (Annegers et al., 1980; Kraus et al., 1984). Other studies have also supported the current findings by reporting that the TBI incidence in males exceeded TBI incidence in females in Australia, France and China with M/F ratios of 2.7:1, 2:1 and 1.3:1 correspondingly (Tate et al., 1998; Tiret et al., 1990; Wang et al., 1986). The reason for this consistent male predominance may be attributed to participation in particular occupational and leisure activities which put the individual at higher risk for sustaining TBI, or may reflect an inherent predisposition to risk taking in males (Yates et al., 2006).

4.1.3 Age-specific incidence

The current study revealed that the highest number of cases were in the second and the third decades comprising 26.7% of the cohort. This is similar to other studies which showed that the peak incidence of TBI was seen in the age group between 15-19 years old in the United Kingdom (Yates et al., 2006) and 15-24 years old in the United States (Thurman et al., 1999). The incidence was also reported to be highest in the 16-25 year age group in San Diego (Kraus et al., 1984), 15-24 year age group in Olmsted Country (Annegers et al., 1980), and 16-30 year group in the Bronx, NY (Cooper et al., 1983). The age-specific TBI incidence peaked in those aged 16-25 years in France (Tiret et al., 1990) and 15-24 years in Australia (Tate et al., 1998). This peak incidence suggests that the age group 20-39 engages in activities that make them more vulnerable to accidents.

A second peak of TBI incidence in the present results was seen in the elderly (25.7%), namely those above 60 years of age. This is in keeping with the findings of a large number of previous studies in this field. Specifically, several research groups have indicated that the second most common age group sustaining TBI is the over

65-year-old age group (Bruns and Hauser, 2003; Yates et al., 2006; Chua et al., 2007; Summers et al., 2009). The study reported by Chua et al has suggested that the incidence of TBI in those aged 65 years or older increases with increasing age (Chua et al., 2007). In those older than 74 years, an increased frequency of TBI has been reported in France and Australia (Tiret et al., 1990; Tate et al., 1998), and a recent publication (Bener et al., 2010) further supports these findings, reporting that, in a cohort of 1919 cases, the peak rate of TBI was amongst the over 65 years old group. There are several explanations for these results among elderly people. A possible explanation for this might be the medical co-morbidities and frailty associate with elderly people (Chua et al., 2007), or may be due to a combination of sensory and motor decline, de-conditioning and cognitive or conscious impairments (Bruns and Hauser, 2003). Most of the episodes of TBI in this older age group are attributed to falls and motor vehicle accidents (Thurman et al., 1999; Bruns and Hauser, 2003). It has been reported that falls are the most common cause of TBI in older adults since approximately 10% of falls in older people result in injuries such as TBI (Thompson et al., 2006). Other studies have confirmed that falls were the leading mechanism of TBI for older adults, accounting for 51% of cases (Langlois et al., 2004).

In the current study, the rate of TBI-associated cases from the ages of 40-59 years was observed to include the third largest group with an incidence of 20.5%. The lower incidence in this age group is in keeping with other published studies, since incidence rates for TBI have been reported to decrease throughout adulthood to a lower point in middle-aged individuals after the high-risk adolescent and young adult years (Bruns and Hauser, 2003). Despite the slight variation in age groups studied between this study and previous publications, a similar trend was seen in

several studies done in Olmsted Country, France, Australia, and the Bronx (Annegers et al., 1980; Tiret et al., 1990; Tate et al., 1998; Cooper et al., 1983). It seems that the trend of lower rates in adult and middle-aged cohorts is almost universal, although TBI incidence varies with population and locale. It precedes the increase in TBI seen in the geriatric population and may results from a decline in the impulsivity of the younger years (Bruns and Hauser, 2003).

The incidence rate of TBI in the age group 1-19 years was observed to be 18.3% of the cohort. This finding is consistent with other studies assessing the risk of TBI in children and adolescents (Guerrero et al., 2000; Greenwald et al., 2003). The rates of TBI in children have been observed to be generally low in Northern Manhattan as well as Australia (Tiret et al., 1990; Durkin et al., 1998). Rates of brain injury have been reported to be mostly steady during childhood, but to increase significantly at the age of 15 years (Adekoya et al., 2002; McCarthy et al., 2002; Kraus et al., 1990). Childhood injuries may be attributed to various important risk factors including behavioural features, levels of physical and sporting activity, socioeconomic status, neighbourhood characteristics and family type (Lalloo and Sheiham, 2003).

The Lothian and Borders cohort revealed that the number of infant cases (<1 year) were the lowest (4 cases, 1%), and consequently the lowest rate when compared to the overall population. This is in accord with previous studies performed in Northern Manhattan, France, and Olmsted Country, all of which found low incidence rates of TBI in those younger than 1 year (Durkin et al., 1998; Annegers et al., 1980). Infants tend not to be exposed to high risk situations and society tends to protect this vulnerable group. However, we also see the situation of

non-accidental injury within this age group, although the incidence of this type of injury is low.

Collectively, brain injury has generally been accepted, in population-based studies, to show a trimodal distribution: early childhood; late adolescence/early adulthood; and the elderly. Although these same general trends described in previous studies are seen in the Lothian and Borders cohort, comparison of age-specific incidence between studies is often difficult. This might be because of non-consistency of age grouping throughout studies; different case-ascertainment schemes; and confounders like gender, socioeconomic status and race. Therefore, a unified system of categorization should be adopted for future studies in order to help the health and safety authorities in developing an understanding of the external causes accountable for TBI and to assess vulnerable populations (Tagliaferri et al., 2006). This goal may be achieved through creating a standard protocol which then can be published in the international journals and presented in the international meetings.

4.1.4 Mechanism of injury

A World Bank report entitled “The Global Burden of Disease” has stated that traffic injuries are expected to become the third highest disease burden by the year 2020 (Dash et al., 2010). Road traffic accident (RTA) was revealed in the Lothian and Borders study to be the most common cause of TBI (48.5%). This was followed by a fall from height (24.5%) and assault (5.9%). Gunshot wound was the mechanism of injury in only four cases (1%). These findings are in line with about thirteen European studies reviewed in 2006, which found in most reports that motor vehicle related causes were the most common event leading to a TBI, but with

considerable variations from place to place (Tagliaferri et al., 2006; Murray et al., 1999). The same review also revealed that falls were second in frequency although in some reports falls were found to be similar in incidence to RTA as cause of TBI. However, violence/assault was reported to be a very common cause in Glasgow, Scotland (Thornhill et al., 2000; MacCallum et al., 2000). This high incidence of violence-related TBI in may be attributed to alcohol use and social deprivation (Wright and Kariya, 1997; Hutchison et al., 1998; Midford et al., 1998). The Lothian and Borders cohort results provide further evidence that gunshot wound to the head is uncommon in the UK as well as Europe in contrast to some other continents (Kay and Teasdale, 2001). In contrast, according to the National Center for Injury Prevention and Control (NCIPC) in the United States, falls were responsible for the largest proportion (28%) of TBI, followed by motor vehicle-traffic crashes (20%), struck by/against events (19%) and assaults (11%) (Langlois et al., 2006). In Australia, 40% of TBI was attributed to RTA, 25% to sports or recreation injuries and 21% to falls (Tate et al., 1998). In China, RTA accounted for 32% of TBI, occupational accidents 24%, falls from height 22%, recreational activities 16%, and gunshot wounds 1.4% (Wang et al., 1986).

Taken together, it seems that there are variations between centres and regions around the world when identifying the causes of TBI. This variation is likely to be due to the individual's demographic, reflecting the surrounding environment and culture. Although there is a high incidence of RTA-related TBI in western cultures, the actual numbers are numerically much lower than those seen in developing countries, presumably reflecting a better attitude towards traffic flow, maintenance of vehicles, driving under the influence of alcohol or drugs and obedience of traffic

laws. One should consider the fact that, in the UK, it has been reported that 60% of those with fatal head injuries from road accidents are often pedestrians and 40% are vehicle occupants (Jennett, 1996). In Glasgow, Scotland, pedestrian victims of head injury were found to be more intoxicated than injured drivers (Galbraith et al., 1976) and, therefore, the influence of alcohol might also be considered to contribute to RTA-related TBI. Falls and assaults were seen to be other significant causes of head injury amongst the Lothian and Borders cohort. This may be also related to the alcohol consumption in some cases, or may reflect an older population more prone to falls. It has been reported that alcohol is a common characteristic of victims of assaults and of falls. Alcohol excess was observed four times more often in falls and assaults than after road accidents in admissions for head injury in Scotland (Jennett, 1996).

4.1.5 Survival time

In one third to one half of all trauma-related deaths, TBI is estimated to be the primary cause of death (Sosin et al., 1995). A number of studies have reported the association between TBI and significant mortality in the acute period after head injury (Kraus et al., 1984; Masson et al., 1997; McGarry et al., 2002; Thurman, 1999; Vazquez-Barquero et al., 1992, Pentland et al., 2004; McMillan and Teasdale, 2007; Harrison-Felix et al., 2009). This view has been supported by the current findings, which have demonstrated that more than half of the cases (51.4%) had a survival time of less than 24 hours. In 65 cases the survival period ranged from more than 24 hours to 28 days, and a few individuals (7 cases) in the Lothian and Borders cohort survived between one month and 12 months. Similarly, other studies have shown that death occurs at the scene, during ambulance transport phase or during the emergency

medical stage of treatment in about half of all TBI fatalities (Bruns and Hauser, 2003). This high rate of mortality in the acute period may be explained by the severe neurological and functional deficits in TBI victims that are sustained from the primary injury to the brain, such as cerebral contusions, lacerations and diffuse vascular injury. In addition, there is often associated non-neurological trauma which can be associated with various injuries such as fractures with associated hypotension, cardiopulmonary or visceral injuries, spinal cord injuries and peripheral nerve injuries in 70% of cases affected (Chua et al., 2007). The association between mortality in the acute period after head injury and secondary insults due to evolving pathology or delayed treatment has also been demonstrated in multiple studies (Luerssen et al., 1988; Conroy and Kraus, 1988; Klauber et al., 1989; Marshall et al., 1991; Lang et al., 1997; Signorini et al., 1999; Fiedler et al., 2000; Lannoo et al., 2000; Peek-Asa et al., 2001; Jiang et al., 2002; Mosenthal et al., 2002; Schreiber et al., 2002; Fabbri et al., 2008).

17 cases in the Lothian and Borders study were found to have survival time of more than 12 months; 3 survived less than 5 years, 4 survived from 5 up to 10 years, and 10 survived over 10 years. In contrast, all 479 patients included in the study of Lewin and colleagues (Lewin et al., 1979) had experienced coma or post-traumatic amnesia and most cases died within 1 year and no cases survived beyond 10 years. Previous studies have also confirmed the significant reduction in survival after moderate to severe injuries (Kraus et al., 1984; Thurman, 1999; Masson et al., 2001; Adekoya et al., 2002) when compared to mild injuries. In persons who survived six months after moderate to severe injury, most studies reported a decrease in long-term survival when compared to mildly injured cases (Walker et al., 1971; Weiss et al.,

1982; Rish et al., 1983; Baguley et al., 2000; Shavelle et al., 2001). It is difficult to make a direct comparison in our long-term survivors with previously published studies in that we had very limited clinical information from our cases and, in particular, little information relating to the severity of injury on hospital admission and subsequent clinical state.

An Australian study has supported the current view by demonstrating that the length of time between injury and death ranged from 45 days to 9 years (Baguley et al., 2000). The existing literature relating to long-term survival after TBI has revealed that the mortality rates were reported to be 6.7% after 15 years survival (Rish et al., 1983) and 4% with 9 years survival (Strauss et al., 1998). Therefore, there is a degree of correlation between the Lothian and Borders data and some previous publications. An increased mortality rate has been documented in survivors of mild TBI at 1 year post-injury (Thornhill et al., 2000), although the mortality rate in this group is, as expected, much lower than that seen during the acute period (Shavelle et al., 2001; Pentland et al., 2004). In the literature, risk factors for later mortality have also been proposed including old age and male sex, as for the general population (Harrison-Felix et al., 2009). Alcohol use, time since injury, verbal and cognitive functions, education, initial TBI severity, and maladaptive behaviours such as drug use and criminal behaviour are among other factors studied in relation to later mortality (Shavelle et al., 2007). After head injury, the primary causes of death were similar to those seen in the general population (Pentland et al., 2004; Shavelle et al., 2007; McMillan and Teasdale, 2007; Harrison-Felix et al., 2009), although sudden unexpected death in epilepsy (SUDEP) has a high incidence in the post-traumatic seizure group.

4.1.6 Skull fracture

Skull fracture can be an additional cause of morbidity and mortality, especially when associated with complications like haemorrhage or contusion to the underlying brain or spinal cord (Kalimo et al., 2004). Almost a third (30%) of all TBI cases reviewed in this study had evidence of fractures of the skull. The present findings were in contrast to study carried out in Glasgow, which reviewed a series of 151 cases and showed an incidence of 79% for skull fracture in fatal head injury (Adams et al., 1980a). Data from a consecutive series of 635 fatal non-missile head injuries over a 25-year period (1968-1982) revealed that fracture of skull was seen in 75% of cases (Graham et al., 1988). In clinical practice, the incidence of skull fracture was 3% in mild head injury presenting to the emergency room, rising to 65% in those requiring neurosurgical admission (Masters et al., 1987). Although the present results differ from the published studies mentioned above, they are to some extent in line with those described by Osborn and co-workers who reported an incidence of 25% of skull fractures in fatal head injuries at autopsy (Osborn et al., 2002). In Edinburgh, Miller reviewed the head injury admissions in 1981, 1986, and 1989, and he found that 20% of cases had skull fracture (Miller, 1993). The explanation of the different incidence rates between studies may be related to the severity of injury. It has been reported that the frequency of fracture of the skull is associated with the severity of the head injury (Graham and Gennarelli, 2000). Equally, it may be a reflection of the study populations; the Lothian and Borders study was all fatalities with pathological evidence of head injury, whereas the Glasgow studies were all fatalities in which there had been an admission to a neurosurgical unit. This second population is much more selective, and is focused on patients whose primary pathology is head injury

rather than individuals who have some degree of head injury potentially in association with significant systemic pathology which has been the cause of the death.

4.1.7 Contusion

In the present study, analysis of the results showed that contusions were observed in 219 (53.9%) cases, while in 187 cases they were absent. The findings are in contrast to the findings published by Adams and colleagues (Adams et al., 1985). They demonstrated that contusions were seen in more than 90% of fatal cases of TBI and they were absent in some 6% of fatal cases. This inconsistency may be related to different indexes in skull fractures between the Lothian and Borders study and published Glasgow studies. Another possible explanation for this discrepancy might relate to the broad range of severity of the cases used in the current research, rather than focusing on TBI cases which had neurosurgical admission. Despite these differences it is still obvious that contusions are one of the more commonly seen hallmarks of brain damage secondary to head injury.

4.1.8 Diffuse ischaemic brain damage (global ischaemia)

Ischaemic brain injury is a common pathology seen in autopsy series of fatal traumatic brain injury, being seen in 91% of cases in one study (Graham et al., 1978). In the current Lothian and Borders study, it has been shown that 130 (33.5%) cases were associated with global ischaemic brain damage. Global ischaemic was found in the brains of 80% and 92% of two series of fatal cases (Graham et al., 1989). A possible explanation for the lower incidence in the current study may be related to the modern measures of the management of head injured patients which perhaps have an effect in avoiding the occurrence of ischaemic brain damage. However,

another potential reason for this may be related to the fact that ischaemic damage can be identified microscopically only after a survival of at least several hours. The hallmark of ischaemic injury is so-called "ischaemic cell change". There is shrinkage of the nucleus and cytoplasmic eosinophilia. Several hours survival post-ischaemic injury is required for histological changes to develop (Adams et al., 1980; Graham et al., 1978; Graham et al., 1989). As a consequence it is important to note that not all the cases in the present study survived their head injury long enough to be transferred to the hospital, and as such did not survive long enough for histological changes to develop. Therefore, the current low incidence of ischaemic brain injury in this study when compared to other studies where patients are transferred to hospital and ventilated prior to death would be expected.

4.1.9 Intracranial haematomas

One of the most frequent and best known complications of head injury is intracranial haematoma (Jennett et al., 1977; Adams et al., 1980a). The current Lothian and Borders study revealed that 270 cases (66%) had intracranial haematomas; they were supratentorial in 255 (62%) and infratentorial in 4 (1%). These findings are consistent with those of Adams and colleagues who found that supratentorial haematoma was seen in 58% of patients whereas infratentorial haematoma was observed in 5 cases (3.3%) (Adams et al., 1980a). Data from a consecutive series of 635 fatal non-missile head injuries over a 25-year period has also supports the current analysis with a reported incidence of 60% of intracranial haematomas (Adams et al., 1986).

When considering the distribution of the supratentorial haematomas previous studies have reported an incidence of 10% for extradural haematomas, between 18%

and 20% for subdural haematoma, between 16% and 22% for intracerebral haematoma, and burst lobe with an incidence of between 22% and 23% (Adams et al., 1980a; Adams et al., 1986). In line with these earlier studies, subdural haematoma was reported in approximately 18% of the Lothian and Borders cases. However, in the current study extradural and intracerebral haematomas were seen in about 7% and 13% consecutively. It seems also that the lower incidence (1%) of burst lobe in the current study differs from previous publications.

In patients with head injury, traumatic subarachnoid haemorrhage has been reported to be a common autopsy finding (Freytag, 1963; Lindenberg and Freytag, 1970; Tatsuno and Lindenberg, 1974). It is also clear from the present study that subarachnoid haemorrhage is common in patients sustaining brain injury, being seen in approximately 46% of cases. Although medical and surgical advancements are improving survival rates, subarachnoid haemorrhage has been reported to be the fourth most common intracranial cause of death (Leon-Carrion et al., 2005). The current findings seem to fall within the range of other studies which showed an incidence of traumatic subarachnoid haemorrhage ranging from 5 to 57% (Martin et al., 1992; Eisenberg et al., 1990; Kakarieka et al., 1993; Abraszko et al., 1996; Weber et al., 1990). The variation in these studies probably represents different methods of reporting and assessing subarachnoid blood.

4.1.10 Raised intracranial pressure

For many intracranial problems, raised ICP is known to be the final common pathway and has a profound influence on outcome (Pickard and Czosnyka, 1993). The current study revealed that almost 186 (45%) out of 406 cases had some form of raised ICP, 217 did not have evidence of any such pathology and no information was

available in 3 cases. This was in contrast with earlier work carried out in Glasgow which found that 75% of 434 patients dying with TBI had such lesions (Graham et al., 1987). Other studies have shown that there was evidence of a high ICP in 125 (83%) cases of fatal head injury (Adams et al., 1980a). It has been reported that fifty percent of patients with head injury seen in Accident and Emergency Departments in the United Kingdom every year have an ICP greater than 20 mm Hg (Jennett and Teasdale, 1981; Miller et al., 1992). A possible reason for the lower incidence of ICP in the current Lothian and Borders study may be related to the much lower incidence of severe contusions and intracranial haematomas in the current cases when compared to previous publications.

Brainstem haemorrhage is reported to be a common autopsy finding among severely head injured patients (Adams et al., 1977; Tomlinson, 1970). In craniocerebral trauma victims, secondary haemorrhage of the brainstem has been reported to occur at a later stage as a result of axial displacement of diencephalic structures (Graham et al., 1987). In the present study, there was evidence of secondary brainstem haemorrhage in 34 (18%) cases and in four of these the damage could be seen only microscopically. Previous studies have shown that raised ICP was associated with secondary brainstem damage in 51% (221cases) of an autopsy series of 434 patients with non-missile head injury (Graham et al., 1987). They also report 44 (20%) cases where such lesions were identified only microscopically. In addition, in a post mortem study of 132 fatal head injuries, brainstem haemorrhage was observed in 37% of cases (Tandon, 1964).

4.1.11 Diffuse traumatic axonal injury

TAI is believed to be an important cause of morbidity and mortality in the absence of any other obvious intracranial lesions. The analysis of two previous cohorts in Glasgow revealed that the incidence of TAI among cases with head injuries was approximately 14% (Adams et al., 1980a) and 22% (Graham et al., 1989). In one series of 434 fatal non-missile head injuries which were subjected to comprehensive histological studies, TAI was identified in 122 (28%) cases in which 10 had grade 1, 29 had grade 2 and 83 had grade 3 (Adams et al., 1989). However, analysis of TAI in the present series showed a different incidence (46 cases, 11.4%) when compared to those published previously. Grade 1 was seen in 41 cases, grade 2 was seen in 1 case and in 4 cases there was evidence of grade 3. Prior to the advent of immunohistochemistry axonal pathology was identified as eosinophilic axonal bulbs which could be highlighted by silver stains. However, with the advent of immunohistochemistry it became apparent that there was greatly increased sensitivity with this technique. In the current group, immunohistochemistry was used for the diagnosis of TAI in only 73 cases (18%) between 1994 and 2005 and all cases identified between 1982 and1993 having TAI diagnosed using Palmgren's silver impregnation technique. So, it is possible that through the histological examination of the current cases the diagnosis of TAI has been underestimated in cases where classic silver stain is only employed. As a result of this, there was an overall decrease in the total number of cases diagnosed with TAI. This is supported by previously published data, which showed that immunohistochemistry resulted in a marked increase in the number of cases of Grade 1 TAI when compared to the silver impregnation technique (Gentleman et al., 1995). In the present Lothian and Borders

study, it is additionally noted that there was a reduction in the number of cases diagnosed with Grade 3 TAI. This may be in part due the greater sensitivity afforded by immunohistochemistry since this technique was used in 18% of the cases. However, it may be that there is increased awareness that much of the axonal pathology seen in TBI is in fact secondary to ischaemia, as discussed earlier.

4.1.12 Brain swelling

Brain swelling is particularly feared by neurosurgeons as it results in a significant increase in the volume of the intracranial contents and is very difficult to control. It is well recognized to be an important complication of head injury (Adams et al., 1980a), and can be either diffuse, involving one or both hemispheres, or focal as in relation to contusions. Ischaemia is the most common underlying pathology of diffuse brain swelling, although diffuse TAI can be associated with such swelling (Smith, 2005). The present results indicated that 275 out of 406 cases had evidence of bilateral brain swelling whereas only 3 cases had unilateral brain swelling. Brain swelling was considered to be a consequence of contusions (5.2%), intracranial haematoma (22.7%), hypoxic brain damage (2.7%) or a combination of pathologies (24.1%). In the other cases it was not possible to assign a cause. Other studies have reported that unilateral enlargement of a cerebral hemisphere was seen in 11% of cases (Sarabia et al., 1988), in contrast to the current study. However, the study by Sarabia and colleagues was a CT based study, and examined a series of 589 severe head injury cases of adults, while the current study was autopsy based and included cases with different degrees of severity amongst all age groups. In the Sarabia et al study, the cerebral swelling was seen to be associated in 88% of cases with an ipsilateral haematoma, in 7% with a large epidural haematoma, and with isolated

cerebral swelling in 4% of cases. Another study performed by Kobayashi et al reported that the incidence of diffuse brain swelling was 16% in severely head-injured patients of all ages (Kobayashi et al., 1983). CT and MRI scans will identify brain swelling with greater sensitivity than autopsy studies, particularly focal low volume areas of swelling and, therefore, results are not directly comparable.

In a study of 151 cases of fatal head injury, unilateral brain swelling was seen in 17 cases whereas in 9 cases there was evidence of bilateral brain swelling (Adams et al., 1980a). A review of fatal non-missile head injury without clinically documented raised ICP showed that brain swelling was seen in 13 cases out of a total cohort of 434, and the swelling was bilateral in 10 cases and unilateral in 3 cases (Graham et al., 1988). In the same study, bilateral brain swelling was attributed to contusions in 3 cases, to ischaemic damage in 2 cases and in 5 cases it was thought to be caused by a combination of ischaemic damage and contusions. The unilateral hemispheric swelling was related to contusions in the remaining three cases.

4.2 Axonal pathology in piglet brains

4.2.1 Sham vs injured piglets

In this study, a significant increase of β-APP immunoreactivity has been demonstrated in the brains of the 4 week old injured piglets (6hour survival) when compared to the sham group. β-APP is transported by fast transport mechanisms (Koo et al., 1990), and is shown to accumulate within axons at the site of injury when there is axonal transport interruption (Yam et al., 1997). The current results are consistent with previous studies in that there was no significant positive staining of β-APP in any of the sham piglets tested (Raghupathi and Margulies, 2002; Raghupathi et al., 2004; Friess et al., 2007). Also, evidence of traumatic axonal

injury, as demonstrated by β-APP, has been reported following diffuse brain trauma in the 3-5 day old piglets (Raghupathi and Margulies, 2002; Raghupathi et al., 2004) and 17 day old rat (Adelson et al., 2001; Raghupathi and Huh, 2007). The present findings are therefore consistent with previous studies which have shown the ability of this marker to detect axonal injury after trauma in the brains of human (Cochran et al., 1991 Gentleman et al., 1993) and experimental animals (Otsuka et al., 1991; Nakamura et al., 1992; Kawarabayashi et al., 1993).

The accumulation of the medium subunit of neurofilaments, NF160, has been reported after inertial brain injury in the pig (Chen et al., 1999). The current study revealed that, when compared to the baseline levels of NF-160 immunoreactivity in the 3-5 day old control animals surviving for 6 hours, piglets sustaining low or high velocity injury showed a reduced expression of NF-160. This is at odds with a previous study carried out by Yaghmai and Povlishock (1992) which found that there was modest enhancement of immunoreactivity associated with the medium NF subunit in comparison to the control situation. Unfortunately, little is known in the literature about the response of NF-160 with TAI in the brains of humans and animals. However, there are some potential reasons which may explain the observation that there is reduced expression of NF-160 after rotational non-impact trauma. It may be that an acute response in the axons to the evolving axonal injury is down-regulation of NF-160 expression. A second possibility is that a survival time of 6 hours was not enough for up-regulated NF-160 to be immunohistochemically detected in the injured animals. This potential is supported by previous studies which have not demonstrated the accumulation of NFM and NFH until after at least 24 hours following trauma (Chen et al., 1999; Smith et al., 1999). The current study has

also shown no significant difference between sham and injured piglets with regard to the level of expression of NF-200.

The regulation of NF phosphorylation and dephosphorylation has been reported to occur mainly within the axonal compartment, utilising different phosphate acceptor sites on the NFs (Dewaegh et al., 1992; Nguyen et al., 2001). Sternberger and Sternberger (1983) have suggested that the compactness and order in NF structure can be increased by phosphorylation. However, the degree of phosphorylation is correlated inversely with the velocity of axonal transport in that the dephosphorylated forms of NF are more easily transported along the microtubule tracks (Watson et al., 1989). After rotational acceleration trauma, the phosphorylated heavy NFs have been shown to decrease significantly within the axonal compartment (Hamberger et al., 2003). In contrast to earlier findings, however, the current study revealed a significant increase of the hyperphosphorylated NF marker, SMI-34, at 6 hours post-injury in the piglet group aged between 3 and 5 days compared to the sham animals. It is not clear why the immunostaining of SMI-34 increased after injury. The metabolism of NF-200 has been shown to be affected by its degree of phosphorylation (Goldstein et al., 1987; Greenwood et al., 1993). Greenwood et al (1993) observed that there was a significant increase in the rate of degradation of NF-200 by calpain if the protein was dephosphorylated, suggesting that phosphorylation state can alter the activity of calpain. Therefore, one could speculate that degradation of the hyperphosphorylated NF-200 in the current study may have been reduced and, if the rate of synthesis remains unchanged, this could lead to an increased accumulation, or that rapid hyperphosphorylation of NF-200 is a protective mechanism after TBI.

It is thought that axonal injury can lead to misalignment or compaction of NFs, and studies of experimental TAI have shown a decrease of axonal NFs after injury secondary to sidearm modification (Yaghmai and Povlishock, 1992; Okonkwo et al., 1998; Maxwell et al., 2003). The findings of the current study are consistent with previous studies which show decreased levels of NF protein. Whether this is due to proteolysis or the pathological reorganization of NF protein is uncertain. The results of this study have provided further evidence of the complexity of the pathology of NFs and the difficulty in their assessment particularly when TAI is evaluated.

4.2.2 Effect of age, velocity, and survival time

The effect of different variable factors including age of animals, velocity of injury and survival time was also examined in the current study among injured piglets. No significant effect of age on the accumulation of the various proteins was observed between injured neonatal (3-5 day old) and juvenile (4 week old) pigs. Data from animal studies have shown the considerable decrease of the rate of fast anterograde axonal transport in old age (Geinisman et al., 1977; Viancour and Kreiter, 1993). It has been suggested that β-APP may concentrate more quickly and in greater quantity in younger cases (Sherriff et al., 1994a; Hortobágyi et al., 2007). Although this has not been observed in the current animal study, this may be a reflection of the relatively young age of both groups.

The velocity of the injury did not significantly influence the expression of the markers used in this study. Although some brains of piglets receiving high velocity injury appeared to have greater accumulation of β-APP compared to piglets receiving low velocity injury, this failed to reach significance.

In paediatric TBI cases, β-APP has been shown to be detected within 35-45 minutes after injury (Gorrie et al., 2002). Hortobágyi and co-workers demonstrated β-APP immunoreactivity in adult cases who survive as little as 35 minutes (Hortobágyi et al., 2007). β-APP was also detectable in patients surviving as long as 99 days after mild head injury (Blumbergs et al., 1994), although the potential effect of perimortem ischaemia was not considered in this report. In animal models, accumulation of β-APP can be detected as early as 30 minutes to 1 hour (Pierce et al., 1996; Stone et al., 2004). The immunopositive axons highlighted by β-APP antibody have been identified for several months to a year following injury in rodents (Pierce et al., 1998) and pigs (Chen et al., 2004), suggesting an evolving pathology over many months. The immunostaining of NF has been also reported after TAI in different studies. It has been demonstrated in humans with survivals as short as 6 hours in damaged swollen axons, and by 1 week in axonal bulbs (Grady et al., 1993; Christman et al., 1994). Robust NF immunoreactivity within several hours after TBI has been reported in numerous animal models (Yaghmai and Povlishock, 1992; Povlishock et al., 1997; Saatman et al., 1998; Chen et al., 1999; Smith et al., 1999). Therefore, the short and long term accumulations of β-APP and NF markers shown in previous studies have been supported by the current study which found no significant effect of survival time when the proteins were assessed.

4.2.3 Association between the antibodies and the presence of axonal swellings and terminal bulbs

Axonal swelling and axonal bulbs have been observed in the injured piglet brain after TAI (Raghupathi and Margulies, 2002; Raghupathi et al., 2004). These axonal pathologies were also seen in both porcine and non-human primate models of

diffuse brain trauma and in human cases (Smith et al., 1997; Smith et al., 2000; Maxwell et al., 1993; Maxwell et al., 1997; Adams et al., 1982; Gennarelli et al., 1982; McKenzie et al., 1996). The current study revealed that accumulation of β-APP was observed commonly in association with both contiguous and disconnected axons, being seen in almost 11 out of the 16 cases of injured piglets examined. The remaining 5 cases are amongst animals aged between 3 and 5 days in which 4 survived 6 hours and 1 had a survival time of 6 days. These results are consistent with previous animal and human studies of TAI, which found β-APP immunostaining in most injured axons throughout the brains (Smith et al., 1999; McKenzie et al., 1996). Axonal bulbs and varicose axonal swelling have been also demonstrated but are less commonly seen when the antibodies to NF-160 and NF-200 were used. Similar observations have been reported when antibodies specific for the sidearm domains of NFM and NFH were used following brain injury in the pig (Chen et al., 1999). Raghupathi et al (2004) have evaluated the brains of the injured piglets for the presence of TAI using the heavy subunit of the neurofilament (NF-200). They reported an increase of the NF-200 immunoreactivity in both axonal swellings and axonal bulbs at 6 hours after injury in all injured animals. More interestingly, evidence of axonal injury was further demonstrated using the antibody against the hyperphosphorylated NF-200, SMI-34, but to a much lesser extent compared to other markers. This finding corroborates the concepts highlighted in the literature which suggest that the accumulating NF in damaged axons is non-phosphorylated (Postmantur et al., 1994; Wang et al., 1994; Nixon and Sihag, 1991) since transported forms of NF are predominately non-phosphorylated (Hollenbeck, 1989; Nixon and Sihag, 1991; Meller et al., 1993). Other studies have also reported

similar results in which the accumulated NF is mainly but not exclusively dephosphorylated (Chen et al., 1999; Saatman et al., 2003). This part of the current study showed the usefulness of antibody targeting β-APP for detecting changes in axonal function in brain-injured piglets when compared to NF-160, NF-200 and SMI-34 markers. However, caution must be exercised in interpreting the presence of β-APP immunoreactivity as the heterogeneity of the responses of individual cases after injury has previously been demonstrated in animal and human studies (Yaghmai and Povlishock, 1992; Grady et al., 1993).

4.3 Axonal pathology in human brains

4.3.1 Trauma vs ischaemia

This study has assessed β-APP immunoreactivity in different anatomical regions in ischaemic cases and in cases with TAI. No significant difference was observed between ischaemic and traumatic cases. Immunostaining for β-APP is the most widely used and most reliable method in detecting axonal injury (Gentleman et al., 1993; Sherriff et al., 1994a).

Axonal injury is a frequent finding in cases of fatal head injury (Gentleman et al., 1995). Identification of axonal injury through utilizing antibody against β-APP has also allowed damage axons to be seen in mild head injured cases (Blumbergs et al., 1994) and in severely disabled or vegetative patients (Adams et al., 1999; Kinney and Samuels, 1994). Therefore, the current study further supports previous studies which found evidence of axonal injury by means of β-APP immunopositive staining after traumatic brain injury in humans. Also in keeping with the present findings, previous studies revealed β-APP immunoreactivity in non-mechanically injured tissue of patients who sustained cerebral ischaemia or hypoxia (Kaur et al., 1999;

Oehmichen et al., 1999; Shannon et al., 1998). Another study has reported positive axonal staining in control cases with ishaemia and other metabolic insults such as hypoglycaemia (Dolinak et al., 2000a). An explanation of this may be related to the fact that the accumulation of β-APP is an active, energy-dependent process (Ferreira et al., 1993). Thus, we now know that β-APP will accumulate in the absence of TAI in cases of severe metabolic dysfunction (Harrington et al., 2000; Hortobagyi et al., 2007). It may even be that β-APP expression in axons may be transient and may represent a population of axons that can be salvaged with appropriate metabolic intervention.

The presence of axonal injury is important in forensic practice (Geddes, 1997; Oehmichen et al., 1998; Sherriff et al., 1994b). However, caution must be used as β-APP appears to be a useful general marker of axonal damage and is specifically a marker indicative of axonal transport interruption, but is not a marker of traumatic injury *per se*.

Neurofilaments are composed of three subunits (NF-68, NF-160, and NF-200) and are found in high concentrations in axons (Nixon and Sihag, 1991). The current study revealed that there were no differences in the staining of NF-200 or NF-160 between ischaemic and traumatic cases. This is in agreement with previous reports which showed that antibody to NF-200 labelled injured axons as well as un-injured axons, in human brain (Grady et al., 1993). Although NF-160 antibody has not been tested using autopsy materials in earlier studies, it seems that this protein has the tendency also to stain normal axons. This observation is supported by previous studies which found that NF-68 can stain most axons (Sherriff et al., 1994a), although NFs are different in their characteristics. Regional increases in NF

immunostaining may be seen due to accumulation of NF proteins resulting from impaired axonal transport. This can be caused by alterations in phosphorylation or structural changes of sidearm structures that increase antigenicity (Serbest et al., 2007). Thus, it can be postulated that antibodies to NF-160 and NF-200 might underestimate the extent of axonal injury, when evaluated in human cases.

The current study demonstrated no significant differences between traumatic and ischaemic cases in the expression of hyperphosphorylated heavy neurofilament subunit. This contrasts with a previous report by Hamberger et al (2003). Hamberger et al reported dramatic decrease of the phosphorylated form of the heavy neurofilament subunit in the axons after rotational trauma, while axons were immunopositive for the same antibody throughout the brain in control animals.

Although there is a significant difference in the amount of phosphorylation for each subunit of NFs (Jones and Williams, 1982), they are the most highly phosphorylated proteins in the brain (Nixon, 1993). Various studies have shown that antigenicity can be altered by changes in the degree of phosphorylation of NF (Carden et al., 1985; Shaw et al., 1986; Schmidt et al., 1987; Banerjee et al., 1990). One could suggest that phosphorylation-specific PCR can be used to find evidence to support this explanation. Hence, some of the discrepancies between the current and previous findings may be linked to the selection of the antibody used to detect the amount of NF. When the NF is either dephosphorylated or the degree of phosphorylation is altered, antibodies that bind to phosphorylated NF are unable to bind. Other antibodies are phosphate independent, that is, they will not be affected by the amount of protein phosphorylation (Mink and Johnston, 2000). This might affect NF-200 most significantly because of its high level of phosphorylation (Carden et al.,

1985). It is likely that the reduced levels of NF-200 that were reported actually represent a change in phosphorylation, rather than a true reduction in the amount of the protein, since the specificity of the antibody is unknown or is not described in most studies (Mink and Johnston, 2000). McCracken et al (1999) reported reductions in NF-200 immunoreactivity by 80% and 40% after traumatic injury of human brain, when employing a phosphate-dependent antibody and a phosphate-independent antibody respectively. They hypothesised that there was dephosphorylation as well as a decrease in the amount of NF-200.

4.3.2 Anatomical distribution of axonal injury

Sherriff et al (1994b) reported that the corpus callosum was the most commonly affected region, but the extent and the distribution of axonal β-APP immunoreactivity varied between cases when examined in different brain areas. The current data however provided evidence that the most severely affected region was the brain stem, which was shown to have significantly greater immunoload of β-APP compared with corpus callosum or internal capsule. This was in agreement with a previous report by Niess et al (2002) which found that injured axons were seen in the pons in almost 89% of traumatic cases, although much of the aetiology was likely to be ischaemic (Smith et al., 2002). In addition, other authors reported that the incidence of axonal injury, as demonstrated by β-APP positivity, was found through qualitative and quantitative measurements to be most prominent in the pons when compared to the corpus callosum, consistent with the present findings (Oehmichen et al., 1998).

4.4 Expression of caspase-3 and calpain

The antibodies used in the current study had been well characterised for western blotting and are specific to the activated forms of caspase-3 (active caspase-3 antibody) and calpain (Ab37 and Ab38 antibodies). However, despite multiple attempts with different protocols, there was no significant immunostaining associated with TAI in the brains of the piglet and human. In addition, a control section of human lymph node with widespread apoptosis showed no positive staining. Therefore it was not possible to assess caspase-3 or calpain activity in the formalin fixed paraffin-embedded tissues studied.

Previously published data was based on immunoblotting studies and frozen sections only. The activity of calpain was first reported in traumatically injured axons after lateral fluid percussion brain injury in rats (Saatman et al., 1996). They employed a combination of immunoblotting and immunohistochemical analyses of frozen sections to detect calpain-specific cleavage of the cytoskeletal protein spectrin. Calpain activation has also been reported in injured axons using impact acceleration injury in the rat and optic nerve stretch in the mouse, with calpain-mediated SBDPs being seen within minutes after injury (Buki et al., 1999; Saatman et al., 2003). In human tissues, McCracken et al (1999) have demonstrated the detection of SBDPs specifically generated by calpain in the corpus callosum after blunt head injury. Injured axons immunoreactive for active caspase-3 have been demonstrated in the brainstem following impact acceleration brain injury in rats (Buki et al., 2000). The current negative findings for active caspase-3, Ab37 and Ab38 antibodies almost certainly relates to the tissue fixation, with formalin fixation

presumably cross-linking important epitopes, hiding them from primary antibodies and, therefore, preventing immunohistochemical staining.

4.5 Co-localisation of β-APP and different neurofilament isoforms, in piglet and human cases associated with TAI

The results of the double-labelling immunofluorescence studies revealed that, although there was some co-localisation between β-APP immunopositive axons and the various NF subtypes studied, there were, in addition, populations of damaged axons which did not co-localise. In general, most β-APP positive axonal swellings co-localised with NF immunopositive swellings, but there was a substantial population of NF (NF-160, NF-200, SMI-34) immunostained axonal swellings which did not show any expression of β-APP staining.

While in this study both piglet and human brain tissues revealed strong evidence of co-localisation of β-APP and each form of NFs, there were, in addition, different and distinct classes of TAI in all the cases examined. Previous studies used β-APP and RM014 markers to study the relationship between impaired axonal transport and neurofilament compaction in adult and paediatric rat models of diffuse brain injury. This is the first study to examine the association between β-APP accumulation and the immunoreactivity of different subunits of neurofilament (NF-160, NF-200 and NFH phosphoform) in an experimental model of non-impact head injury (piglet model) and in human autopsy material.

Traumatically induced axonal damage is thought to cause subtle intra-axonal changes that can lead consistently to local cytoskeletal alterations and axonal transport disruption, resulting in local axonal swelling, detachment and bulb formation (Povlishok, 1992; Povlishock et al., 1992; Povlishock, 1993; Povlishock

and Christman, 1995; Maxwell et al., 1997; Saatman et al., 1998; Smith et al., 1999; Maxwell et al., 2003). A correlation between neurofilament compaction and swelling has been shown in previous reports (Maxwell et al., 1991; Yaghmai and Povlishock, 1992; Grady et al., 1993). It has been hypothesized that impaired axonal transport can result from structural changes in the axon such as axolemmal disruption and/or NFC (Maxwell and Graham, 1997).

The present findings are similar to previous studies that showed, through quantitative analysis, that 25% of the total population of damaged axons in the medial lemniscus of the brain stem of the adult rat exhibited co-localisation of impaired axonal transport and neurofilament compaction (Marmarou et al., 2005), but no correlation between neurofilament compaction and impaired axonal transport in the majority of damaged axons. Using two antibodies, β-APP to detect impaired axonal transport and RM014 to identify neurofilament compaction, Stone et al demonstrated that neurofilament compaction could take place independently of impaired axonal transport in pyramidal and corticospinal tracts (Stone et al., 2001). The relationship between impaired axonal transport and neurofilament compaction has also been investigated recently in the immature rat but no evidence of co-localisation of compacted neurofilament and β-APP was found (Dileonardi et al., 2009). Administration of FK506 treatment, the calcineurin inhibitor, was shown to decrease the extent of impaired axonal transport (Marmarou and Povlishock, 2006) but no effect was noticed on NFC (Reeves et al., 2007).

Based upon the present findings, it appears that cytoskeletal alterations can occur in piglets and in humans in the absence of subsequent disruption of axonal transport. This observation was unexpected since these distinct forms of intra-axonal

change have been postulated to represent stages in the progressive axonal failure that would eventually result in delayed disconnection (Stone et al., 2001); RMO14 was thought to correspond to areas of NFC, mitochondrial swelling and microtubular loss, whereas β-APP accumulation was thought to correspond to areas of organelle accumulation. Although distinct these changes were thought to form part of continuum in the disconnection and ultimate degeneration of the axon. Dramatic cytoskeletal misalignment such as NFC would be expected to cause local anterograde transport disruption and upstream swelling. However, it is a possible that more severely damaged axons may undergo the process of anterograde to retrograde conversion of axonal transport (Martz et al., 1989; Sahenk and Lasek, 1988), a process thought to be associated with the activation of the cysteine protease calpain (Nevin, 1967; Sahenk and Lasek, 1988; Buki et al., 1999a; Buki et al., 1999b). Therefore, it is possible that the severe structural damage caused by NFC results in conversion to retrograde transport within the axon, such that β-APP does not accumulate (Stone et al., 2001), although accumulation of this protein is expected in the soma. An additional explanation for the present observation is that there is axonal damage at different sections of the same axon.

In the current investigation, some forms of TAI did not seem to undergo the established sequence of axonal demise including focally impaired axonal transport, swelling and disconnection of a proximal axonal bulb. Although the mechanism that may underlie these distinct events remains unclear, other issues should be taken into consideration. It appears in the current study that β-APP as a marker may underestimate the full extent of traumatically induced axonal injury. This is in keeping with the previous reports which have raised this possibility (Stone et al.,

2001; Marmarou et al., 2005). Buki and colleagues also demonstrated through a quantitative single labelling strategy that β-APP antibody was able to stain only 30-50% of axons from the total axonal numbers identified by the use of the antibody to a calcium-activated calpain-mediated proteolytic break-down product of α-spectrin (Ab38) (Buki et al., 1999). This suggests that either there is some functional recovery taking place within the axon prior to organelle accumulation and disconnection which may, in part, contribute to the absence of β-APP staining within some regions exhibiting neurofilament immunopositive axons (Stone et al., 2001), or that there is a temporal pattern of marker expression, with β-APP accumulation being a terminal event.

The present data appear to have important implications for therapeutic studies focusing on TAI. It is recognized that antibodies to β-APP are often used to measure the efficacy of therapeutic interventions to attenuate TAI, or to detect and then determine the full extent of axonal pathology following TBI (Bramlett et al., 1997; Stone et al., 2000; Singleton et al., 2001). The current observations, however, offer convincing proof that using this will underestimate the full extent of TAI and, therefore, will provide an incomplete insight into the full therapeutic potential for any given pharmacological agent.

Of further importance is the implication of the results of this study in evaluating diffuse axonal injury in humans. β-APP has been used as the only marker of axonal injury in the majority of post-mortem and forensic assessments of TBI (Gentlman et al., 1993; Sherriff et al., 1994a; Sherriff et al., 1994b; Stone et al., 2000). Since the antibody to β-APP stains only a subset of traumatically injured axons as demonstrated in the current study, a broader panel should be employed

utilizing multiple markers of TAI to evaluate the full extent of axonal damage following TBI. This needs to be correlated with detailed clinical studies to assess which populations of damaged axons are actually clinically relevant, and which axons are potentially salvageable by therapeutic intervention. Developing broader antibody panels to more completely assess axonal injury in experimental and diagnostic forensic neuropathology may result in more reliable diagnoses.

In summary, our data has indicated that co-localisation of β-APP immunoreactivity with axonal accumulation of NFs was not a consistent feature of TAI in piglets and humans. These findings raise the possibility that several mechanisms may be involved in TAI due to the heterogeneity of traumatically induced axonal injury in piglet and human brains. As our understanding of the intrinsic mechanisms associated with "programmed axonal death" increases we may develop a fuller understanding of the different molecular pathways defining different populations of damaged axons in TAI.

5. Conclusion

In summary, in rrelation to the hypotheses outlined at the beginning of this thesis:

1. A database of autopsy human traumatic brain injury cases from Lothian and Borders has been developed and critically evaluated against published data. The current neuropathological findings seem to be different from those observed in the west of Scotland. This will serve as a valuable resource for all future human TBI research studies undertaken in Edinburgh. The published data relating to human traumatic axonal injury has been critically evaluated. This has demonstrated how the concept of TAI has evolved over time, and how introduction of immunohistochemsitry has altered our understanding of the diagnosis of TAI, initially by improving diagnostic sensitivity and then by identifying different patterns associated with trauma and metabolic insults. Interpretation of β-APP immunoreactivity continues to evolve and it is likely that a wider panel will be introduced to assess axonal injury.

2. Immunohistochemical analysis of β-APP and a range of NF antibodies was undertaken in both an experimental model of non-impact head injury (piglet model) and in human autopsy material. These studies demonstrated some co-localisation between β-APP, a marker of axonal flow dysfunction, and NF subtypes, as markers of structural changes to the axon. However, there were clearly demonstrable separate populations of axons that showed structural alterations, but with no axonal flow dysfunction. This is in line with previously published rodent models and points towards a much more complex process than simply mechanoporation with subsequent axonal degeneration. The use of β-APP alone in

diagnostic assessment of TAI is likely to under-represent the population of damaged axons, and supports the concept of the use of an antibody panel to fully assess axonal injury. The suggested antibody panel include β-APP as a marker of axonal transport dysfunction, three antibodies targeting various subunits of NF (NFL, NFM and NFH) and an antibody that recognises the hyperphosphorylated form of NFH.

6. Future studies

A disappointing aspect of this study was the inability to successfully immunostain formalin fixed paraffin embedded sections with antibodies to markers of calpain and caspase activation. To fully address the issue of activation of these enzymes in human tissues in trauma a prospective archive of fresh human TBI tissue will need to be developed to allow frozen section immunostaining and western blotting. In addition such a resource would allow for proteomics studies to provide greater detail at a protein level as to the mechanisms activated by mechanical trauma.

In addition, with increased understanding of the orchestrated death pathways within the axonal component (Figure 6.1) there is an opportunity to carefully assess at which points trauma activate the pathway and at which points other injurious stimuli produce axonal injury.

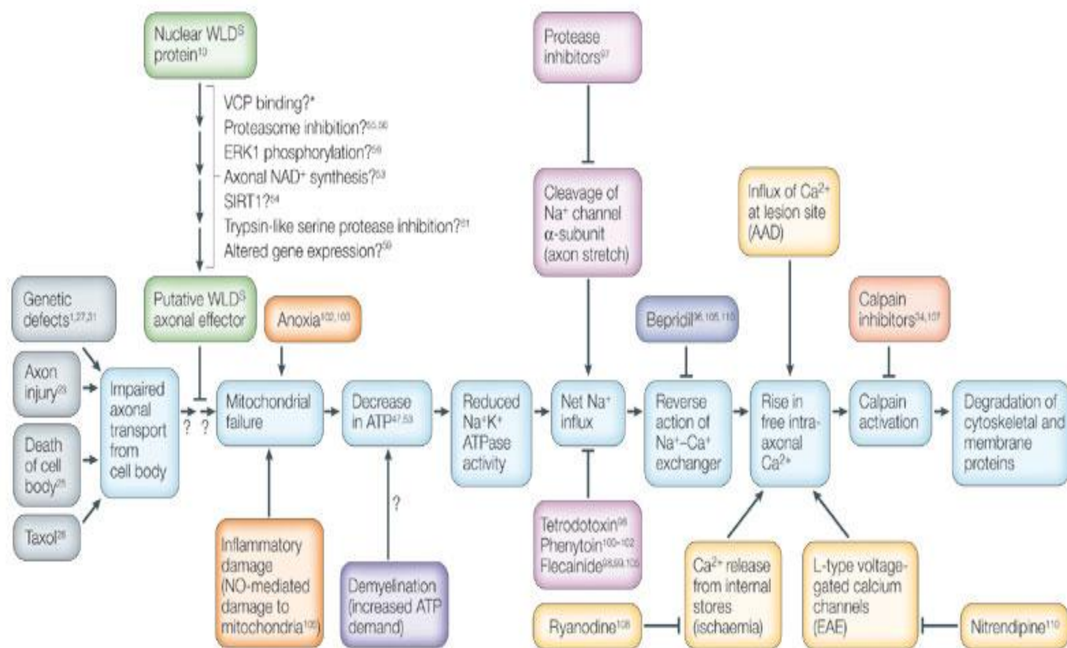


Figure 6.1: A pictorial representation of the possible molecular mechanism underlying programmed axonal degeneration. In addition, the point of activation of many toxic insults are highlighted, although where trauma initiates the death programme is uncertain. Adapted from (Coleman, 2005).

The roles of Nmnat-1, Nmnat-2 and nicotinamide deaminase (NAD) in TAI are of particular interest (Figure 6.2). Mitochondria clearly play a major role in axonal degeneration and a range of antibodies are now available to assess mitochondrial function in axonal compartments, although again fresh frozen tissue is optimal in this assessment. Novel drugs are being developed which can modify these pathways and potentially alleviate axonal degeneration and possibly limit symptoms associated with TAI. The piglet model can be used as a model to assess the

molecular pathways and to subsequently test the effects of pharmacological intervention targeted to focal points of the molecular pathway to alleviate the functional impact of TAI in a closely controlled physiological model.

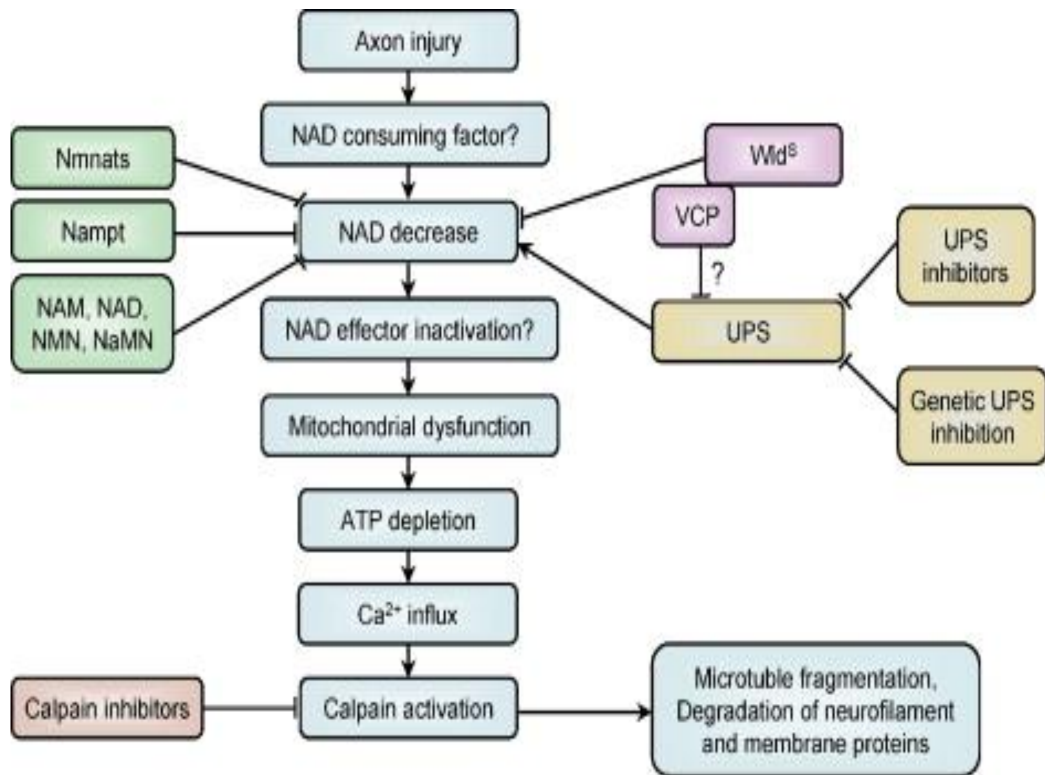


Figure 6.2: The potential role of NAD and NAD-consuming factors in axonal death programmes. Adapted from (Yan et al., 2010).

7. References

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8. Appendices

8.1 Appendix 1

Study Proforma

NEUROPATHOLOGIES AFTER TRAUMATIC BRAIN INJURY

AMONGST EDINBURGH COHORT

Division of Pathology (Forensic Medicine)
The Wilkie Building
Teviot Place
Edinburgh
EH8 9AG

1.PM Number

2.Histological study

No:	1
Limited:	2
Comprehensive:	3

3. Sex

Male:	1
Female:	2

4. Age

<12 months:	1
1-19 years:	2
20-39 years:	3
40-59 years:	4
60-79 years:	5
80+ years:	6

5. Mechanism of Injury

RTA:	1
Fall:	2
Assault:	3
Not Known:	9

6. Survival

≤24 hours:	1	≤3 months:	7
≤48 hours:	2	≤6 months:	8
≤72 hours:	3	≤12 months:	9
≤7 days:	4	>12 months:	10 (specify)
≤14 days:	5	Not known:	99
≤28 days:	6		

7. If survival <24 hours, specify hours

Instantaneous death:	00
Not known/not applicable:	99

8. Fracture of skull

No:	1
Yes:	2
Not known:	9

9. Contusion Index

Absent:	1
Present:	2
Not known:	9

10. Diffuse ischaemic brain damage (global ischaemia)

Absent:	1
Present:	2
Not known:	9

11.Intracranial Haematoma

Code		Location	L	R
None:	1	Supratentorial-Extradural	☐	☐
≤2 cm diameter:	2	Subdural	☐	☐
>2 cm diameter:	3	Subarachnoid	☐	☐
Present, size unknown:	4	Intracerebral- Frontal	☐	☐
Removed at operation:	5			
Not known:	9	- Temporal	☐	☐
		-Parietal	☐	☐
		-Occipital	☐	☐
		-Basal ganglia	☐	☐
		Burst lobe -Frontal	☐	☐
		-Temporal	☐	☐
		-Parietal	☐	☐
		-Occipital	☐	☐
		Infratentorial- Extradural	☐	☐

Subdural ☐ ☐

Intracerebellar ☐ ☐

Burst lobe ☐ ☐

12.Raised Intracranial Pressure

Absent:	1
Macroscopic:	2
Macro plus micro:	3
Microscopic only:	4
Not known:	9

	L	R
Supracallosal hernia	☐	☐
Tentorial hernia	☐	☐
Tonsillar hernia	☐	☐
Secondary brainstem haemorrhage/infarction	☐	☐

13.Diffuse traumatic axonal injury

Absent:	1
DAI grade 1:	2
DAI grade 2:	3
DAI grade 3:	4

☐

14. Diffuse vascular injury

Absent:	1
Present:	2
Not known:	9

15. Brain swelling

L R

Absent:	1	Related to ischaemia: brain damage	4
Related to contusions:	2	Related to combination:	5
Related to intracranial: haematoma	3	Other:	6

8.2 Appendix 2

Database

1982

Case Number	Sex	Age	Survival Time	Type of trauma	Fracture of skull	Contusions	ICH	Swelling	Ichaemia/ Hypoxia	TAI
NA127/82	M	54 Y	NA	NA	-	-	-	-	-	-
NA042/82	M	66 Y	3 W	NA	+	-	LEDH	-	-	-
NA143/82	M	20 Y	1 Y and 4 Mo	RTA	-	-	-	-	-	-
NA185/82	F	93 Y	16 D	Fall	+	+	RSDH	+	-	-

1983

Case Number	Sex	Age	Survival Time	Type of trauma	Fracture of skull	Contusions	ICH	Swelling	Ichaemia/ Hypoxia	TAI
NA009/83	M	47 Y	NA	NA	-	-	-	+	-	-
NA025/83	F	33 Y	NA	Fall	-	-	-	-	-	-
NA128/83	M	25 Y	10 D	Fall	-	-	LEDH,RSAH	+	+	-
NA148/83	F	5 Y	NA	NA	-	-	ICH	+	+	-
NA190/83	M	45 Y	NA	NA	-	-	-	-	-	-
NA218/83	F	95 Y	NA	NA	-	-	-	-	-	-
NA241/83	M	84 Y	NA	Fall	+	-	RSDH	+	-	-
NA248/83	F	63 Y	NA	NA	-	-	SDH	+	-	-
NA251/83	F	21 Y	NA	NA	-	-	-	-	-	-

1984

Case Number	Sex	Age	Survival Time	Type of trauma	Fracture of skull	Contusions	ICH	Swelling	Ichaemia/ Hypoxia	TAI
NA064/84	M	75 Y	NA	NA	-	-	-	-	-	-
NA112/84	F	78 Y	NA	NA	-	-	-	-	-	-
NA113/84	F	77 Y	NA	NA	-	-	-	-	-	-

1985

Case Number	Sex	Age	Survival Time	Type of trauma	Fracture of skull	Contusions	ICH	Swelling	Ichaemia/ Hypoxia	TAI
NA019/85	F	67 Y	NA	NA	+	+	EDH,SDH,SAH	-	-	-
NA147/85	M	73 Y	NA	NA	-	+	SAH,SDH	+	-	-
NA202/85	M	68 Y	NA	NA	-	-	SDH	-	-	-

1986

Case Number	Sex	Age	Survival Time	Type of trauma	Fracture of skull	Contusions	ICH	Swelling	Ichaemia/ Hypoxia	TAI
NA009/86	M	72 Y	NA	NA	-	-	-	-	-	-
NA065/86	M	NA	NA	NA	-	-	ICH	-	-	-
NA124/86	M	NA	NA	NA	-	-	-	-	-	-
NA139/86	M	63 Y	NA	NA	-	-	-	-	-	-
NA172/86	M	26 Y	NA	RTA	-	-	-	-	-	-
NA202/86	M	68 Y	NA	NA	-	-	-	-	+	-

1987

Case Number	Sex	Age	Survival Time	Type of trauma	Fracture of skull	Contusions	ICH	Swelling	Ichaemia/ Hypoxia	TAI
NA076/87	M	64 Y	NA	NA	-	-	-	-	-	-
NA140/87	M	73 Y	NA	Fall	-	-	-	-	-	-
NA177/87	M	67 Y	NA	NA	-	-	SDH	+	-	-
NA328/87	M	28 Y	NA	NA	-	-	SAH	+	-	-
NA332/87	F	26 Y	NA	NA	-	-	-	-	-	-

1988

Case Number	Sex	Age	Survival Time	Type of trauma	Fracture of skull	Contusions	ICH	Swelling	Ichaemia/ Hypoxia	TAI
NA009/88	M	50 Y	6 D	Fall	+	-	EDH	+	-	-
NA011/88	F	27 Y	NA	NA	-	+	-	+	-	-
NA012/88	F	75 Y	NA	Fall	+	+	SAH,SDH	-	-	-
NA047/88	M	75 Y	NA	NA	-	-	-	-	-	-
NA075/88	F	81 Y	4 H and 15 Min	RTA	-	+	SAH	+	-	-
NA076/88	M	79 Y	8 Y	W.Accident	-	-	-	-	-	-
NA093/88	F	59 Y	2 D	Fall	+	+	SDH,SAH,EDH	+	-	-
NA137/88	M	88 Y	NA	NA	-	-	-	-	-	-
NA174/88	M	12 Y	1 D	RTA	-	+	SAH,REDH	+	-	-
NA213/88	F	59 Y	6 D	Fall	-	+	SAH	-	-	-
NA214/88	M	61 Y	7 H	RTA	+	+	SAH	+	-	-
NA215/88	M	64 Y	16 H	Fall	+	+	SDH,SAH	-	+	-
NA226/88	M	20 Y	4 H and 25 Min	W.Accident	+	+	SAH	+	-	-
NA259/88	M	74 Y	NA	NA	-	-	SDH,EDH	+	+	-
NA261/88	M	4 M	50 Min	RTA	+	-	-	-	-	-
NA282/88	M	74 Y	NA	NA	-	-	-	-	-	-
NA294/88	M	47 Y	NA	NA	-	-	-	-	-	-
NA304/88	F	57 Y	NA	NA	-	+	SDH	+	-	-
NA305/88	F	56 Y	9 H and 20 Min	RTA	-	+	-	+	-	-
NA306/88	M	62 Y	NA	RTA	-	+	SAH	+	-	-
NA307/88	M	45 Y	NA	RTA	-	-	-	-	-	-
NA359/88	M	54 Y	40 Min	Fall	+	+	-	+	-	-
NA406/88	M	52 Y	4 D	Gunshot	-	-	SAH,SDH	+	-	-
NA408/88	M	44 Y	NA	RTA	-	+	SAH,EDH	+	-	-
NA409/88	M	3 Y	17 H and 50 Min	RTA	-	-	SAH,SDH	+	-	-

1989

Case Number	Sex	Age	Survival Time	Type of trauma	Fracture of skull	Contusions	ICH	Swelling	Ichaemia/ Hypoxia	TAI
NA017/89	F	69 Y	2 Min	Fall	-	-	SAH	-	-	-
NA018/89	M	40 Y	18 D	Fall	-	-	SDH	+	+	-
NA021/89	F	29 Y	2 D	RTA	+	+	SAH,SDH	+	-	-
NA025/89	M	74 Y	NA	NA	-	-	-	-	-	-
NA049/89	F	79 Y	12 D	RTA	-	+	SDH,SAH	+	+	-
NA051/89	M	64 Y	NA	Fall	-	+	SAH,LSDH	+	-	-
NA070/89	M	67 Y	1 D and 6 H	Fall	-	-	SDH,EDH,SAH	+	-	-
NA088/89	M	74 Y	NA	NA	-	-	-	-	-	-
NA091/89	F	88 Y	6 W	Fall	-	+	SDH	+	-	-
NA114/89	M	78 Y	6 D	RTA	+	-	SAH,ICH	+	-	TAI
NA118/89	M	53 Y	NA	Gunshot	+	-	SAH	-	-	-
NA120/89	F	19 Y	NA	RTA	-	-	-	+	-	-
NA137/89	M	8 Y	25 Min	RTA	-	-	-	-	-	-
NA138/89	F	78 Y	NA	Collapse	+	+	-	-	-	-
NA140/89	F	34 Y	NA	NA	-	-	-	-	-	-
NA182/89	F	65 Y	NA	Assault	-	+	-	+	-	-
NA183/89	M	20 Y	2 H	Fall	+	+	-	+	-	-
NA185/89	F	57 Y	2 H	Assault	+	-	SAH,SDH	+	-	-
NA186/89	M	33 Y	2 D	Assault	-	+	EDH,SDH	+	-	-
NA212/89	M	21 Y	1 H and 30 Min	RTA	-	+	SAH	+	+	-
NA231/89	F	42 Y	50 Min	Assault	-	-	SAH	+	-	-
NA232/89	F	70 Y	4 H and 10 Min	RTA	+	+	-	+	-	-
NA251/89	F	40 Y	NA	Assault	+	-	-	+	-	-
NA252/89	M	84 Y	1 D	Fall	-	+	SAH	+	-	-
NA260/89	M	77 Y	NA	Collapse	-	-	-	-	-	-
NA262/89	M	21 Y	0 M	RTA	-	-	-	+	-	-
NA265/89	M	22 Y	0 M	Fall	-	+	-	+	-	-

NA268/89	F	82 Y	5 D	RTA	-	+	SAH,SDH	-	-	-
NA278/89	M	78 Y	1 D	RTA	+	-	SAH	+	-	-
NA279/89	M	21 Y	0 M	RTA	-	-	SAH	+	-	-
NA280/89	F	25 Y	12 H	RTA	-	+	SAH,SDH	+	-	TAI
NA281/89	F	63 Y	3 D	Fall	-	+	SAH,SDH	+	-	-
NA282/89	M	23 Y	0 M	RTA	-	+	-	+	-	-
NA295/89	F	41 Y	0 M	RTA	+	-	-	+	-	-
NA296/89	M	42 Y	3 D	RTA	-	-	SAH,SDH	+	+	TAI
NA297/89	M	25 Y	0 M	RTA	+	-	SAH	+	-	-
NA309/89	M	35 Y	0 M	Fall	-	+	SAH,SDH	+	-	-
NA312/89	F	65 Y	20 M	RTA	-	+	SAH	+	-	-
NA318/89	M	18 Y	18 H	RTA	-	+	-	+	-	-
NA334/89	M	29 Y	55 Min	Fall	-	+	-	+	-	-
NA335/89	M	21 Y	18 H	RTA	-	+	SAH,SDH	+	-	-
NA339/89	M	27 Y	1 H	RTA	+	+	-	+	-	-
NA340/89	F	22 Y	0 M	RTA	-	-	-	+	+	-
NA362/89	M	18 Y	18 H	RTA	-	+	-	+	-	-
NA371/89	M	28 Y	0 M	RTA	-	+	SAH	+	+	-
NA372/89	F	62 Y	0 M	RTA	+	-	-	+	-	-
NA373/89	F	21 Y	0 M	C.building	-	-	-	+	+	-
NA374/89	M	35 Y	0 M	C.building	-	-	SAH,SDH	+	+	-
NA381/89	F	86 Y	4 D	RTA	+	-	ICH	+	-	-
NA382/89	M	25 Y	3 D	RTA	-	-	ICH,SDH	+	+	-
NA384/89	M	72 Y	24 H	RTA	-	-	SDH	-	+	-
NA407/89	F	54 Y	0 M	F.Wall	-	+	SDH	+	+	-
NA408/89	F	63 Y	NA	RTA	-	-	-	-	+	-
NA409/89	F	26 Y	1 H	Fall	-	+	SAH	+	+	-
NA410/89	M	17 Y	15 H	RTA	-	+	SAH,ICH	+	+	-

1990

Case Number	Sex	Age	Survival Time	Type of trauma	Fracture of skull	Contusions	ICH	Swelling	Ichaemia/ Hypoxia	TAI
NA015/90	M	73 Y	NA	Gunshot	-	-	-	-	-	-
NA019/90	F	68 Y	NA	Fall	-	-	SDH,SAH	-	-	-
NA020/90	M	69 Y	18 H	RTA	+	-	SAH,SDH,ICH	+	+	-
NA025/90	M	19 Y	8 H	RTA	-	-	SAH	+	-	-
NA050/90	F	16 Y	1 H	RTA	+	-	-	+	+	-
NA053/90	M	44 Y	18 H	Fall	-	+	SAH	+	-	-
NA055/90	M	57 Y	18 H	RTA	-	+	SAH	+	+	-
NA056/90	F	73 Y	NA	RTA	+	+	SAH	+	-	-
NA065/90	F	74 Y	0 M	Assault	-	-	SAH	+	-	-
NA066/90	F	2 Y	0 M	RTA	-	+	SAH	+	+	-
NA067/90	M	30 Y	0 M	RTA	-	+	SAH	+	-	-
NA070/90	M	77 Y	40 H	Fall	-	-	SDH	+	-	-
NA071/90	M	36 Y	1 H	RTA	-	+	SAH	+	-	-
NA072/90	M	77 Y	11 D	RTA	-	+	SAH	+	-	-
NA077/90	M	34 Y	NA	NA	-	-	SAH	+	-	-
NA078/90	M	21 Y	0 M	RTA	+	+	SAH,SDH	+	+	-
NA079/90	M	40 Y	0 M	Fall	+	-	SAH	+	-	-
NA080/90	M	68 Y	1 H	RTA	+	-	SAH,SDH	+	-	-
NA089/90	F	66 Y	1 D	NA	-	+	SAH,ICH	+	-	-
NA110/90	M	75 Y	NA	Dementia	-	+	-	-	-	-
NA132/90	M	18 Y	5 D	RTA	-	+	RSDH,SAH,ICH	+	-	TAI
NA133/90	M	66 Y	1 D	Fall	+	+	RSDH,SAH	+	+	-
NA136/90	M	24 Y	0 M	RTA	-	+	SAH	+	+	-
NA155/90	M	39Y	20 H	Assault	-	-	RSDH	+	+	-
NA158/90	M	25 Y	0 M	RTA	-	-	-	-	-	-
NA160/90	F	83 Y	24 H	RTA	+	+	RSDH,SAH	+	-	-

NA161/90	M	80 Y	13 Y	RTA	+	-	-	-	-	-
NA162/90	M	61 Y	9 H	Fall	+	+	SAH,IVH	+	+	-
NA163/90	F	75 Y	0 M	RTA	+	+	SAH	-	+	-
AN164/90	M	20 Y	1 D and 2 H	RTA	-	+	ICH,SDH	+	+	-
NA171/90	F	78 Y	Brief	RTA	+	+	SAH	+	-	-
NA172/90	M	20 Y	Minimum	Fall	-	-	BSAH,BSAH	-	+	-
NA174/90	M	32 Y	19 H	RTA	+	-	BSDH,SAH	+	+	-
NA191/90	M	21 Y	30 H	RTA	+	-	BSDH,SAH	+	+	-
NA192/90	M	38 Y	2 H	Fall	+	+	SAH.SDH	+	+	-
NA197/90	M	82 Y	26 D	RTA	-	-	-	-	-	-
NA198/90	F	75 Y	24 H	NA	-	-	SAH	+	-	-
NA199/90	F	17 Y	0 M	RTA	+	+	-	+	+	-
NA200/90	F	51 Y	2 H	RTA	+	-	SAH	+	-	-
NA208/90	M	15 Y	0 M	RTA	-	+	SAH	+	-	-
NA209/90	F	35 Y	NA	Stabbing	-	-	-	+	-	-
NA221/90	M	71 Y	24 H	NA	-	+	-	-	+	-
NA230/90	M	30 Y	8 Y	RTA	-	-	-	-	+	-
NA231/90	F	17 Y	0 M	RTA	-	-	SAH	+	-	-
NA239/90	M	30 Y	24 H	RTA	-	+	SAH,SDH	+	+	-
NA242/90	M	31 Y	45 M	RTA	+	-	-	+	-	-
NA243/90	M	43 Y	1 H	RTA	-	-	-	-	+	-
NA245/90	M	37 Y	1 H	RTA	+	+	SAH	+	+	-
NA260/90	M	18 Y	0 M	RTA	+	+	-	+	-	-
NA262/90	M	41 Y	3 H	Assault	-	-	-	-	+	-
NA280/90	M	77 Y	1 W	Fall	-	+	BSDH,LEDH,SAH	+	-	-
NA281/90	M	47 Y	31 Y	RTA	+	-	-	-	-	-
NA288/90	F	69 Y	0 M	Fall	-	-	SAH	-	+	-
NA297/90	M	28 Y	20 Y	RTA	+	-	-	+	-	-
NA303/90	F	66 Y	NA	NA	-	-	-	-	-	-
NA307/90	M	30 Y	0 M	Fall	+	+	LSDH,SAH	+	+	-
NA308/90	M	51 Y	0 M	RTA	+	+	SAH	-	+	-
NA309/90	M	14 Y	0 M	RTA	+	-	SAH	-	+	-

NA330/90	F	80 Y	10 H and 30 M	Fall	+	+	BSDH	+	+	-
NA336/90	M	59 Y	11 D	NA	+	+	RSDH	+	+	-
NA337/90	M	36 Y	0 M	RTA	-	-	SAH	+	+	-
NA338/90	M	53 Y	10 D	NA	-	+	REDH,SAH	+	+	-
NA339/90	M	33 Y	0 M	RTA	+	-	ICH	+	-	-
NA344/90	M	58 Y	0 M	Fall	+	+	SAH	+	-	-
NA379/90	F	77 Y	NA	NA	+	+	-	-	-	-
NA393/90	F	81 Y	0 M	Fall	-	+	SAH	-	-	-
NA423/90	M	36 Y	0 M	RTA	-	-	-	-	-	-
NA424/90	F	2 Y	1 H and 30 M	Fall	-	+	SAH	+	-	-
NA425/90	M	33 Y	12 Y	RTA	-	-	-	+	-	-
NA426/90	M	23 Y	35 M	RTA	-	+	SAH	+	-	-
NA449/90	M	42 Y	0 M	RTA	-	-	SAH	+	-	-
NA464/90	F	31 Y	6 H	Fall	-	+	RSDH	+	-	-
NA465/90	F	79 Y	20 D	RTA	-	+	ICH	-	+	TAI
NA497/90	M	59 Y	4 H	RTA	-	+	SDH,SAH,ICH	+	-	TAI
NA500/90	M	17 Y	0 M	RTA	+	+	SAH	+	+	-
NA520/90	M	6 Y	40 M	RTA	-	+	-	+	-	-
NA522/90	M	19 Y	0 M	RTA	-	-	-	+	+	
NA523/90	M	54 Y	3 H and 30 M	RTA	+	+	SAH	-	+	-
NA524/90	M	50 Y	5 H and 10 M	Fall	+	+	SAH	+	-	-
NA525/90	M	13 Y	0 M	RTA	-	+	SAH	+	-	-
NA534/90		68 Y	NA	NA	-	-	SDH	-	-	-
NA548/90	M	59 Y	0 M	Fall	-	+	IVH	+	-	-
NA549/90	M	82 Y	5 H	RTA	-	-	SAH	-	-	-
NA551/90	F	41 Y	15 M	RTA	+	+	IVH	-	+	-

1991

Case Number	Sex	Age	Survival Time	Type of trauma	Fracture of skull	Contusions	ICH	Swelling	Ichaemia/ Hypoxia	TAI
NA016/91	F	20 Y	55 M	RTA	-	+	H in brain stem	+	-	-
NA018/91	M	62 Y	31Y	Fall	+	+	-	-	+	-
NA032/91	M	75 Y	6 D	Fall	-	+	SAH,SDH	+	-	-
NA034/91	M	47 Y	12 H	Fall	-	+	SAH	+	-	-
NA035/91	M	20 y	30 M	RTA	-	-	SAH	+	-	-
NA036/91	M	32 Y	3 H	Fall	-	+	ICH	+	-	-
NA050/91	F	70 Y	2 H and 35 M	RTA	+	+	SDH,SAH,ICH	+	-	-
NA053/91	M	68 Y	34 Y	Fall	-	+	-	-	-	-
NA054/91	M	65 Y	8 H	Fall	+	+	ICH,SAH	+	+	-
NA076/91	M	35 Y	NA	RTA	+	+	SAH	+	-	-
NA089/91	M	62 Y	3 D	Fall	+	+	LSDH	+	-	-
NA090/91	F	72 Y	13 H	RTA	-	-	LEDH,SAH	+	-	-
NA092/91	M	61 Y	NA	NA	-	-	LSDH	+	-	-
NA112/91	M	26 Y	24 D	Assault	-	+	-	+	-	TAI
NA121/91	M	24 Y	1 H	RTA	-	+	-	+	-	-
NA143/91	M	8 Y	3 D	RTA	-	+	SDH	+	-	TAI
NA151/91	M	20 Y	5 H	RTA	-	+	SAH	+	-	-
NA154/91	M	76 Y	2 H	RTA	+	+	SAH	+	-	-
NA168/91	M	64 Y	2 D	Fall	-	+	SAH,SDH	+	-	-
NA177/91	M	7 Y	5 H and 15 M	RTA	-	-	SAH	+	+	-
NA189/91	M	38 Y	0 M	By train	-	-	SAH	+	+	-
NA207/91	M	49 Y	NA	NA	-	+	SAH,PVH	+	+	-
NA221/91	M	19 M	2 H	Fall	-	+	-	+	-	-
NA222/91	M	76 Y	30 M	RTA	-	-	SAH,PVH,IVH	-	-	-
NA229/91	F	19 Y	1 H	RTA	-	-	SAH,H in BS	+	+	-
NA232/91	M	68 Y	35 Y	RTA	+	+	-	-	-	-
NA256/91	M	55 Y	NA	NA	-	+	-	-	-	-
NA262/91	M	35 Y	20 H	RTA	-	-	-	+	+	-

NA273/91	F	65 Y	45 M	RTA	+	-	SAH	+	+	-
NA288/91	M	58 Y	0 M	RTA	-	+	SAH	+	+	-
NA289/91	M	17 Y	45 M	RTA	+	+	-	+	+	-
NA291/91	M	27 Y	9 Y	NA	-	-	-	+	+	-
AN292/91	M	41 Y	10 D	RTA	+	+	ICH	-	+	TAI
NA301/91	M	77 Y	NA	NA	-	+	-	-	+	-
NA319/91	M	16 Y	0 M	RTA	-	+	SAH	+	+	-
NA323/91	F	43 Y	0 M	Fall	-	+	SAH	+	-	-
NA324/91	M	54 Y	NA	NA	-	+	-	+	-	-
NA337/91	M	20 Y	0 M	RTA	-	+	SAH	+	-	-
NA344/91	F	63 Y	2 D	Fall	+	+	SDH,SAH,H in brain stem	+	+	-
NA345/91	F	51 Y	0 M	RTA	+	+	SAH,Stem H	+	+	-
NA350/91	M	73 Y	0 M	Fall	-	+	-	+	-	-
NA352/91	M	52 Y	NA	NA	-	-	SDH	-	-	TAI
NA357/91	M	38 Y	5 Y	Fall	+	+	ICH	+	-	-
NA358/91	F	75 Y	1 Y	NA	-	-	SAH	-	-	-
NA360/91	M	52 Y	4 H and 45 M	RTA	-	+	SAH,IVH	+	-	-
NA362/91	M	30 Y	2 D	RTA	-	+	SDH,SAH	+	-	TAI
NA377/91	M	18 Y	1 H	RTA	-	-	SAH.IVH	+	-	-
NA383/91	M	81 Y	1 M	Fall	+	-	RSDH,LEDH	-	-	TAI
NA384/91	M	21 Y	0 M	RTA	+	+	-	+	+	-
NA387/91	M	26 Y	0 M	Fall	+	+	SAH,IVH	+	+	-
NA398/91	F	67 Y	1 H and 40 M	Assault	-	-	SAH	+	-	-
NA401/91	M	20 Y	1 H and 15 M	RTA	+	+	SAH,IVH	+	-	-
NA413/91	F	23 Y	12 H	RTA	-	+	SDH	+	+	-
NA417/91	F	26 Y	0 M	RTA	+	+	SAH	+	+	-
NA418/91	F	13 Y	0 M	RTA	-	-	SAH	+	-	-
NA419/91	F	3 Y	1 H	RTA	-	-	-	+	+	-
NA446/91	F	46 Y	NA	NA	-	+	SAH,ICH,IVH	+	+	TAI
NA447/91	F	5 Y	1 H	RTA	-	+	SAH	+	-	-
NA448/91	M	2 Y	1 H	RTA	-	+	-	+	-	-
NA449/91	M	6 Y	1H	RTA	-	-	-	+	-	-

NA454/91	M	63 Y	0 M	Fall	-	+	SDH,SAH,ICH,EDH	+	+	-
NA456/91	M	20 Y	23 H	Gunshot	-	+	SAH,ICH	+	-	-
NA461/91	F	38 Y	40 M	RTA	-	-	SAH	+	+	-
NA470/91	F	21 Y	30 M	RTA	+	+	SAH	+	+	-
NA472/91	M	25 Y	0 M	RTA	+	-	-	+	-	-
NA484/91	M	39 Y	0 M	RTA	-	+	SAH,PVH	+	-	-
NA489/91	M	28 Y	0 M	RTA	-	+	SAH,SDH	+	-	-
NA490/91	F	15 y	0 M	RTA	-	-	-	+	+	-
NA491/91	F	44 Y	0 M	RTA	-	-	-	+	+	-
NA492/91	M	19 Y	NA	RTA	-	-	SAH	+	-	-
NA494/91	F	28 Y	NA	RTA	-	+	H in Pons	+	-	-
NA495/91	M	19 Y	1 D	RTA	-	+	SAH	+	-	-

1992

Case Number	Sex	Age	Survival Time	Type of trauma	Fracture of skull	Contusions	ICH	Swelling	Ichaemia/ Hypoxia	TAI
NA060/92	M	16 Y	1 H and 45 M	NA	-	+	-	+	-	-
NA061/92	M	14 Y	40 M	Fall	-	+	SAH	+	-	-
NA062/92	F	39 Y	NA	Fall	-	+	SAH	+	-	-
NA064/92	M	89 Y	9 D	RTA	-	+	ICH	+	-	-
NA066/92	M	19 Y	10 H	Fall	+	+	SAH	+	-	TAI
NA067/92	F	86 Y	5 H	Fall	-	+	SAH,IVH	-	-	-
NA068/92	M	64 Y	40 M	RTA	-	-	SAH	+	-	-
NA069/92	F	82 Y	1 H	RTA	-	+	SAH	+	-	-
NA070/92	F	19 Y	1 D	RTA	-	+	SAH	+	-	-
NA089/92	M	25 Y	18 M	RTA	-	+	SAH,IVH	+	-	-
NA091/92	F	76 Y	2 H	RTA	-	-	-	-	-	-
NA102/92	F	14 Y	17 H	RTA	-	+	ICH	-	+	TAI
NA105/92	M	47 Y	50 M	RTA	-	-	SAH	+	+	-
NA114/92	M	54 Y	NA	Fall	+	+	SDH,H in BS	+	-	-
NA117/92	M	70 Y	NA	NA	-	-	-	+	-	-

NA120/92	F	15 Y	NA	RTA	-	+	SAH,ICH	+	-	-
NA121/92	F	71 Y	3 H and 15M	Fall	-	+	SAH,H in Pons	+	-	TAI
NA122/92	M	20 Y	NA	RTA	-	+	SAH	-	-	-
NA195/92	F	13 Y	45 M	RTA	+	+	SAH,SDH	+	+	-
NA126/92	F	75 Y	40 M	RTA	-	-	SAH	+	+	-
NA129/92	M	73 Y	30 M	RTA	+	+	SAH,IVH	+	+	-
NA132/92	M	42 Y	50 M	Fall	-	+	SAH	-	-	-
NA133/92	M	72 Y	45 M	RTA	+	+	SAH	-	-	TAI
NA134/92	M	35 Y	NA	NA	-	+	SAH	+	-	-
NA145/92	M	3 M	NA	Fall	-	-	BSDH,SAH	-	-	-
NA172/92	M	59 Y	14 Y	Fall	+	+	SDH,SAH	+	+	-
NA174/92	M	24 Y	35 M	RTA	+	+	SAH	+	+	-
NA176/92	M	64 Y	19 D	RTA	+	+	RSDH,ICH	+	-	-
NA178/92	M	65 Y	2 D	RTA	-	-	SAH	-	-	-
NA179/92	F	19 Y	1 Month	Fall	+	+	-	+	-	-
NA206/92	M	54 Y	25 M	RTA	-	-	SAH	-	+	-
NA210/92	M	47 Y	3 H and 30 M	RTA	-	+	SAH	+	+	TAI
NA211/92	M	28 Y	1 D	RTA	-	+	SAH,IVH	+	+	-
NA212/92	M	16 Y	2 D	RTA	+	+	SAH	+	+	-
NA213/92	M	44 Y	0 M	RTA	-	+	LSDH,SAH,IVH	-	+	-
NA238/92	M	23 M	NA	NA	-	-	LSDH,SAH, H in BS	+	-	-
NA240/92	M	20 Y	1 H	RTA	-	+	H in Pons	+	-	-
NA242/92	M	54 Y	NA	NA	-	+	-	+	-	-
NA245/92	M	67 Y	7 D	Fall	+	-	SDH,SAH,H in BS,ICH	+	+	TAI
NA246/92	M	25 y	0 M	RTA	+	+	SAH,H in BS	+	+	-
NA250/92	F	30 Y	30 M	RTA	-	+	SAH	-	-	-
NA251/92	F	65 Y	5 D	Fall	-	-	RSDH	-	-	-
NA286/92	F	90 Y	1 Month	RTA	-	-	LSDH,SAH	-	+	TAI
NA290/92	M	23 Y	0 M	RTA	-	-	SAH,H in BS	+	-	-
NA325/92	M	19 Y	1 H	RTA	-	-	-	-	+	-
NA331/92	F	86 Y	5 D	RTA	+	+	SAH,ICH	+	+	TAI
NA346/92	M	20 Y	45 M	RTA	-	+	SAH	-	-	-

NA347/92	M	27 Y	2 H and 40 M	RTA	-	-	SAH,IVH	+	-	-
NA348/92	M	49 Y	1 D and 6 H	Fall	+	+	LSDH,SAH,ICH	+	-	-
NA350/92	M	27 Y	2 D	Fall	-	+	SAH,SDH	+	-	-
NA359/92	M	13 Y	2 D	Fall	-	+	SAH,IVH	+	-	-
NA366/92	M	18 Y	35 M	RTA	-	-	IVH	+	-	-
NA411/92	M	18 Y	2 H and 10 M	RTA	+	-	SAH,ICH	+	+	-
NA442/92	M	27 Y	0 M	Gunshot	-	-	SAH	+	-	-
NA449/92	M	88 Y	3 D	Fall	+	+	SDH,SAH,ICH	-	-	-
NA456/92	M	19 Y	1 H	RTA	-	-	SAH,H in Pons	+	-	-
NA457/92	F	12 Y	1 H and 13 M	RTA	-	-	-	+	-	-

1993

Case Number	Sex	Age	Survival Time	Type of trauma	Fracture of skull	Contusions	ICH	Swelling	Ichaemia/ Hypoxia	TAI
NA003/93	M	43 Y	35 M	RTA	-	+	SAH	+	-	-
NA004/93	M	15 Y	1 D and 10 H	RTA	+	+	-	+	-	-
NA0047/93	F	84 Y	2 H	RTA	-	-	-	-	-	-
NA072/93	M	51 Y	40 M	RTA	-	-	SAH	+	+	-
NA130/93	M	38 Y	0 M	RTA	+	+	H in BS	+	-	-
NA131/93	M	54 Y	NA	RTA	-	-	-	-	-	-
NA132/93	M	21 y	4 H	Fall	-	-	-	+	-	-
NA177/93	M	57 Y	NA	NA	-	+	-	-	-	-
NA229/93	M	2 M	NA	NA	-	-	SDH,SAH	-	-	-
NA235/93	F	18 M	NA	NA	-	+	SAH,ICH	+	+	-
NA289/93	F	83 Y	1 Y	Fall	-	-	-	-	-	-

1994

Case Number	Sex	Age	Survival Time	Type of trauma	Fracture of skull	Contusions	ICH	Swelling	Ichaemia/ Hypoxia	TAI
NA131/94	M	71 Y	NA	RTA	-	-	-	-	-	-
NA156/94	M	21 M	NA	Fall	-	-	SAH,RSDH	+	-	TAI
NA175/94	F	69 Y	2 D	Fall	-	-	-	+	-	-
NA189/94	F	57 Y	15 D	Fall	+	+	SAH,H in BS	-	-	-
NA211/94	M	52 Y	3 Y	W.Accident	-	-	-	-	+	-
NA266/94	M	66 y	1 D	Fall	+	+	BSDH,SAH,ICH,IVH	+	+	TAI

1995

Case Number	Sex	Age	Survival Time	Type of trauma	Fracture of skull	Contusions	ICH	Swelling	Ichaemia/ Hypoxia	TAI
NA026/95	M	87 Y	NA	NA	-	-	LICH,SAH	+	-	TAI
NA038/95	M	70 Y	9 D	Fall	-	-	IVH	+	-	TAI
NA114/95	M	75 Y	NA	NA	-	-	-	-	+	-

1996

Case Number	Sex	Age	Survival Time	Type of trauma	Fracture of skull	Contusions	ICH	Swelling	Ichaemia/ Hypoxia	TAI
NA006/96	F	9 Y	1 D and 7 H	RTA	+	+	ICH,LSDH,REDH	+	-	TAI
NA032/96	M	46 Y	NA	Fall	+	+	SAH,BSDH	+	+	-
NA055/96	M	8 Y	NA	RTA	-	+	ICH,SAH	+	-	TAI
NA149/96	F	3 Y	9 H	Fall	+	+	EDH,SAH,IVH,ICH,H in BS	+	+	-
NA197/96	M	54 Y	NA	Fall	+	+	SDH,SAH,IVH	+	+	TAI
NA202/96	F	3 Y	9 H	RTA	-	-	-	+	+	-
NA382/96	M	26 Y	12 D	Fall	-	+	ICH	+	+	TAI
NA408/96	M	85 Y	NA	NA	+	+	-	-	-	-

1997

Case Number	Sex	Age	Survival Time	Type of trauma	Fracture of skull	Contusions	ICH	Swelling	Ichaemia/ Hypoxia	TAI
NA102/97	M	50 Y	NA	RTA	-	+	SDH,EDH,SAH	+	+	-
NA103/97	M	55 Y	NA	Fall	-	+	SDH,SAH,H in BS	+	+	TAI
NA211/97	M	65 Y	26 Y	RTA	-	-	-	-	-	-
NA262/97	M	48 Y	10 D	Fall	-	+	LSDH,SAH,IVH	+	+	-
NA268/97	M	26 Y	NA	Assault	-	-	SAH,SDH	+	-	TAI
NA269/97	F	3 M	NA	NA	-	-	SAH	+	-	-
NA322/97	F	11 Y	NA	Fall	+	+	EDH,SDH	+	+	-
NA338/97	M	51 Y	19Y	RTA	-	+	-	-	-	TAI

1998

Case Number	Sex	Age	Survival Time	Type of trauma	Fracture of skull	Contusions	ICH	Swelling	Ichaemia/ Hypoxia	TAI
NA036/98	M	57 Y	NA	NA	-	+	-	-	-	-
NA047/98	M	10 Y	NA	Fall	+	-	EDH,SAH	+	+	TAI
NA197/98	F	11 Y	1 D	RTA	+	+	SAH,IVH	+	+	-
NA326/98	M	51 Y	NA	NA	-	+	-	-	-	-

1999

Case Number	Sex	Age	Survival Time	Type of trauma	Fracture of skull	Contusions	ICH	Swelling	Ichaemia/ Hypoxia	TAI
NA074/99	M	41 Y	NA	NA	-	-	SDH	-	+	-
NA103/99	M	32 Y	1 D	Assault	-	+	-	+	+	TAI
NA280/99	M	26 Y	5 D	Assault	-	-	-	+	+	-
NA314/99	M	89 Y	NA	Fall	-	-	SDH	-	-	-
NA380/99	M	40 Y	14 D	Assault	+	-	SDH,ICH,SAH	+	+	-

2000

Case Number	Sex	Age	Survival Time	Type of trauma	Fracture of skull	Contusions	ICH	Swelling	Ichaemia/ Hypoxia	TAI
NA101/00	M	30 Y	14 D	Assault	-	+	-	+	+	-
NA143/00	M	12 Y	NA	RTA	-	-	-	-	-	-
NA151/00	M	3 Y	NA	RTA	+	+	SAH	-	-	-
NA161/00	M	9 Y	1 D	RTA	+	+	EDH,SDH,SAH	+	+	TAI

2001

Case Number	Sex	Age	Survival Time	Type of trauma	Fracture of skull	Contusions	ICH	Swelling	Ichaemia/ Hypoxia	TAI
NA116/01	F	9 Y	7 D	RTA	-	+	SDH,SAH	+	+	TAI
NA203/01	M	65 Y	7 D	Fall	+	+	LSDH,EDH,SAH,ICH	-	-	-

2002

Case Number	Sex	Age	Survival Time	Type of trauma	Fracture of skull	Contusions	ICH	Swelling	Ichaemia/ Hypoxia	TAI
NA043/02	F	3 Y	1 H and 30 M	Assault	-	-	SDH,SAH	+	+	TAI
NA056/02	M	67 Y	5 D	Fall	+	+	SAH,H in BS,IVH	+	-	TAI
NA057/02	F	37 Y	1 D	Assault	-	-	SAH	+	-	-
NA060/02	M	40 Y	2 D	RTA	-	-	-	-	-	TAI
NA129/02	M	4 Y	1 H	RTA	+	+	SAH	+	+	TAI
NA150/02	M	43 Y	NA	Assault	-	-	LSDH	-	+	-
NA160/02	M	82 Y	2 D	Assault	-	-	LSDH,ICH	-	-	-
NA188/02	M	26 Y	1 D	NA	+	+	SDH,SAH	+	+	-
NA189/02	F	84 Y	NA	NA	-	-	LSDH	-	+	-
NA198/02	F	48 Y	NA	NA	-	-	SAH	-	+	TAI
NA222/02	M	3 Y	NA	NA	+	+	LSDH,SAH	+	-	-

2003

Case Number	Sex	Age	Survival Time	Type of trauma	Fracture of skull	Contusions	ICH	Swelling	Ichaemia/ Hypoxia	TAI
NA058/03	F	75 Y	3 H and 30 M	RTA	-	+	SAH	-	-	-
NA113/03	M	38 Y	15 H	Assault	-	-	SAH	-	-	-
NA176/03	M	69 Y	10 Y	NA	+	+	-	-	-	-
NA179/03	M	10 Y	2 H	RTA	+	+	PVH	+	-	-
NA182/03	M	64 Y	5 H and 45 M	Fall	-	+	RSDH,SAH,EDH	+	-	TAI
NA185/03	F	51 Y	NA	Fall	-	-	RSDH	-	-	-
NA210/03	M	86 Y	NA	Assault	-	-	SAH,LICH	-	-	-
NA211/03	M	36 Y	1 M and 24 D	Assault	-	-	LSDH,SAH,H in BS	-	+	-
NA244/03	F	95 Y	NA	NA	+	+	SAH	-	-	-

2004

Case Number	Sex	Age	Survival Time	Type of trauma	Fracture of skull	Contusions	ICH	Swelling	Ichaemia/ Hypoxia	TAI
UA095/04	M	22 Y	2 H	RTA	-	-	SAH	+	+	TAI
UA149/04	M	9 Y	1 H and 15 M	Fall	+	+	LSDH,SAH	+	+	TAI
UA218/04	M	65 Y	1 Y	Fall	-	+	-	-	+	-
UA274/04	M	51 Y	NA	Assault	-	-	SAH	-	+	TAI
UA275/04	M	56 Y	0 M	Fall	+	+	SAH	+	-	-
UA386/04	M	41	0 M	Assault	+	+	H in Pons	-	-	TAI
UA390/04	M	64 Y	3 W	Fall	-	+	SAH,LSDH	-	-	-
UA392/04	F	9 Y	45 M	RTA	-	-	-	+	-	-
UA400/04	F	49 y	26 D	RTA	-	+	-	-	+	TAI
UA401/04	M	21 Y	1 H	Fall	+	+	SAH	+	+	-

2005

Case Number	Sex	Age	Survival Time	Type of trauma	Fracture of skull	Contusions	ICH	Swelling	Ichaemia/ Hypoxia	TAI
UA103/05	F	10 Y	40 M	RTA	-	+	-	+	+	-
UA121/05	F	27 M	2 D	Fall	-	-	RSDH	+	-	-

8.3 Appendix 3

Immunohistochemistry protocols

8.3.1 Cutting of paraffin-embedded tissue sections

[1] Preparing the water bath-

1/ Fill the water path and heat to 40ºC.

2/ Remove trapped air bubbles with designated ‘wet’ brush.

3/ Skim a piece of paper across water surface to remove any dust particles.

[2] Attaching the tissue sample-

1/ Make sure that the wheel brake is applied.

2/ Secure the tissue block into its clamp.

[3] Positioning the blade-

1/ Ensure that the microtome wheel is in locked position.

2/ Remove the microtome blade from its cassette and place in the blade clamp.

3/ Lock the blade into position and raise the blade guard.

[4] Adjusting the tissue block-

1/ Lower the blade guard and release the wheel brake and slowly line the block up with the blade, advancing the block with the wheel.

2/ Use the horizontal and vertical adjustment levers on the block holder to ensure that the block face is exactly perpendicular to the blade.

3/ Clamp the block into position, apply the wheel brake, and replace the blade guard.

[5] Cutting sections-

1/ Adjust the microtome to the desire thickness setting (5 µm thick sections)

2/ Use the designated ‘dry’ brush to flatten out any imperfections.

3/ Cut sections consecutively and in rapid succession creating a ribbon.

[6] Floating sections onto slides-

1/ Dip the 'wet' brush into the water bath and use it to gently lift the cut sections from the microtome and float onto the water surface.

2/ Lift each section from the water bath onto the surface of a Superfrost plus slide, label, and place into a slide rack.

3/ Incubate slides to dry at 37ºC overnight in a fan assisted cabinet.

8.3.2 Immunohistochemistry protocol (ABC method)

[1] Deparaffinization and rehydration-

1/ Put the slides in a slide rack and place in Xylene for 5 minutes [x2].

2/ Rehydrate the sections by placing in graded alcohol solutions, 74OP (99% alcohol) for 5 minutes [x2] followed by 70% alcohol for 5 minutes [x2].

3/ Immerse the slides in picric acid for 20-30 minutes to ensure all residual formalin has been removed from the sections.

4/ Rinse in running water until the sections are clear of picric acid.

[2] Antigen retrieval and immunohistochemical staining-

1/ Perform antigen retrieval pre-treatment if required.

2/ Quench endogenous peroxidases by placing slides in hydrogen peroxide (3%) for 10 minutes.

3/ Rinse in water.

4/ Attach slides to coverplates and insert into Sequenza rack.

5/ Rinse sections with TBS.

6/ Incubate sections in serum (1 in 5 dilution in TBS) for 10 minutes.

7/ Incubate sections with primary antibody (diluted in serum/TBS) for 30 minutes.

8/ Rinse with TBS.

9/ Incubate sections with biotinylated antibody (diluted in serum/TBS) for 30 minutes.

10/ Rinse with TBS.

11/ Incubate with ABC for 30 minutes.

12/ Rinse with TBS.

13/ Remove slides from Sequenza and incubate with DAB (or other chromogen) until the desired intensity is achieved, usually 30-60 seconds.

14/ Rinse slides in running water.

15/ Counterstain sections by dipping briefly in haematoxylin.

16/ Rinse in running water.

17/ Dip in Lithium carbonate.

18/ Dehydrate the sections by briefly immersing in graded alcohol (70%, 99%, and absolute alcohol).

19/ Immerse in xylene [x3].

20/ Coverslip sections using Pertex mounting medium.

8.3.3 Double immunofluorescent labelling protocol

[1] Staining for first antigen-

1/ Wash sections in buffer (PBS) for 2 minutes [x2].

2/ Perform Avidin/Biotin blocking if required.

3/ Incubate sections for 5 minutes in working solution of M.O.M.™ diluent.

4/ Incubate section in diluted primary antibody for 30 minutes (dilute primary antibody in M.O.M.™ diluent to the appropriate concentration).

5/ Wash sections in buffer for 2 minutes [x2].

6/ Apply working solution of M.O.M.™ Biotinylated Anti-Mouse IgG Reagent for 10 minutes.

7/ Wash sections in buffer for 2 minutes [x2].

8/ Incubate sections with Fluorescein Avidin DCS for 5 minutes.

9/ Wash sections in buffer for 5 mintes [x2].

[2] Staining for second antigen-

1/ Perform Avidin/Biotin blocking step to prevent the interaction of the second set of labelling reagents with the first set of labelling reagents.

2/ Incubate sections for 5 minutes in working solution of M.O.M.™ diluent.

3/ Incubate section in diluted primary antibody for 30 minutes (dilute primary antibody in M.O.M.™ diluent to the appropriate concentration).

4/ Wash sections in buffer for 2 minutes [x2].

5/ Apply working solution of M.O.M.™ Biotinylated Anti-Mouse IgG Reagent for 10 minutes.

6/ Wash sections in buffer for 2 minutes [x2].

7/ Incubate sections with Texas Red® Avidin DCS (at a concentration of 15-20 µg/ml in buffer) for 5-10 minutes.

8/ Wash sections in buffer for 5 minutes [x2].

9/ Mount with appropriate VECTASHIELD® mounting media.

10/ Store stained slides in the dark at 4ºC if not view immediately.

8.4 Appendix 4

Manuscripts in preparation

Al-Hasani OH, Smith C. Traumatic white matter injury and Toxic leukoencephalopathies. Expert Review in Neurotherapeutics

Al-Hasani OH, Smith C. The evolution of TAI diagnosis in neuropathology practice over a 40 year period. (Submission to Neuropathol Applied Neurobiol)

Al-Hasani OH, Ibrahim NG, Eucker SA, Ralston J, Margulies SS, Smith C. Sub-populations of traumatically injured axons in a gyrencephalic animal model and human. (Submission to J Neurotrauma)