STUDIES ON UPREGULATION OF AMYLOID PRECURSOR PROTEIN IN RESPONSE TO TRAUMATIC BRAIN INJURY

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Lives of great men all remind us
We can make our lives sublime,
And, departing, leave behind us
Footsteps on the sands of time.

Henry Wadsworth Longfellow,
-A Psalm of Life-

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APPENDUM

Page	Paragr.	Line	
	3	4	Replace "There was a 2.3. fold increase in APP mRNA levels in impacted sheep following $MgSO_4$ therapy" with "Post-traumatic administration of $MgSO_4$ 30 minutes after head impact resulted in a 2.3. fold increase in APP mRNA in the thalamus of treated impacted animals compared to untreated impacted animals".
4	Fig. 1.2.2	2-4	Replace "The isoforms produced by 'classic' APPmRNA's containing exon 15 but lacking exons 7 and/or 8 are shown in the upper left" with "The isoforms produced by 'classic' APP mRNAs all contain exon 15 with or without exon 7 and/or 8 are shown in the upper left.
24	2	5	Replace "Cleavage at the 2 sites either side of the βA4 molecule by an enzyme designated β-secretase" with "β-secretase cleaves at both N and C-terminals of βA4".
25	2	4	Replace "The βA4 [1-42] are preferentially deposited within senile plaques of patients with AD, Down's syndrome and familial AD" with "Both βA4 [1-42] and βA4 [1-40] are deposited within senile plaques of patients with AD, Down's syndrome and familial AD".
26	3	5	Add "It has been shown that in some families with late onset AD which shows a linkage to chromosome 21, there is a point mutation in APP causing an amino acid substitution (Val-Ile) close to carboxyl terminus of the βA4 (Goate *et al.*, 1991).
27	1	9	Replace "APP mediates NGF in cell culture" with In vivo studies by Milward *et al.*, (1992) demonstrated that APP is required for NGF to exert its full effects on neurite length and branching".
27	3	1	Replace "and have" with "that have".
35	1	4	Remove "interest".
35	2	1-7	Replace "Head injury in transgenic mice, which have an APP mutation that normally results in AD-like pathology at 6 months of age, produces memory dysfunction before the appearance of AD-like pathology (Hsiao *et al.*, 1996; Nalbantoglu *et al.*, 1997; Smith *et al.*, 1998). Head injury also accelerates the onset of βA4 deposition in transgenic mice with levels at 4 months of age normally seen in uninjured mice at 1 year of age (Kawabata *et al.*, 1991; Games *et al.*, 1995; Johnson-Wood *et al.*, 1997)" with "Head injury in transgenic mice, which have an APP mutation that normally results in AD-like pathology at 6 months of age, produces memory dysfunction before the appearance of AD-like pathology (Smith *et al.*, 1998).
35	2	8	Add "The possible reason that these two studies did not produce plaques is due to the fact that they were using wild type i.e. non-humanised rodents, which do not produce βA4".
39	1	4	Insert "half" before "life".

27	3	3	Insert "been" after "have".
35	1	3	Add Nicoll *et al.*, 1995 as the first reference before Graham *et al.*, 1996.
78	3	8	Add "Overall there was a greater NCB response in the impacted compared to the non-impacted hemisphere".

ABSTRACT

Traumatic brain injury (TBI) affects neuronal cell bodies (NCBs), axons and dendrites in a complex fashion, producing a spectrum of damage dependent on the initial injury and secondary effects. Accumulation of amyloid precursor protein (APP) in NCBs and axons is a feature of TBI. This accumulation may be due to impairment of the axonal transport of APP and/or upregulation of APP mRNA synthesis.

We hypothesized that mechanical deformation, which is not severe enough to cause immediate cell death, results in increased APP mRNA and antigen expression as an acute phase response to injury. We used APP immunocytochemistry, in-situ hybridization (ISH) and northern blotting to study the APP antigen and mRNA response in experimental sheep and lamb head impact models and in a selection of human TBI cases. We also hypothesized that the upregulation of APP which follows head injury may be related to a possible neuroprotective function of APP and therefore, some neuroprotective agents, such as magnesium sulphate ($MgSO_4$) may act by increasing APP mRNA and antigen expression.

It was found that trauma induced a significant increase in APP antigen and APP mRNA as early as 30 minutes after injury which increased over time in injured sheep. Human studies also showed an increase in APP positive NCBs 2.5 hrs to at least 7 days post-impact. There was a 2.3 fold increase in APP mRNA levels in impacted sheep following $MgSO_4$ therapy demonstrating for the first time a link between a putative neuroprotective agent and APP.

DECLARATION OF ORIGINALITY

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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

Corinna Van Den Heuvel

ACKNOWLEDGMENTS

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ABBREVIATIONS

AD	Alzheimer's disease
AI	axonal injury
AP-1	activator protein-1
AP-2	activator protein-2
AP-4	activator protein-4
APLP	amyloid precursor-like-protein
APP	amyloid precursor protein
ASDH	acute subdural haematoma
bp	base pair
BP	blood pressure
Ca^{2+}	calcium
cAMP	cyclic AMP
cDNA	complementary deoxy ribonucleic acid
CBF	cerebral blood flow
C/L	contusions and/or lacerations
CNS	central nervous system
CO_2	carbon dioxide
cm	centimetres
cpm	counts per minute
DAB	3,3' - diaminobenzidine
DAI	diffuse axonal injury
DDW	double distilled water
DNA	deoxy ribonucleic acid
DTT	dithiothreitol

EDH	extradural haematoma
EDTA	ethylene diamine tetra acetic acid
GC	gliding contusions
GCS	Glasgow coma scale
H&E	haematoxylin and eosin
HIS	hypoxic injury score
hr	hour
HR	heart rate
HSE	heat shock element
ICH	intracranial haematoma
ICP	intracranial pressure
il-1	interleukin-1
ISH	in situ hybridization
IVH	intraventricular haemorrhage
kDa	kilodalton
kg	kilogram
KPI	Kunitz type protease inhibitor
L	litre
LOC	loss of consciousness
M	molar
Mg^{2+}	magnesium
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
min	minute
ml	millilitre

mm	millimetre
mM	millimolar
mRNA	messenger ribonucleic acid
N_2	nitrogen
NCB	neuronal cell body
NCBs	neuronal cell bodies
ng	nanogram
NFκB	Nuclear Factor kappa B
NMDA	N-Methyl-D-aspartate
O_2	oxygen
PM	post-mortem
sAPP	secreted APP
SAH	subarachnoid haemorrhage
SDH	subdural haematoma
SDS	sodium dodecyl sulphate
SP-1	signal peptide-1
SSC	standard saline citrate
TAE	tris acetate EDTA
tRNA	transfer ribonucleic acid
TSAH	traumatic subarachnoid haemorrhage
VIS	vascular injury score
w/v	weight per volume
%G+C	percentage guanosine and cytidine
µg	microgram

µl	microlitre
µm	micrometre
°C	degress celcius

PUBLICATIONS AND PRESENTATIONS

Publications

Van Den Heuvel, C., Lewis, S., Wong, M., Manavis, J., Blumbergs, P., Jones, N., Reilly, P. (1998) Diffuse neuronal perikaryon amyloid precursor protein immunoreactivity in a focal head impact model. *Acta. Neurochir. (Suppl.),* **71,** 209-211.

Finnie, J.W., Lewis, S.B., Manavis, J., Blumbergs, P.C., **Van Den Heuvel, C.**, Jones, N.R. (1999) Traumatic axonal injury in lambs: a model for paediatric axonal damage. *J Clin. Neurosci,* **6,** 38-42.

Manuscripts in preparation

Van Den Heuvel, C., Blumbergs, P.C., Finnie, J.W., Manavis, J., Jones, N.R., Reilly,P.L. and Pereira, R.A. (1999) Upregulation of amyloid precursor protein messenger RNA in response to traumatic brain injury: An ovine head impact model. (Submitted Exp. Neurol.)

Van Den Heuvel, C., Blumbergs, P., Finnie, J., Manavis, J., Lewis, S., Jones, N., Reilly, P. and Pereira, R. Upregulation of amyloid precursor protein mRNA in an experimental model of paediatric head injury. (Submitted J. Clin. Neurosci.)

Van Den Heuvel, C., Finnie, J., Blumbergs, P., Manavis, J., Jones, N., Reilly, P. and Pereira, R. Upregulation of neuronal amyloid precursor protein (APP) and APP mRNA following magnesium sulphate ($MgSO_4$) therapy in traumatic brain injury. (Submitted J. Neurotrauma)

Scientific communications

Van Den Heuvel,C., Blumbergs,P.C., Finnie,J., Manavis,J., Reilly,P., Jones,N., Pereira,R. Upregulation of neuronal amyloid precursor protein (APP) and APP mRNA following magnesium sulphate ($MgSO_4$) therapy in traumatic brain injury. *Australian and New Zealand Society for Neuropathology, Annual Scientific Meeting.* Hobart, Australia, May 29, 1999.

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Finnie,J.W., Lewis,S.B., Manavis,J., Blumbergs,P.C., **Van Den Heuvel,C.,** Jones,N.R. An experimental lamb model of paediatric traumatic head injury. *Proceedings of the 49th Annual Meeting of the American college of Veterinary Pathologists,* St Lois MO, USA, November 15-20, 1998. (Abstract in Veterinary Pathology).

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protein in traumatic brain injury. *Australian Society for Medical Research, National Annual Scientific Meeting,* Adelaide, Australia, November 23-26, 1997.

Van Den Heuvel,C., Lewis,S.B., Blumbergs,P.C., Scott, G., Manavis,J., Reilly,P.L., Jones,N.L. Diffuse neuronal perikaryon APP immunoreactivity in a focal head impact sheep model. *X111 International Congress of Neuropathology,* Perth, Australia, September 7-12, 1997. (Abstract in *Brain Pathol.*, 7(4), 1325.)

Lewis,S., Wong,M., Finnie,J., Blumbergs,P., Manavis,J., **Van Den Heuvel,C.,** Reilly,P., Jones,N. Prediction of axonal injury by a physiological scoring system in a head impact model. *10th International Symposium on Intracranial Pressure and Neuromonitoring in Brain Injury,* Williamsburg, Virginia, USA, May 25-29, 1997.

Van Den Heuvel,C., Lewis,S.B., Wong,M., Manavis,J., Finnie,J., Blumbergs,P.C. Widespread Neuronal Perikaryon APP immunoreactivity in a focal head impact sheep model. *10th International Symposium on Intracranial Pressure and Neuromonitoring in Brain Injury,* Williamsburg, Virginia, USA, May 25-29, 1997.

Van Den Heuvel,C., Lewis,S.B., Blumbergs,P.C., Scott,G., Manavis,J., Brown,C., Reilly,P.L., Jones,N.L. Axonal injury in a sheep head impact model. *Australian and New Zealand Society for Neuropathology, Annual Scientific Meeting,* Sydney, Australia, November 10-11, 1996.

CHAPTER 1

Literature review

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1.1- TRAUMATIC BRAIN INJURY (TBI)

In industrialized countries, trauma is the leading cause of death in people 45 years and under and in particular head injuries account for 25-33% of all deaths from trauma (Kraus, 1993) constituting an important medical problem for modern society.

1.1.1- Classification of human TBI

Head injuries vary widely in their aetiology, clinical presentation and pathophysiology (Graham *et al.*, 1995a). The nature and distribution of traumatic lesions are diverse and vary significantly in their complexity, location and severity (Blumbergs, 1997).

There is increasing evidence that mechanical loading and deformation of neural and vascular tissue initiates cascades of biochemical and structural changes which progressively evolve over time (Povlishock, 1992; Faden, 1993).

1.1.1.1- Primary and secondary traumatic brain damage

In **primary traumatic damage** mechanical forces produce tissue deformation at the moment of injury to directly damage the blood vessels, axons, neuronal cell bodies (NCBs) and glia in a focal, multifocal or diffuse pattern of involvement and initiate dynamic and evolving processes which differ for each component part (Blumbergs, 1997). Examples of diffuse primary injury are diffuse axonal injury (DAI) and diffuse vascular injury (DVI) and focal primary injury lesions include brain lacerations, contusions, intracerebral, subdural and extradural haemorrhages and focal axonal injury (AI) (Blumbergs, 1997; Graham and Gennarelli, 1997).

Secondary brain damage results from secondary complications such as ischaemia, hypoxia, cerebral swelling, cerebral oedema, elevated intracranial pressure (ICP), infection, fat embolism and hydrocephalus (Graham *et al.*, 1989a; Graham, 1996; Blumbergs, 1997). Secondary brain injury may also occur in focal (local) or diffuse distributions. Focal lesions include focal hypoxic-ischaemic injury and focal brain swelling. Diffuse effects are associated with widespread dysfunction and include diffuse hypoxic-ischaemic damage and diffuse brain swelling.

This classification based on traditional neuropathologic examination of human post mortem brains is under review as the result of the rapid explosion of information produced by the application of new immunocytochemical and molecular biologic techniques. These new techniques allow examination for the first time of such cell components as dendrites and synapses and provide the means to study particular molecules of interest in-situ. This is well exemplified by the impact that amyloid precursor protein (APP) immunocytochemistry has had in changing some of the traditional concepts relating to traumatic axonal injury (AI).

1.1.2- Axonal injury (AI)

Damage to the axon interferes with the normal axoplasmic transport and the flow of electrical information leading to failure of interneuronal communication. Mechanical forces producing deformation of axons leads to a spectrum of AI varying from primary axonal disruption or transection termed primary axotomy to secondary axotomy where there is progressive evolution of cytoskeletal collapse and eventual axonal disconnection (Maxwell *et al.*, 1991, 1995; Povlishock, 1992; Yaghmai and Povlishock, 1992; Sherriff *et al.*, 1994a, 1994b; Povlishock and Christman, 1995; Povlishock and Jenkins, 1995; Pettus and

Povlishock, 1996; Povlishock and Pettus, 1996; Povlishock *et al.*, 1997). At a microscopic level there is a spectrum of AI ranging from axonal swellings to axonal retraction balls (ARBs) representing axonal disconnection (Adams *et al.*, 1984; Adams *et al.*, 1982; Adams, 1992; Gentleman *et al.*, 1993b; Blumbergs *et al.*, 1989; 1994; 1995; Slazinski and Johnson, 1994; McKenzie *et al.*, 1996; Abou-Hamden *et al.*, 1997).

Recent studies have shown that secondary axotomy is the predominant type of AI seen in head injury cases that survive for any length of time (Pettus *et al.*, 1994; Povlishock and Jenkins, 1995; Pettus and Povlishock, 1996) whereas primary axotomy occurs in settings where the magnitude of forces is such that the victims usually die at the scene of the accident before any reactive changes can develop (Blumbergs, 1997). Secondary axotomy has been extensively studied in experimental models of mild to moderate TBI (Povlishock and Becker, 1985; Pettus *et al.*, 1994; Povlishock and Jenkins, 1995; Pettus and Povlishock, 1996) and these have shown that mechanical deformation produces an alteration of axolemmal permeability (Povlishock *et al.*, 1997) which allows normally excluded Ca^{2+} ions to enter the intra-axonal compartment and activate proteases (Povlishock and Christman, 1995) resulting in neurofilament sidearm loss and microtubule disruption. This sequence of changes lead to eventual disconnection or secondary axotomy after about 2 to 3 hrs (Povlishock and Christman, 1995). This progressive evolution of axonal damage from the time of injury is of great importance as it creates a potential window for therapeutic intervention (Povlishock, 1992; Graham *et al.*, 1997).

1.1.2.1- Markers of AI

Standard silver impregnation methods have been classically used to demonstrate the axonal swellings and ARBs which, however, require survival times in excess of 12 hrs to be reliably demonstrated (Adams, 1992). Immunological techniques which use antibodies to low molecular weight non-phosphorylated neurofilament proteins have also been used successfully to demonstrate AI as early as 6 hrs after injury in humans (Grady *et al.*, 1993) and 1 hr after experimental head injury in rats (Yaghmai and Povlishock, 1992). More recently, monoclonal and polyclonal antibodies to the different isoforms of the amyloid precursor protein (APP) have successfully demonstrated AI much earlier than previous methods in human studies (Gentleman *et al.*, 1993b, 1999; Blumbergs *et al.*, 1994, 1995; McKenzie *et al.*, 1994, 1996; Roberts *et al.*, 1994; Sherriff *et al.*, 1994a, 1994b; Abou-Hamden *et al.*, 1997) and experimental studies (Otsuka *et al.*, 1991; Kawarabayashi *et al.*, 1991; Nakamura *et al.*, 1992; Shigematsu and McGeer, 1992a, 1992b; Stephenson *et al.*, 1992; Kalaria *et al.*, 1993a, 1993b; Hajimohammadreza *et al.*, 1994; Pluta *et al.*, 1994; Lewen *et al.*, 1995, 1996; Lewis *et al.*, 1996; Pierce *et al.*, 1996; Bramlett *et al.*, 1997; Lin *et al.*, 1998; Finnie *et al.*, 1999). The sensitivity and specificity of APP as a marker of AI will be discussed in detail in Section 1.2.6.

1.1.2.2- Diffuse axonal injury (DAI)

DAI is characterized by widespread morphological changes to axons throughout the cerebral hemispheres, corpus callosum, brainstem and cerebellum. With traditional staining methods, it was originally thought that DAI was present in approximately 30% of fatal head injury cases and that it was the most common structural basis of severe disability and the vegetative state after head injury (Graham *et al.*, 1983). However, with the introduction of

sensitive immunocytochemical techniques for APP, it seems that AI is a universal consequence of fatal head injury (Gentleman *et al.*, 1995).

1.1.3- Neuronal cell body injury

During the course of studies using APP as a marker of AI, it was noted that more NCBs showed APP immunoreactivity than axons and as these changes were so widespread and striking throughout the brain we wished to investigate this phenomenon as the subject of this thesis.

The NCB response to TBI has been little studied in human TBI but considerable advances have been made experimentally in understanding some of the neurochemical mechanisms involved in traumatic neuronal injury (for review see Tymianski and Tator, 1996; Mattson, 1996). As is the case with AI, there is a spectrum of neuronal damage and most experimental studies have focused on the neurochemical changes associated with changes in ionic homeostasis and metabolism (McIntosh *et al.*, 1998).

1.1.3.1- The deleterious effects of calcium (Ca^{2+}) and excitatory amino acids (EAAs)

It is now widely believed that TBI produces a deregulation of intracellular calcium (Ca^{2+}) homeostasis by numerous mechanisms, including energy depletion, cell membrane depolarization, excessive synaptic activity and organelle dysfunction (Choi,1988; Shapira *et al.*, 1989; Ikeda and Long, 1990;; Nilsson *et al.,* 1990, 1993; Soares *et al.*, 1992; Fineman *et al.*, 1993; Nakamura *et al.*, 1993; Smith *et al.*, 1993a, 1993b) and that this deregulation is a fundamental pathological mechanism initiated by injury (refer Figure 1.1.1 for proposed mechanisms by which trauma and ischaemia cause intracellular Ca^{2+} increases).

This pathological increase in intracellular Ca^{2+} ions triggers further injury to neurons (Feldman *et al.*, 1996) by stimulation of enzymes such as calpains and other proteases, protein kinases and phopholipases leading to the production of toxic reaction products such as free radicals (Malorni *et al.*, 1993) leading to degradation of enzymes, membrane associated proteins, adhesion molecules and neurotrophin receptors. If the response is prolonged irreversible cellular structural and functional alterations may occur (for review see Tymianski and Tator, 1996).

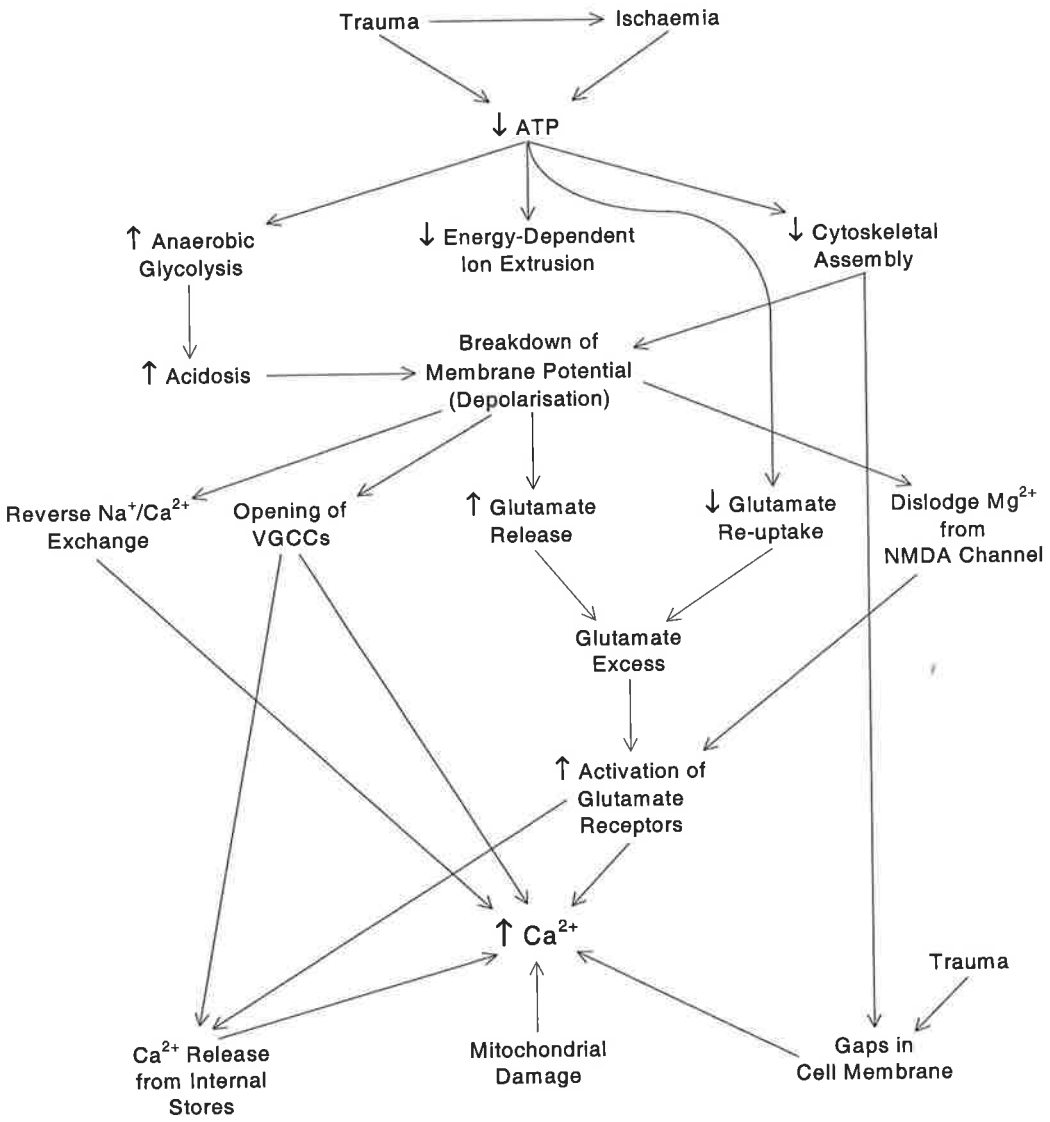


Figure 1.1.1: Mechanisms by which trauma and ischaemia produce intracellular Ca^{2+} elevations. VGCCs = voltage-gated Ca^{2+} channel. (Adapted from Tymianski and Tator, 1996).

There is also evidence that excitatory amino acids (EAAs) such as glutamate and aspartate, which are essential for normal brain functioning, are released into the extracellular space in excessive amounts from terminals of ischaemic or traumatically injured neurons (Faden *et al.*, 1989; Katayama *et al.*, 1990; Pellegrini-Giampietro *et al.*, 1990) resulting in excitotoxic neuronal cell death in regions with a preponderance of glutamate sensitive receptors (Rothman and Olney, 1986). EAAs may also promote free radical production (reviewed by Smith and McIntosh, 1996).

1.1.3.2- The production of immediate early genes (IEGs), heat shock proteins (HSPs) and cytokines

Transient changes in ionic balance, in particular Ca^{2+} and alterations in cell membranes are able to trigger the transcription of IEGs such as c-*fos,* c-*jun* and JunB that regulate the expression of a variety of target genes that include growth factors, cytoskeletal proteins and metabolic enzymes (Morgan and Curran, 1986; Hengerer *et al.,* 1990; Lowenstein *et al.*, 1994). TBI and ischaemia also induce the 72kDa heat-shock protein (hsp72) (Uney *et al.,* 1988; Chopp, 1993) and cytokines such as interleukin-1 (il-1) (Rothwell and Relton, 1993). Although the precise role of these molecules after trauma are not known it is thought that they may initiate or modulate the expression of an array of other genes and proteins, including growth factors regulating cell proliferation and that in the long term they may bestow a protective effect on the damaged brain (Raghupathi *et al.*, 1995).

Alternatively it has been found that short-term expression of these molecules provide a protective effect and persistence in the long term causes problems (Griffin *et al.,* 1989).

1.2- AMYLOID PRECURSOR PROTEIN (APP)

Within the brain, APP is expressed in high abundance and one of its proteolytic products, the 4kDa βA4, is the main protein component of senile plaques in the cerebral cortex of patients afflicted with AD (Glenner and Wong, 1984; Masters *et al.*, 1985). The partial amino acid sequencing of βA4 enabled four independent laboratories to clone DNAs encoding part or all of APP (Goldgaber *et al.*, 1987; Kang *et al.*, 1987; Robakis *et al.*, 1987; Tanzi *et al.*, 1987).

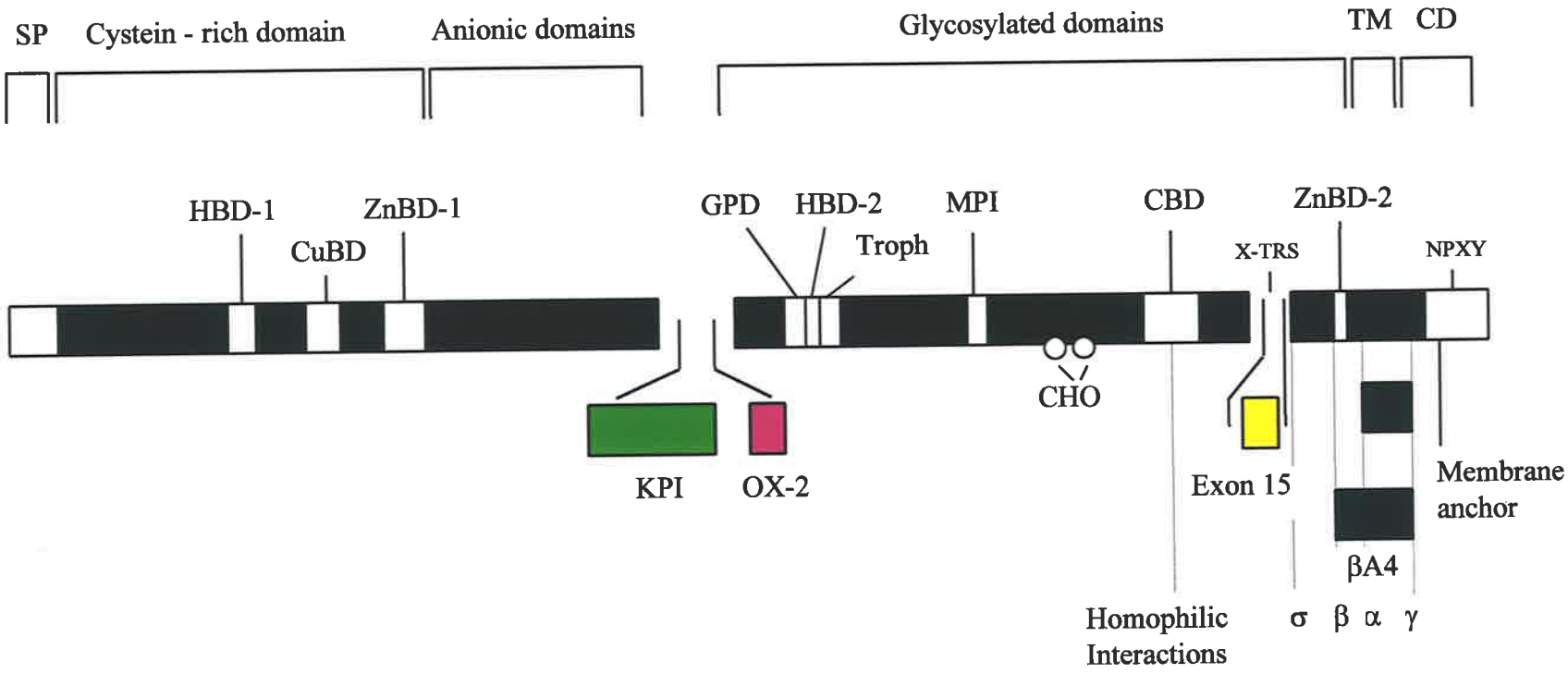
1.2.1- Structural properties of APP

APP is a transmembrane protein with a single membrane-spanning domain (Kang *et al.*, 1987). APP is a ubiquitous highly conserved secretory glycoprotein and its structure consists of a single membrane-spanning segment, an extracellular N-terminal segment (amino terminal) and a short intracellular C-terminal tail (carboxyl terminal) (Kang *et al.*,1987; Hardy and Allsop, 1991) (Figure 1.2.1). The extracellular domain of APP carries binding sites for extracellular matrix glycoproteins including laminin (Kibbey *et al.*, 1993), glycosaminoglycans and collagen (Beher *et al.*, 1996), heparin (Small *et al.*, 1994), zinc and copper (Bush *et al.*, 1993). The APP domain also interacts with G protein (Go) by a binding site in the cytoplasmic domain of APP (Ikezu *et al.*, 1998). There are also putative proteoglycan glycosylation sites within the APP nucleotide sequence suggesting that APP may also be a proteoglycan (PG) (Salinero *et al.*, 1998). APP has domains responsible for growth promotion (GPD) and metalloprotease inhibition (MPI) and it also has glycosylation sites (CHO). There is also an NPXY tetrapeptide sequence at residues 759-762 of the

cytoplasmic domain of APP which is a signal for coated pit mediated endocytosis (Chen *et al.*, 1990). (Figure 1.2.1).

The molecular weight of APP varies from 91 to 135 kDa depending on the isoform and post-translational modifications (Selkoe *et al.,* 1989; Dyrks *et al.,* 1988; Weidemann *et al.,* 1989).

Figure 1.2.1: Schematic representation of APP-695 *(black)* with the spliced regions which are found in other isoforms shown in colour. The molecule is characterized by a large extracellular domain, a transmembrane domain (TM) and a short intracellular C-terminal tail (CD). The extracellular domain of APP molecule is characterized by a 17 residue signal peptide (SP) followed by a cystein-rich domain (cys) and an anionic domain. A KPI and MRC-OX2-like domain (OX-2) are also found in this region and are subject to alternative splicing events (refer Figure 1.2.3). The next region of APP contains a domain responsible for growth promotion (GPD), trophic activity (troph), a metalloprotease inhibitor domain (MPI), glycosylation sites (CHO) and the β-amyloid fragment (βA4), which extends into the membrane. The α-secretase site occurs within the βA4 domain. β and γ sites define the βA4 region. The γ-secretase releases the C-terminal of βA4 which is within the TM. TM is anchored in the phospholipid bilayer and is followed by the CD which contains a Go binding site and the NPTY tetrapeptide sequence. The extracellular portion of APP also contains binding domains for copper (CuBD), zinc (ZnBD-1 & 2), heparin (HBD-1 & 2) and collagen (CBD). The 18 residues of exon 15 are spliced out in L-APP isoforms.


SP
Cystein - rich domain
Anionic domains
Glycosylated domains
TM
CD
HBD-1
CuBD
ZnBD-1
GPD
HBD-2
Troph
MPI
CBD
ZnBD-2
X-TRS
NPXY
CHO
KPI
OX-2
Exon 15
Membrane anchor
βA4
Homophilic Interactions
σ β α γ

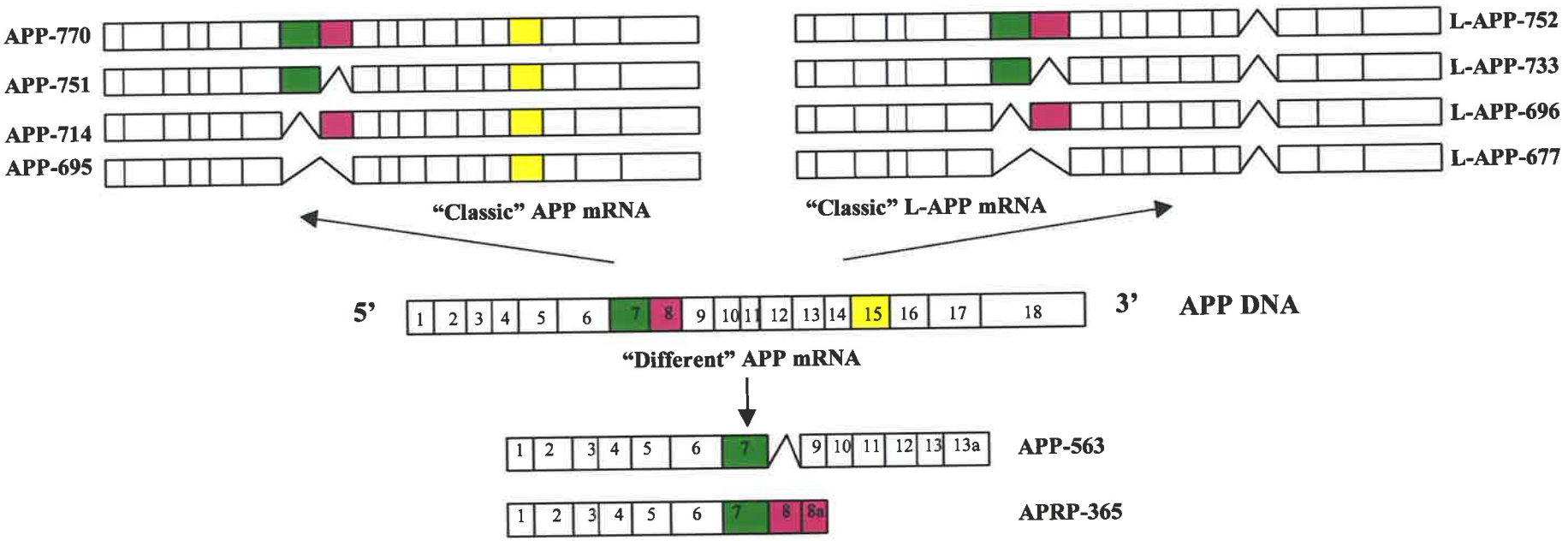
1.2.1.1- APP isoforms and APP-like-proteins

APP constitutes a family of isoforms formed by alternative splicing of exons 7, 8 and 15 (Yoshikai *et al.*, 1990; Sandbrink *et al.*, 1994, 1996). Exon 7 encodes a 57 amino acid domain called the Kunitz type protease inhibitor (KPI) (Kitaguchi *et al.*, 1988) which has extensive homology to protease inhibitors (Ponte *et al.*, 1988). Exon 8 codes for the 19 amino acid Ox-2 domain.

The different isoforms are named according to their amino acid length and are shown in Figure 1.2.2. APP-695 lacks the amino acid sequences coded by exon 7 and 8 and is the predominant isoform found in neurons (Sandbrink *et al.*, 1994; 1996). APP-751 has the KPI domain but lacks the OX-2 domain, APP-770 contains both the KPI domain and the OX-2 domain coded by exons 7 and 8 (Tanzi *et al.*, 1987). Within the central nervous system, the isoforms containing the KPI domain are found mostly in glial cells (Siman *et al.*, 1989; deSilva *et al.*, 1997; Garcia-Ladona *et al.*, 1997). Another isoform of APP is APP-714 which lacks the KPI domain but has the OX-2 domain (for review see Sandbrink *et al.*, 1994).

Exon 15 comprises 54 base pairs and encodes for 18 amino acids, which precede the βA4 region by 16 amino acids, and is an alternatively used splice site in the APP gene (Konig *et al.*, 1992). The APP transcripts excluding the amino acid sequences coded by exon 15 were first observed in peripheral leukocytes and microglial cells and therefore called leukocyte-derived APP (L-APP) mRNAs (Konig *et al.*, 1992). These APP transcripts are designated L-APP-752, L-APP-733, L-APP-696 and L-APP-677 depending on the alternative splicing of exons 7 and 8. These isoforms have been found in glial cells and astrocytes, but have not been observed within neurons (Monning *et al.*, 1992) (Figure 1.2.2).

Figure 1.2.2: APP isoforms. Alternative splicing variants of APP cDNA (centre). The APP gene contains 18 exons and is spliced into 10 different isoforms. The isoforms produced by "classic" APP mRNA's containing exon 15 but lacking exons 7 and/or 8 are shown in the upper left and the isoforms resulting from "classic" L-APP's lacking exons 15 and/or exons 7 and 8 are shown in the upper right. The non-amyloidogenic APP isoforms are represented in the bottom of the figure (adapted from Roβner *et al.*, 1998).


APP-770
APP-751
APP-714
APP-695
"Classic" APP mRNA
L-APP-752
L-APP-733
L-APP-696
L-APP-677
"Classic" L-APP mRNA
5'
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
3'
APP DNA
"Different" APP mRNA
1 2 3 4 5 6 7 9 10 11 12 13 13a
APP-563
1 2 3 4 5 6 7 8 8a
APRP-365

It has been suggested that APP is part of a multigene family (Sprecher *et al.,* 1993) as other APP–like-transcripts have been discovered. A family of APP-like proteins (APLP1 and APLP2) were first identified in the mouse by Wasco *et al* (1992). APLP1 is encoded by a gene on human chromosome 19, mapped to 19q13.2 and shows a high degree of sequence conservation to APP and L-APP in the extramembranous and cytoplasmic regions but divergence in the βA4 and transmembrane domains (Wasco *et al.*, 1992). The APLP2 family, the best characterized of the APLPs, are highly homologous to the APP and L-APP isoforms particularly in the amino- and carboxy-terminus regions but due to a 125 amino acid divergent region are lacking the βA4 domain (Slunt *et al.*, 1994). Four isoforms of APLP2 exist due to alternative splicing of a KPI-containing exon (analogous to exon 7 of the APP gene) and an exon coding for 12 amino acids located on the amino-terminal side of the transmembrane domain (Sandbrink *et al.*, 1996). The resultant isoforms are APLP2-763, APLP2-707, L-APLP2-751 and L-APLP2-695.

An Ox-2 related domain is not found in any of the APLP2 isoforms. They have a region very homologous to the APP KPI domain which begins at amino acid 290 and spans approximately 55 amino acids (Wasco *et al.*, 1992). Human APLP2 isoforms exhibit a very similar cellular and subcellular distribution as APP isoforms (Crain *et al.*, 1996; Sisodia *et al.*, 1996) and appear to mature through the same secretory-cleavage pathway as APP (Slunt *et al.,* 1994).

Two rare poorly characterized non-amyloidogenic C-terminal truncated APP transcripts have also been identified. These are denoted amyloid precursor-related protein (APRP) 563 and APRP365. They both incorporate the rare 13A exon and do not contain the βA4 domain (Yoshikai *et al.*, 1990). Another APP homologue was found after sequencing the human

placental library (Sprecher *et al.*, 1993). This APP homologue 763 encodes a signal peptide in brain, heart, lung, liver, kidney and placenta. The significance of these APP related proteins is not known.

1.2.2- Distribution of APP

Northern blot hybridization and in-situ hybridization (ISH) have demonstrated that APP is expressed in virtually all neural and non-neural mammalian tissues (Kang *et al.*, 1987; Card *et al.*, 1988; Mita *et al.*, 1989; Selkoe *et al.*, 1989; Ikeda and Tomonga, 1990; Oltersdorf *et al.*, 1990; Zhao *et al.*, 1995; de Silva *et al.*, 1997). The highest levels of expression of APP appear in the kidney and brain (Selkoe, 1996) and neurons are the principle producers of APP in the brain (Bendotti *et al.*, 1988). However, different isoforms of APP are found in different cells, for example, cells in non-neural tissues express APP isoforms containing the KPI domain and have much lower levels of the 695 amino acid isoform (for review see Mattson, 1997; Roβner *et al.*, 1998).

1.2.3- APP gene and transcription of APP mRNA

The APP gene consists of 18 exons on the long arm of chromosome 21 at the locus 21q21.3-21q22.1 (Goldgaber *et al.*, 1987; Yoshikai *et al.*, 1990). The APP gene has been found in many different animal species including dogs (Okuda *et al.*, 1994), guinea pigs (Beck *et al.*, 1997), rats, mice (Yamada *et al.*, 1987) and monkeys (Martin *et al.*, 1993). Sequencing of these APP genes in other species has shown that they are highly homologous to the human APP gene (for review see Hilbich *et al.*, 1993).

1.2.3.1- The APP promoter

The APP promoter lacks a typical TATA box and CCAAT box (LaFauci *et al.*, 1989) and therefore transcription is initiated at multiple sites (Salbaum *et al.*, 1988). The promoter of the APP gene has some characteristics of "housekeeping" genes (Yoshikai *et al.*, 1990) and there is conservation of sequence homology in non-human primates including gorilla, orang-utan and monkey (Adroer *et al.*, 1997). The 5' end of the APP gene contains a promoter sequence which contains numerous binding sites for regulatory transcription factors such as activator protein-1, 2 and 4 (AP-1, 2 and 4) (Lee *et al.*, 1987; Mermod *et al.*, 1988), signal peptide–1 (SP-1), Nuclear factor kappa B (NF-κB) (Lenardo and Baltimore, 1989; Grilli *et al.*, 1996) and Hox1.3 (Odenwald *et al.*, 1989). The APP promoter also has many other sequences which are important for regulation of APP transcription. These include antioxidant responsive elements (ARE-like) (Yang *et al.*, 1998), a heat shock element (HSE) (Wu *et al.*, 1987) and GC rich boxes (Pollwein *et al.*, 1992) (see review Quitscke and Goldgaber, 1992) (Figure 1.2.3).

1.2.3.2- Proposed regulators of APP transcription

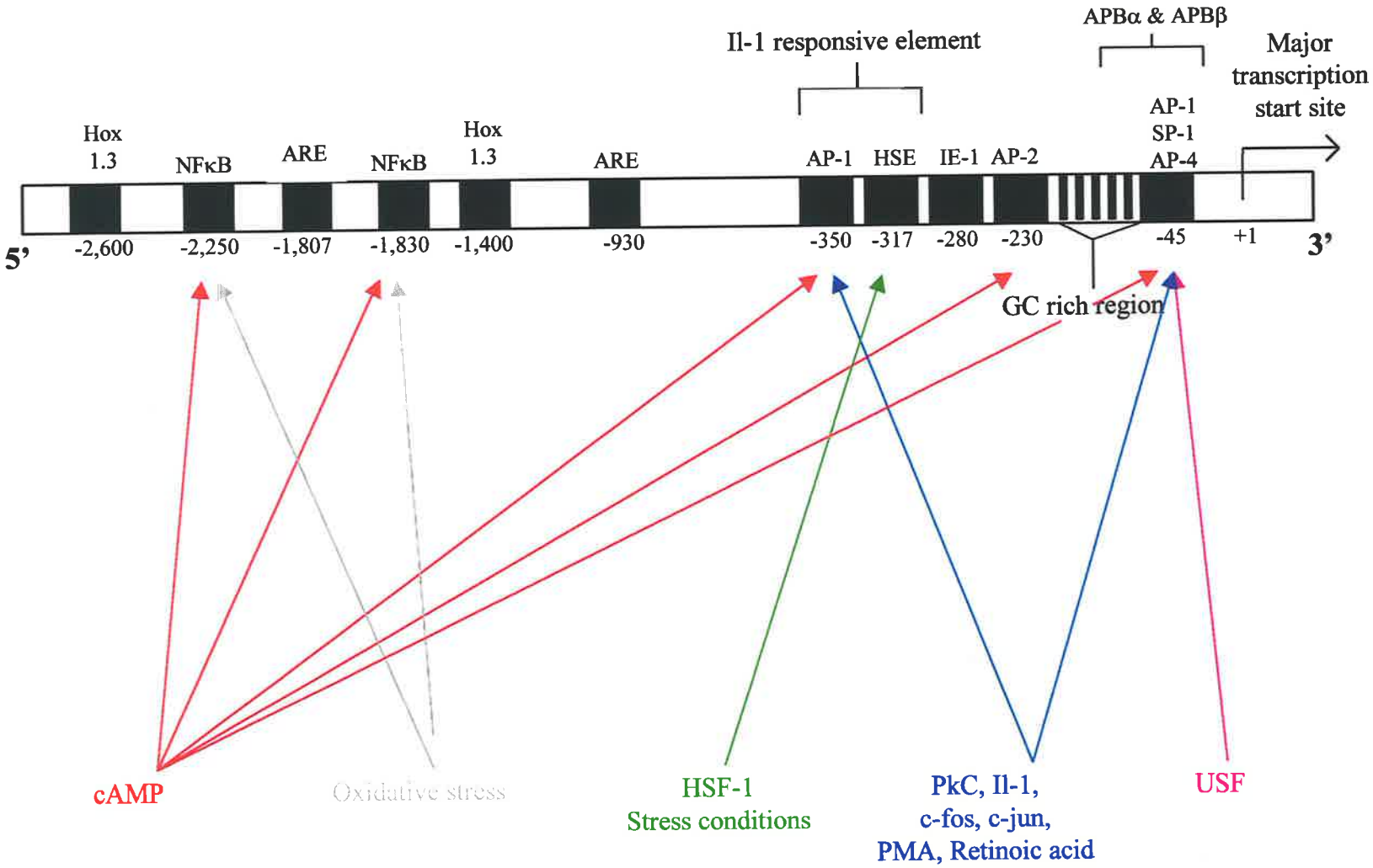
As described above, there is a complex set of genes and proteins which interact with the binding sites on the APP promoter to initiate transcription of the APP gene. Many of these transcriptional regulators are affected and upregulated after cellular stress and injury. Most transcription factors have been studied in cell culture or in vivo using the rat. AP-1 binding sites on the APP promoter at –350 and –344 are responsive elements for the PKC signalling pathway (Lenardo and Baltimore, 1989). The PKC pathway is a complex pathway activated by a variety of agents such as phorbol ester (PMA) and interleukin-1 (il-1) (Trejo *et al.*, 1994; Yang *et al.*, 1998). Il-1 is capable of increasing APP mRNA expression in cell culture through the AP-1 responsive element at position –350 (Goldgaber *et al.*, 1989; Donnelly *et*

al., 1990; Forloni *et al.*, 1992; Griffin *et al.*, 1998; Panegyres and Hughes, 1998; Yang *et al.*, 1998). Cyclic AMP (cAMP) can also cause upregulation of APP mRNA in neuroblastoma cells (Bourbonniere *et al.*, 1997) and cortical astrocyte cultures (Lee *et al.*, 1997) possibly by inducing NFkB (Serkkola and Hurme, 1993) or AP-2 binding activity (Shekarbi *et al.*, 1997) by increasing c-*fos* transcription or PKC activation of c-*jun* (Imagawa *et al.*, 1987). The c-*fos*/c-*jun* heterodimer has consensus binding sites in the APP promoter (Salbaum *et al.*, 1988) and therefore activation of c-*fos* and c-*jun* may act as switches to regulate APP gene expression (Bohmann *et al.*, 1987).

APP can be classified as a heat shock protein (HSP) as it is transcriptionally activated following stress (Ciallella *et al.*, 1994) possibly by binding of inducible nuclear factors to the HSE at position –317 on the APP promoter (Dewji *et al.*, 1995). Cell culture studies have shown that Heat Shock Factor – 1 (HSF-1) produced following cellular stress binds to the heat shock element on APP (Dewji and Do, 1996).

SP-1 has been implicated in mediating transcriptional activity from TATA less promoters such as APP and in determining the selection of transcriptional start sites. SP-1 is associated with retinoic acid (RA) which induces neuronal differentiation by increasing APP expression in neuro-blastoma cells (Konig *et al.*, 1990). Other binding sites include APBα and APBβ which account for 70 to 90% transcriptional activity in PC-12 cells and HeLa cells (Quitscke, 1994; Quitscke *et al.*, 1996). The human upstream stimulatory factor protein (USF) binds to the APBα and APBβ binding sites and is essential in regulating APP gene expression (Hoffman and Chernak, 1995; Vostrov *et al.*, 1995). NFkB is an oxidative stress-responsive transcription factor which can be activated by hydrogen peroxide in T lymphocytes (Yang *et al.*, 1998) (see Figure 1.2.3).

Figure 1.2.3: Structure of the proximal APP promoter. The general structure of the APP promoter is schematically illustrated. The approximate position of consensus sequences for known transcription factors are indicated (black boxes). These include Hox 1.3 (Odenwald *et al.*, 1989), NFκB (Lenardo and Baltimore, 1989), AP-1 (Lee *et al.*, 1987), HSE (Wu *et al.*, 1987), AP-2 and AP-4 (Mermod *et al.*, 1988) and ARE (Yang *et al.*, 1998). The major transcription start site is located at position +1. Also shown are some of the proposed transcription factors which have been shown to directly or indirectly affect APP synthesis and metabolism.


APBα & APBβ
Il-1 responsive element
Major transcription start site
Hox 1.3
NFκB
ARE
NFκB
Hox 1.3
ARE
AP-1
HSE
IE-1
AP-2
AP-1 SP-1 AP-4
5'
-2,600
-2,250
-1,807
-1,830
-1,400
-930
-350
-317
-280
-230
-45
+1
3'
GC rich region
cAMP
Oxidative stress
HSF-1
Stress conditions
PkC, Il-1,
c-fos, c-jun,
PMA, Retinoic acid
USF

1.2.4- Translation of APP and proteolytic processing

1.2.4.1- Passage of APP through the cell

After synthesis translation of APP occurs in the endoplasmic reticulum via the signal peptide on APP and maturation of APP occurs in the Golgi complex where it undergoes tyrosine sulphation, O-N-linked glycosylation and the addition of sialic acid (Caporaso *et al.*, 1994). It is not exactly clear how APP is post-translationally modified, metabolized and processed. However it is known that APP is cleaved by at least 3 different proteolytic activities. In vitro studies have shown that processing of APP by either of the enzymes designated α, β or γ secretases occurs only in mature APP after O-glycosylation has occurred in the Golgi complex (Tomita *et al.*, 1998). The exact pathways APP takes within the cell is still under investigation but the majority of literature suggests that APP travels through the central vacuolar system en-route to the plasma membrane for secretion of its amino-terminal domain and/or lysosomal degradation within the endosomal compartments (Weidemann *et al.*, 1989; Haass *et al.*, 1992).

1.2.4.2- Non-amyloidogenic processing

Following maturation, APP may be cleaved by α-secretase on the way to (in secretory vesicles, or trans Golgi) or at the cell membrane (Koo and Squazzo, 1994). α-secretase cleaves APP within the βA4 domain (Mills and Reiner, 1996) to release a secreted form of APP (sAPP) which is approximately 100 kDa in weight (Boseman-Roberts *et al.*, 1994). This precludes the release of intact βA4 and is therefore non-amyloidogenic (Haass *et al.*, 1992; Sisodia, 1992; Busciglio *et al.*, 1993; Evin *et al.*, 1994). sAPP is then transported anterogradely by fast axoplasmic transport (Koo *et al.*, 1990). APP may then be inserted into the axolemma (Sapirstein *et al.*, 1994) and partially colocalizes with clathrin (Norstedt

et al., 1993; Allinquant *et al.,* 1994). APP may also be transported distally to pre-synaptic nerve terminals (Lyckman *et al.*, 1998) where it colocalizes with synaptophysin in vesicles (Schubert *et al* 1991). It has also been proposed that internalized APP from the axolemmal surface may undergo retrograde transport (Simons *et al.*, 1995; Yamazaki *et al.*, 1995, 1996; Marquez-Sterling *et al.*, 1997). However, the fate of APP that has been delivered retrogradely to the somatodendritic domain is poorly understood.

Following α-cleavage a 10 kDa C-terminal fragment of APP is left within the membrane (reviewed by Suh *et al.,* 1996). Whether this fragment, which comprises the transmembrane and intracellular domains, is potentially amyloidogenic or degraded by the cell is not known. It is possible that this fragment may undergo endocytosis via clathrin-coated vesicles and targeted to endosomes and lysosomes (Yamazaki *et al.*, 1996). However, this internalized APP may also be rapidly recycled to the cell surface for later secretion or re-internalization (Yamazaki *et al.*, 1996; Koo *et al.*, 1996).

The proportion of APP which is proteolytically processed by this pathway varies and can be as high as one third of the total APP (Hung *et al.,* 1992). However, it is now believed that this secretory pathway is only a minor route for APP and that the bulk of the APP is degraded by the cell and may potentially produce amyloidogenic derivatives (Weidemann *et al.*, 1989; Caporaso *et al.*, 1992a).

(a) Proposed factors which affect non-amyloidogenic processing

In vivo and cell culture studies have shown that a variety of extracellular and intracellular signals can modulate APP processing to favour the secretion of sAPP (for review see Mills and Reiner, 1999). For example, PKC is able to increase sAPP and reduce βA4 levels

(Gandy *et al.*, 1988; Caporaso *et al.*, 1992b; Gillespie *et al.*, 1992; Slack *et al.*, 1993; Caputi *et al.*, 1997; Ulus and Wurtman, 1997). Other signals including glutamate (Lee *et al.*, 1995; Kirazov *et al.*, 1997) and Ca^{2+} are also able to increase sAPP release possibly by increasing net tyrosine phosphorylation (Buxbaum *et al.*, 1994; Slack *et al.*, 1995; Wolf *et al.*, 1995; Petrniak *et al.*, 1996). Muscarinic acetylcholine receptors play a significant role in the regulation of APP processing. Nitsch *et al.*, (1992) first showed that agonist activation of muscarinic receptors by carbachol increased sAPP release from transfected cells expressing M1 and M3 receptor subtypes and further observations strongly suggested that only selective activation of the M1 subtype effectively increased α-secretase cleavage of APP in brain tissue.

1.2.4.3- Amyloidogenic processing

It is unclear whether βA4 is generated by secretory, endosomal or lysosomal pathways or a combination of these. However, in vitro studies have established some of the characteristics of the processing pathway. In the amyloidogenic pathway, often referred to as the endocytic pathway (Haass *et al.*, 1992; Golde *et al.*, 1992; Shoji *et al.*, 1992; Koo and Squazzo, 1994), cleavage at the 2 sites either side of the βA4 molecule by an enzyme designated β-secretase results in an array of C-terminal fragments including those that contain the entire βA4 region (Estus *et al.*, 1991; Golde *et al.*, 1992; Evin *et al.*, 1994; Lai *et al.*, 1995). Further cleavage of these fragments between amino acids 639 and 640 may then occur by γ-secretase to generate the C-terminal end of βA4 (McPhie *et al.*, 1997). This cleavage may occur within the endosomes or in transport vesicles close to the membrane (Knops *et al.*, 1995; Iizuka *et al.*, 1996).

It has been assumed that abnormal proteolytic processing of the APP is required for the deposition of βA4 (Cole *et al.,* 1989; Golde *et al.,* 1992; Sisodia *et al.*, 1990). Reports indicate that internalization into the endocytic compartments of the cell is necessary for βA4 release by either β or γ secretase (Koo and Squazzo, 1994; Fuller *et al.*, 1995; Knops *et al.*, 1995). This internalization may occur from the cell membrane or from the trans Golgi network (Haass *et al.*, 1992). Evidence for this has been obtained by studies where endocytosis is inhibited causing a marked decrease in βA4 production and deposition (Koo and Squazzo, 1994). Agents which inhibit the trafficking of APP through the Golgi complex (e.g., Brefeldin A) or alter the transvesicular pH (e.g., ammonium chloride, chloroquine) block βA4 formation (Caporaso *et al.*, 1992a).

γ secretase cleavage occurs at either valine 711 or isoleucine 713 releasing the βA4 [1-40] or βA4 [1-42] (Hartmann *et al.*, 1997). The βA4 [1-42] are preferentially deposited within senile plaques of patients with sporadic AD, Down's syndrome and familial AD (Selkoe, 1996). βA4 [1-40] and βA4 [1-42] have non-overlapping distributions in COS-7 cells and Kidney 293 cells and therefore the γ secretase may vary in different compartments. It was found that γ secretase may generate βA4 [1-40] in the trans Golgi network or vesicles in its vicinity and βA4 [1-42] generation is favoured in the endoplasmic reticulum (ER) (Hartmann *et al.,* 1997; Wild-Bode *et al.*, 1997).

(a) Proposed factors which affect amyloidogenic processing

APP plays a crucial role in the pathophysiology of AD because it is the immediate precursor of the βA4. There are many factors which have been proposed to lead to βA4 deposition within the brain. These include overexpression of APP as seen in Down's syndrome where an extra chromosome 21 may result in increased transcription of APP (Rumble *et al.*, 1989)

or following head injury (Roberts *et al.*, 1991, 1994; Gentleman *et al.*, 1993a; Sherriff *et al.*, 1994b; Graham *et al.*, 1995b, 1996) where overexpression of APP may result in βA4 deposition.

It has also been proposed that impaired transport of APP inside the ER (Gabuzda *et al.*, 1994) or prolonged residence inside the ER might enhance βA4 [1-42] production (Hartmann *et al.*, 1997).

βA4 deposition may also result from altered processing of APP by mutations in APP (Cai *et al.*, 1993; Lai *et al.*, 1995; Haass *et al.*, 1995; Zhao *et al.*, 1995) which constitute a small percentage of inherited forms of AD. These mutations occur in regions of the APP gene that code for the βA4 sequence or encode amino acids which lie directly adjacent to the β or γ secretase cleavage sites (Haass *et al.*, 1995).

1.2.5- Functional characteristics of APP

The functional properties of APP are still not fully understood and are still being investigated. Some putative functions of APP are listed below:

1.2.5.1- Neurite outgrowth and growth promotion

APP may play a part in neurite extension (Breen *et al.*, 1991; Milward *et al.*, 1992; Roch *et al.*, 1993; Small *et al.*, 1994; Allinquant *et al.*, 1995; Wallace *et al.*, 1997) and preservation of neuronal connections (Saitoh *et al.*, 1989). All APP molecules have high affinity for heparin sulphate proteoglycans, laminin and other extracellular matrix molecules which are important in advancing neurite outgrowth (Klier *et al.*, 1990). APP contains a sequence that

has growth-promoting activity (Ninomiya *et al.*, 1993) and shares structural features with a precursor for epidermal growth factor (EGF) (Allsop, *et al.*, 1988). APP acts as an autocrine growth factor by mediating the growth of thyroid epithelial cells (Pietrzik *et al.*, 1998) and stimulating the growth of fibroblasts (Saitoh *et al.*, 1989; Ninomiya *et al.*, 1993) in cell culture. An increase in APP in the rodent brain during maturation suggests that APP may be important in establishing neuronal connections and in cell differentiation (Loffler and Huber, 1992). APP is a potentiating agent which renders compromised neurons more responsive to low levels of neurotrophins e.g. nerve growth factor (NGF) (Wallace *et al.*, 1997) and is capable of mediating NGF in cell culture (Milward *et al.*, 1992).

1.2.5.2- Neuroprotection

As discussed in section 1.2.2, APP contains a HSE in its promoter sequence and as APP mRNA and protein increase with heat shock it can be classified as a Heat Shock Protein (HSP) (Dewji *et al.*, 1995) and therefore may play a role in protecting cells from the effects of heat and other stresses. APP-695 and APP-751 protect hippocampal neurons against excitotoxic, metabolic and oxidative insults, including glucose deprivation-induced injury and glutamate toxicity both in cell culture (Goodman and Mattson, 1994; Mattson *et al.*, 1993) and in vivo (Smith-Swintosky *et al.*, 1994).

1.2.5.3- Immune response

APP participates in the interaction of cells of the mononuclear phagocytic system, and have an important function in the inflammatory response in relation to tissue maintenance and repair (Beer *et al.*, 1995). CD4+ lymphocytes and a majority of CD8+ lymphocytes have shown to secrete APP upon activation (Monning *et al.*, 1992) suggesting that APP may participate in T-cell-dependent immune responses.

1.2.5.4- Synaptic function and plasticity

Modulation of neuronal excitability may be a major mechanism by which APP regulates developmental and synaptic plasticity in the nervous system (Furukawa *et al.,* 1996). APP plays a role in synaptic function (Schubert *et al.,* 1991) and synapse formation (Moya *et al.*, 1994; Roch *et al.*, 1994; Morimoto *et al.*, 1998). The highest levels of APP were seen in the second postnatal week in rats suggesting an important role of APP in cell differentiation and synaptic contacts (Loffler and Huber, 1992). sAPP modulates NMDA receptor activity in cultured embryonic rat hippocampal neurons suggesting important roles for sAPP in regulating developmental and synaptic plasticity (Furukawa and Mattson, 1998). Reactive gliosis in the brain and a decrease in locomotor activity is found in transgenic mice which lack APP, suggesting that APP may play a role in neural plasticity (Zheng *et al.,* 1995).

1.2.5.5- Reception and Signalling

The structure of APP has characteristics of cell surface receptors (Kang *et al.,* 1987; Nishimoto *et al.,* 1993; Okamato *et al.,* 1995). APP has been described as a novel signalling system that is capable of interacting with both glutamate and neurotrophic factor systems playing an important role in neuronal plasticity and survival (Tominaga *et al.*, 1997). sAPP is localized primarily in the axon and cell body in caveolae-like vesicles raising the possibility that APP serves as a transmembrane signalling molecule for glycosyl phosphatidylinositol (GPI)-linked glycoproteins (Bouillot *et al.,* 1996).

1.2.5.6- Adhesion

Results from adhesion experiments with the neuroblastoma cell line Neuro-2A support early suggestions that APP might participate in cell-cell (Beher *et al.*, 1996), cell-substrate (Storey *et al.*, 1996) and cell-matrix adhesion (Shivers *et al.*, 1988; Schubert *et al.*, 1989; Breen *et*

al., 1991; Beyreuther *et al.*, 1996). The different isoforms of APP have been found to have different adhesive properties (Gillian *et al.*, 1997).

1.2.5.7- Other functions

APP plays a part in coagulation and wound repair where it inhibits coagulation factors Xa, Xla and plasmin (Kido *et al.*, 1990). Interaction with microglia and components of the extracellular matrix affects APP secretion as well as intracellular APP biogenesis and catabolism suggesting that APP plays a role in the regulation of microglial mobility (Banati *et al.*, 1994). (For review of functions see Panegyres, 1997).

1.2.6- APP as a marker of AI

APP is an excellent marker for AI in human TBI studies (Gentleman *et al.*, 1993b, 1999; Blumbergs *et al.*, 1994, 1995; McKenzie *et al.*, 1994, 1996; Roberts *et al.*, 1994; Sherriff *et al.*, 1994a, 1994b; Abou-Hamden *et al.*, 1997) and experimental studies (Koo *et al.*, 1990; Otsuka *et al.*, 1991; Kawarabayashi *et al.*, 1991; Nakamura *et al.*, 1992; Shigematsu and McGeer, 1992a, 1992b; Stephenson *et al.*, 1992; Kalaria *et al.*, 1993a, 1993b; Hajimohammadreza *et al.*, 1994; Pluta *et al.*, 1994; Lewen *et al.*, 1995, 1996; Lewis *et al.*, 1996; Pierce *et al.*, 1996; Bramlett *et al.*, 1997; Lin *et al.*, 1998; Finnie *et al.*, 1999). APP immunostaining is able to detect axonal swellings as early as 1.75 hours after injury in human studies (Blumbergs *et al.*, 1995) and experimentally after 0.5 hrs following needle stab injury in rats (Otsuka *et al.*, 1991) and 1 hr in a sheep head impact model (Lewis *et al.*, 1996) and a fluid percussion model in the rat (Pierce *et al.*, 1996). Axonal APP immunoreactivity has also been demonstrated in patients surviving as long as 99 days after mild head injury (Blumbergs *et al.*, 1995).

Once an axon has undergone an injury (i.e. traumatic, ischaemic or chemical) resulting in disruption and impairment of fast axoplasmic transport, APP may be visualized as "immunoreactive lengths", axonal swellings or ARBs and detected much earlier than with conventional staining methods (Lewis *et al.*, 1996). APP only stains injured axons aiding in their detection and quantitation as background uninjured axons remain unstained. A semi-automated system for quantitation of AI using APP immunostaining has recently been reported (Gentleman *et al.*, 1999). APP immunostaining is not affected by formalin fixation greatly increasing its utility as a method of demonstrating AI in routinely fixed material (Sherriff *et al.*, 1994a). Although APP is the most sensitive current marker for the demonstration of AI, it is not specific for TBI and primary AI related to mechanical deformation cannot be distinguished from secondary AI due to hypoxic-ischaemic damage using APP antibodies.

APP immunoreactive axons have been shown to occur in the following non-traumatic lesions:

1) Haemorrhages and infarcts (Ohgami *et al.*, 1992; Blumbergs *et al.*, 1994, 1995; Suenaga *et al.*, 1994).
2) Hypoxia/ischaemia (Abe *et al.*, 1991b; Stephenson *et al.*, 1992; Kalaria *et al.*, 1993a, 1993b; Pluta *et al.*, 1994; Tomimoto *et al.*, 1995; Yam *et al.*, 1997, 1998).
3) Kuru and senile plaques of AD (Arai *et al.*, 1990).
4) Cerebral abscesses (Ohgami *et al.*, 1992; Gentleman *et al.*, 1995).
5) Hallervorden-Spatz disease (Ohgami *et al.*, 1992).
6) Binswanger Disease (Suenega *et al.*, 1994).

1.2.7- APP as a marker of neuronal cell body injury

Whereas axonal APP immunocytochemical responses have been extensively studied in human and experimental head injury, this is not the same for the NCB APP response. The human and experimental studies that have been reported have used various antibodies to different regions of APP in different settings and have produced conflicting results.

1.2.7.1- Human studies

Summary of human studies which have demonstrated APP immunoreactive NCBs in TBI.

Authors	Methods and APP antibody used	Results	Conclusions
Gentleman *et al.*, 1993b	n=11 head injured cases (survival time range 12 hrs 10 days) Mab 22C11	APP immunoreactivity was found in NCBs in cortical grey matter. Controls showed faint positivity.	APP may be a response to neuronal injury
McKenzie *et al..*, 1994	n=13 head injured cases (survival time range 3-288 hrs) Mab 22C11	Significant increase in the number of APP immuno-reactive layer II pre-α cells in the entorhinal cortex in fatal head injured patients.	Upregulation may represent a "protective" response to neuronal stress or injury.
Roberts *et al.*, 1994	n=152 head injured cases (survival time range 4 hrs-2.5 years). Mab 22C11	Generalized APP immuno-reactivity was found especially in the NCBs of cortical layers III and IV.	Increased APP expression may lead to βA4 deposition and AD.

1.2.7.2- Experimental studies

The tables below summarize the relevant experimental studies which have assessed the NCB APP response following TBI and various other insults.

Head-impact and fluid percussion models

Authors	Methods and antibody used	Results	Conclusions
Lewen *et al.*, 1995	Weight drop using male Sprague-Dawley rats. Mab 22C11	Homogeneous APP immunoreactivity in NCB in control rats. In injured rats 1 to 3 days after injury there was a loss of APP immunoreactivity at the periphery of the lesion.	The reduction of APP immediately following trauma was due to transportation of APP away from the cell body down the axon.
Lewen *et al.*, 1996	Weight drop using male Sprague Dawley rats. Mab 22C11	Similar to above, however some NCBs at the periphery of the lesion showed intense APP immuno-reactivity.	APP immunoreactivity within NCBs could be part of the normal response to neuronal stress as part of an acute phase response to brain injury.
Pierce *et al.*, 1996	Lateral fluid precussion using male Sprague Dawley rats. LN39- N terminal 100 amino acids of APP. 369W- C terminal domain.	Some thalamic and cortical NCB were positive for APP/APLP 2 to 7 days after injury.	The APP immunoreactivity detectable in NCBs and axons after injury was probably caused by an accumulation of APP due to disruption of axonal transport. However upregulation of APP could not be excluded.
Bramlett *et al.*, 1997	Parasagittal fluid-percussion using male Sprague-Dawley rats Mab 22C11	Cortical and thalamic NCB were APP positive at 1, 3 and 7 days after TBI.	APP immunoreactivity in the NCB may be due to continued synthesis of APP and inhibition of the normal flow or increased synthesis of APP.

Head injury models utilizing intraneural injections of neurotoxins

Authors	Methods and antibodies used	Results	Conclusions
Kawarabayashi *et al.*, 1991	Unilateral injection of kainic acid in male Sprague Dawley rat brains. Antibodies to APP:- W36Na- amino acids 18-38 Z31preA- amino acids 577-596 W61CA- amino acids 666-695 U99A- KPI domain 13-28	APP positive NCBs were evident at 3 hrs around lesion site and peaked at 12 hrs and began decreasing in intensity and number by 3 days.	Rapid increase in neuronal expression of APP as APP is a reactive protein involved in brain damage and repair.
Nakamura *et al.*, 1992	Lateral hippocampal injection of ibotenic acid in Wistar rats. Mab 22C11	APP immunoreactivity was found in degenerated hippocampal NCBs 100 days after injection.	Impaired axonal transport of APP might lead to the APP deposition.
Shigematsu and McGeer, 1992a	Injection of aluminium salts in male wistar rats. Polyclonal antibodies to APP:- R36- amino acids 528-540 of amino terminal end and of APP. R37- amino acids 681-695 of carboxyl terminal end.	Increased APP immunoreactivity in cortical pyramidal NCBs 1 to 2 days post-injection lasting up to 7 months.	Interruption of APP transport can lead to accumulation in NCBs.
Shigematsu and McGeer 1992b	Injection of colchicine in male Wistar rats. Antibodies used as above.	APP immunoreactive NCBs were evident around the lesion site 3 hrs after injection and increased in intensity by at 6 hrs, becoming extensive after 24 hrs and remaining positive up to 1 month.	Conclusions as above.
Hajimohammadreza *et al.*, 1994	Ventricular infusion of leupeptin in male Wistar rats. Mab 22C11 R36 and R37 as described above	Ipsilateral CA1 NCBs were initially positive for APP at 24 hrs after injury, other NCB in the contra-lateral hemisphere were positive over time, especially at 48 hrs post-injury.	Leupeptin infusion results in a build up of NCB immuno-reactivity with all antibodies to APP.
Lin *et al.*, 1998	Unilateral infusion of an immunotoxin (IgG-saporin) into the left ventricle of female Sprague Dawley rats. Mab 22C11	Increased APP staining in the frontal cortex and hippocampus, which are the terminal targets of the cholinergic neurons in the basal forebrain.	A reduction in acetylcholine which occurs when the cholingeric system is depleted results in an increase in APP within NCBs in the frontal cortex and hippocampus 6 months after the lesion.

APP immunocytochemistry has also detected increased NCB immunoreactivity in animal models of ischaemia. APP immunoreactive NCBs were found after varying degrees of ischaemia and reperfusion in gerbils especially in the subiculum, layer CA3 of the hippocampus and layers 3, 5 and 6 of the fronto-parietal cortex (Tomimoto *et al.*, 1995). APP immunoreactive NCBs have also been found around the periphery of the infarct following MCA occlusion in rats at 3 days (Kalaria *et al.*, 1993a, 1993b) and after 4 and 7 days using the same protocol (Stephenson *et al.*, 1992). Scattered APP immunoreactive

NCBs have also been found after 6 hrs following global cerebral ischaemia in rats (Pluta *et al.*, 1994).

1.2.8- APP, βA4 and head injury

Head injury is an epidemiological risk factor for AD (Mortimer *et al.*, 1991). Support for this hypothesis has come from early studies of dementia pugilistica, where the brains of boxers suffering from this condition demonstrated AD-like pathology with diffuse βA4 plaque deposition (Roberts *et al.*, 1990). This βA4 deposition apparently resulted from repeated blows to the head over a long period of time and it was thought that it may also occur in brains of head-injured people who are susceptible to developing AD.

Diffuse, widespread βA4 deposits have also been found in head-injured human brains, also suggesting that the large overexpression of endogenous APP, which is seen following head injury and ischaemia, may exceed the limit of normal processing capacity and cause cells to mismetabolize APP into potentially amyloidogenic fragments (Roberts *et al.*, 1991, 1994; Gentleman *et al.*, 1993a; Sherriff *et al.*, 1994b; Graham *et al.*, 1995b, 1996). However, Adle-Biassette *et al.*, (1996) were unable to detect any βA4 deposits below the age of 63 years in head injury cases of similar age range and survival time to those studied by Roberts *et al.*, (1991).

It is now speculated that because βA4 deposition is not found in all head-injured brains, it may be deposited in brains of people who are genetically susceptible to develop AD, for example, those individuals possessing an Apolipoprotein E4 (ApoEε4) allele. ApoEε4 has been linked with AD (Corder *et al.*, 1993) and it has been proposed that βA4 deposition

following head injury may occur more prominently in those who possess an ApoEε4 allele. Studies have found that the frequency of ApoEε4 in βA4 positive cases of fatal head injury is higher than in most studies of AD with no evidence of TBI (Graham *et al.*, 1996). This relationship is currently the focus of intense interest investigation.

Head injury in transgenic mice, which have an APP mutation that normally results in AD-like pathology at 6 months of age, produces memory dysfunction before the appearance of AD-like pathology (Hsiao *et al.*, 1996; Nalbantoglu *et al.*, 1997; Smith *et al.*, 1998). Head injury also accelerates the onset of βA4 deposition in transgenic mice with levels at 4 months of age normally seen in uninjured mice at 1 year of age (Kawabata *et al.*, 1991; Games *et al.*, 1995; Johnson-Wood *et al.*, 1997). Other head-injury models in the rat have been unsuccessful in producing βA4 plaques following head-injury (Lewen *et al.*, 1995, 1996; Pierce *et al.*, 1996).

Thus the human and experimental studies have not resolved the exact mechanisms by which overexpression of APP results in AD and further investigation is required. The studies in this thesis have been limited to the investigation of the early neuronal expression of APP following TBI and have not addressed the possible relationship with βA4 production.

1.3- NEUROPROTECTION AND TBI

The aim of neuroprotective therapies is to use pharmacologic agents to alter the detrimental neurochemical cascades initiated by mechanical deformation of neural tissue for review see Novack *et al.*, 1996; Bullock, 1996). The experimental and clinical trials of these substances have shown very different and inconsistent results and their usefulness in the treatment of TBI is questionable (McIntosh, 1993). Some examples of trialed neuroprotective therapies include EAA antagonists Ca^{2+} channel blockers, free radical scavengers, arachidonic cascade inhibitors, substances to reduce cellular acidosis and hypothermia.

It is not proposed to undertake a comprehensive literature review of all these different therapies but to provide a selective overview as a background to the use of magnesium sulphate ($MgSO_4$) in our studies. The literature review relating to magnesium (Mg^{2+}) is detailed in Chapter 6.

1.3.1- EAA antagonists

EAA antagonists aim to reduce the extracellular concentrations of EAAs by blocking their release or deactivating the EAA receptors. The benefits of EAA antagonists is that administration can take place up to 12 hrs post-injury and still have beneficial effects. The beneficial actions of EAA-receptor antagonists in injury have been generally assumed to result from pharmacological blockade of the postsynaptic NMDA receptor (Albers *et al.*, 1989). Non-competitive NMDA antagonists act at the NMDA-coupled Ca^{2+} channel and prevent Ca^{2+} entry through the channel, whereas competitive NMDA antagonists block access of glutamate to its NMDA receptor.

EAA antagonists have shown promising neuroprotective properties in animal models and cell cultures. MK-801 (dizocilpine), a non-competitive NMDA receptor blocker, induces protective proteins such as HSPs following TBI in rats (Sharp *et al.*, 1991) and reduces the threshold for the suppression of protein synthesis in rats following ischaemia (Mies *et al.*, 1993). MK-801 administration produced an 80% reduction in ischaemic injury when administered post-injury in rats (Lyden and Hedges, 1992). Despite these promising experimental observations, trials of non-competitive NMDA receptor antagonists such as MK-801 produced severe side effects, possibly by activating limbic structures (Novack *et al.*, 1996) and they have subsequently been withdrawn from clinical trials.

A new competitive NMDA antagonist CGS 19755 has been shown to reduce lesion size and oedema in an ischaemic animal model (Perez-Pinzon *et al.*, 1995) and may have a more rapid therapeutic effect then the non-competitive agents (McIntosh and Smith, 1995). Other substances that possess EAA antagonistic actions include dextrophan which reduces extracellular levels of aspartate and glutamate and improves recovery following TBI in rats (Panter and Faden, 1992; Faden, 1993), NBQX which blocks glutamate and AMPA receptor sites and reduces oedema by slowing down the cellular influx of Na^{2+} during ischaemia (Hirose and Chan, 1993) and adenosine which blocks excitatory neurotransmitter release during ischaemia (Evans *et al.*, 1987).

1.3.1.1- Magnesium as a neuroprotective agent

Mg^{2+} plays numerous important roles in cellular biochemical processes and functions as a natural EAA antagonist (Feldman *et al.*, 1996). A number of recent in vivo studies have established that brain intracellular free Mg^{2+} concentrations decline after TBI (McIntosh *et al.*, 1998, 1989; Vink *et al.*, 1987, 1988, 1996; Feldman *et al.*, 1996; Heath and Vink, 1996,

1998a, 1998b) and this decrease has been associated with decreased motor function and exacerbation of neurological dysfunction. Experimental studies of TBI (McIntosh *et al.*, 1989; Smith *et al.*, 1993; Okiyama *et al.*, 1995; Feldman *et al.*, 1996; Heath and Vink, 1998a; Heath and Vink, 1999), ischaemia (Vacanti and Ames, 1984; Izumi *et al.*, 1991), hypoxia (Hoffman *et al.*, 1994) and a variety of other trauma-related pathological entities (Richards, 1982; McDonald *et al.*, 1990; Wolf *et al.*, 1991; May and Stewart, 1998) have shown that post-traumatic administration of Mg^{2+} has neuroprotective effects. The usefulness of Mg^{2+} as a neuroprotective agent will be discussed in greater detail in Chapter 6.

1.3.2- Ca^{2+} channel blockers

Ca^{2+} channel blockers aim to reduce the excessive cellular influx of Ca^{2+} ions following TBI and ischaemia. Some examples of Ca^{2+} channel blockers include nimodipine (Fujisawa *et al.*, 1986; Gelmers *et al.*, 1988; Teasdale *et al.*, 1992) and s-emopamil (Matsumoto *et al.*, 1993).

S-emopamil (a Ca^{2+} channel blocker and serotonin receptor antagonist) reduced motor and cognitive deficits after fluid percussion injury in rats and stabilized cerebral blood flow (Okiyama *et al.*, 1994).

1.3.3- Free radical scavengers and arachidonic cascade inhibitors

Attempts have been made to reduce the concentrations of free radicals produced following injury that contribute to neural and vascular damage associated with TBI. Free radical

scavengers such as ascorbic acid and superoxide dismutase (SOD) have been shown to reduce lesion size in rat models of ischaemia (Ranjan *et al.*, 1993) and reduce infarction and oedema in a transgenic mouse model of brain oedema (Chan *et al.*, 1991). Clinical trials of SOD have been hampered by the short life of SOD and its inability to penetrate the blood-brain-barrier (Turrens *et al.*, 1984). When combined with polyethylene glycol the half life is increased to 38 hrs and clinical trials have shown a positive effect on intracranial pressure (ICP) and outcome 3 to 6 months after severe head injury (Muizelaar *et al.*, 1993). Vitamin E has also been trialled as a free radical scavenger but it is absorbed slowly through the blood-brain-barrier and significant pretreatment prior to the onset of TBI is necessary thereby limiting application (Novack *et al.*, 1996). Corticosteroids such as methylprednisone have protective effects by inhibiting lipid peroxidase and reversing increased intracellular Ca^{2+} caused by free radicals (Hall, 1992). Experimentally, methylprednisone reduces lesion volume after hypoxia-ischaemia when administered prior to an insult in rats. Clinically, methylprednisone minimizes spinal cord injury when administered in high doses (Bracken *et al.*, 1990; Hall, 1992). A new sub-class of glucocorticoids have been developed called lazeroids. These do not have the side effects of other steroids and cell culture studies have demonstrated that they are potent inhibitors of lipid peroxidation (Braughler *et al.*, 1987). However, other experimental studies failed to show any improvement after administration of lazeroids (Chumas *et al.*, 1993).

Arachidonic cascade inhibitors also attempt to reduce the production of free radicals. Experimental studies using these have been limited, but it has been shown that indomethacin reduces the production of prostaglandins and free radicals after hypoxic/ischaemic injury in the rat (Pellegrini-Giampietro *et al.*, 1990).

1.3.4- Substances to reduce acidosis

To date tromethamine has shown some promise in animal studies and reduces intracellular pH and lactic acid formation, oedema and mortality in rats following fluid percussion injury (Rosner and Becker, 1984). However, these results have not been replicated in clinical trials (Muizelaar *et al.*, 1991).

1.3.5- Hypothermia

Hypothermia has a broad impact on neurochemical systems and limited adverse side effects which is why it has received much attention recently as a putative therapeutic strategy. Post-traumatic hypothermia affords substantial brain protection and improves neurological outcome after TBI in rats (Clifton *et al.*, 1991; Dietrich *et al.*, 1994; Globus *et al.*, 1995; Clark *et al.*, 1996, 1997; Bramlett *et al.*, 1997; Koizumi and Povlishock, 1998) possibly by reducing glutamate release and free radical production. Mild hypothermia also reduced glutamate production in rabbits following ischaemia (Matsumoto *et al.*, 1993) and increased neuronal survival after graded ischaemia in rats (Chopp *et al.*, 1991). Exactly how hypothermia confers protection following TBI and ischaemia is not known. Randomized trials of severely head injury patients (Marion *et al.*, 1993) showed that treatment with moderate hypothermia for 24 hrs lowered cerebrospinal fluid glutamate concentrations and improved neurological recovery and outcome.

1.4- AIMS OF STUDY

1) To topographically analyze and semi-quantitate the distribution of APP immunoreactive axons and NCBs by immunocytochemistry at increasing time intervals after a controlled impact producing the equivalent of a mild to moderate head injury in adult sheep and lambs.

2) To use in-situ hybridization (ISH) to topographically assess the APP mRNA response over time in adult sheep and lambs and to quantify the amount of APP mRNA at increasing times after injury using northern blot analysis and compare the results with non-injured controls.

3) To compare results obtained in 1 and 2 above with adult sheep treated with intravenous $MgSO_4$ 30 minutes post impact.

4) To map the distribution of APP immunoreactive axons and NCBs and assess the APP mRNA response at increasing time intervals in a selection of human head injury cases.

CHAPTER 2

Ovine head impact model

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2.1- INTRODUCTION

The pathologic study of the brains of head injury victims highlights many of the inherent problems involved in research with human post-mortem material. In human head injury, the site of head impact and the duration and magnitude of the forces of impact are often unknown. There are often problems in the accurate distinction between the pathological changes occurring as a consequence of the primary mechanical insult and those resulting from secondary complicating factors such as ischaemia and hypoxia. Each patient presents with a unique combination of lesions which vary significantly in their complexity, location and severity (Blumbergs, 1997) making it very difficult to get a cohort of cases with similar impacts, survival times and pathological lesions. Biological differences such as age and sex and pre-existing medical conditions also add to the difficulties in interpretation of the results. There is often a delay between the death of a person and post-mortem and this post-mortem delay further complicates the histological assessment of the injured brain. Age matched, normal control brains with known post-mortem intervals are also difficult to obtain.

2.1.1- Experimental models of TBI

To overcome these limitations in studying human traumatic brain injury (TBI), numerous experimental models have been developed. These models are designed to produce the functional and pathological changes resulting from head injury in a closely monitored setting in sufficient numbers to enable statistical interpretation (Lighthall and Anderson, 1994).

Experimental head injury studies can be classified into 3 broad groups, (i) head-impact

models, (ii) direct non-penetrating brain deformation models and (iii) indirect non-head impact models.

2.1.1.1- Head-impact models

In road traffic accidents most severe head injuries result from impacts to a freely mobile head (McLean, 1995). Of all the described head injury models, head-impact models reflect most closely the real human situation where acceleration of the head is produced by a head impact (Marmarou *et al.*, 1994; Montasser and Marmarou, 1994) and have been shown to produce primary and secondary focal and diffuse changes similar to that observed following TBI in humans (Lighthall *et al.*, 1990). These models allow independent control of the contact velocity of impact and the subsequent brain deformation (Lighthall, 1988). Over the years, a variety of different techniques have been used to deliver impacts to heads of laboratory animals that were either free to respond to the stimulus or were stabilized to allow no motion upon impact. Humane stunners (Grubb *et al.*, 1970; Lewis *et al.*, 1996; Van Den Heuvel *et al.*, 1998; Finnie *et al.*, 1999), pneumatic impactors (Lighthall, 1988; Lighthall *et al.*, 1990; Gallyas *et al.*, 1992; Palmer *et al.*, 1993; Dunn-Meynell and Levin, 1997; Clark *et al.*, 1996, 1997; Matthews *et al.*, 1998) and weight drop methods (Nilsson *et al.*, 1990, 1993; Marmarou *et al.*, 1994; Meaney *et al.*, 1994; Montasser and Marmarou, 1994; Adelson *et al.*, 1996; Povlishock *et al.*, 1997) have been used successfully to produce diffuse brain injury. In the past blasting caps (Govons, 1944), iron paddles (Tedeschi, 1945) and rotary stickers (Chason *et al.*, 1966) have been used to deliver the impact.

The most commonly used head-impact models today involve the use of pneumatic impactors or weight drops and both of these methods have been used extensively in rats to produce

TBI. Controlled cortical impact by pneumatic impactors requires that a craniotomy be performed and the impact delivered directly onto the dura mater. The pneumatic impactor designs have been altered over the years to adapt to particular experimental protocols, but their basic principle remains unchanged. This type of impact produces widespread structural brain damage including haemorrhages, contusions (Dunn-Meynell and Levin, 1997) and functional changes such as memory impairment (Lighthall *et al.*, 1990).

In weight drop models the animal is placed in a stereotactic frame or restraining device and the dura mater is either exposed by a craniotomy and a metal plate placed over it (Nilsson *et al.*, 1990, 1993; Marmarou *et al.*, 1994; Lewen *et al.*, 1995, 1996), or the plate is placed directly on the intact skull (Montasser and Marmarou, 1994; Adelson *et al.*, 1996; Povlishock *et al.*, 1997). Weights of varying sizes are dropped onto the plate from a set height depending on the level of injury severity required. This means that the magnitude of impact can be controlled and reproduced. The weight drop method has also been modified by different research groups however the basic principles remain the same. This method has been shown to produce higher impact-acceleration levels than other head-impact models in the rat (Montasser and Marmarou, 1994). This technique has been used successfully to produce diffuse brain injury including cerebral oedema, cellular ionic disturbances and axonal damage (Nilsson *et al.*, 1990, 1993; Marmarou *et al.*, 1994; Meaney *et al.*, 1994; Montasser and Marmarou, 1994; Adelson *et al.*, 1996; Povlishock *et al.*, 1997).

2.1.1.2- Direct non-penetrating brain deformation models

In fluid percussion injury models a craniectomy is performed and either the exposed brain or the intact dura overlying the brain is compressed by a fluid pressure pulse, which produces

elastic deformation of the underlying brain tissue (Sullivan *et al.*, 1976). This model has been used in the rat (Cortez *et al.*, 1989; Faden *et al.*, 1989; McIntosh *et al.*, 1989; Katayama *et al.*, 1990; Pierce *et al.*, 1996; Yamaki *et al.*, 1994; Okiyama *et al.*, 1995; Bramlett *et al.*, 1997), cat (Sullivan *et al.*, 1976; Povlishock and Becker, 1985; Erb and Povlishock, 1988) and micropig (Povlishock, 1992) and produces vascular disruption, neuronal and glial alterations and axonal injury (AI). Although fluid percussion injury does reproduce some aspects of the biochemical, physiological, neurological and morphological responses observed in human head injury (Sullivan *et al.*, 1976) it usually produces very focal lesions and limited diffuse injury. Early fluid percussion studies did not produce contrecoup contusive injuries and haemorrhages were only present in the ipsilateral injured hemisphere (McIntosh *et al.*, 1989). However, recent advances in the technique have produced both focal and diffuse pathologies reminiscent of clinical TBI (Pierce *et al.*, 1996).

2.1.1.3- Indirect non head-impact models

Early studies on sub-human primates attempted to minimise direct head impacts and maximise rotation and angular acceleration of the head (Ommaya *et al.*, 1964, 1968; Unterharnscheidt and Higgins, 1969) because it was believed that it was these latter forces which were most important in producing diffuse brain injury. These non-head impact models produced pressure gradients within the primate skull and the resultant shear tensile and compressive strains affecting the brain (Adams *et al.*, 1981; Gennarelli *et al.*, 1981, 1982). These researchers produced intracranial haematomas (ICH), increased intracranial pressure (ICP), diffuse axonal injury (DAI), diffuse vascular injury (DVI), cerebral swelling and contusions and showed that inertial loading and the degree, direction and duration of acceleration and/or deceleration forces were the critical determinants of head injury outcome.

2.1.1.4- Head injury models utilizing intraneural injections of neurotoxins

These have been used to study the pathological responses to injections of neurotoxins which affect axonal transmission and function by damaging axons, glial cells and neuronal cell bodies (NCBs). Examples include injections of aluminium salts which cause chronic changes in neuroskeletal organization (Shigematsu and McGeer, 1992a), colchicine which is a reversible inhibitor of axoplasmic transport (Shigematsu and McGeer, 1992b), kainic acid which is also an inhibitor of axonal transport (Kawarabayashi *et al.*, 1991; Siman *et al.*, 1989; Salinero *et al.*, 1998) and leupeptin which is a potent serine protease inhibitor interrupting lysosomal protein degradation and fast axoplasmic transport (Hajimohammadreza *et al.*, 1994). Excitotoxic neuronal degeneration at the lesion site have been produced by ibotenic acid (Nakamura *et al.*, 1992) and quinolinic acid (Topper *et al.*, 1995).

2.1.2- The ovine head impact model

One factor common to many of the models described above is that they often use laboratory rodents whose brains are small and almost lissencephalic. Brain mass is important in head injury and inertial loading and shearing forces are related to brain mass, so animals with smaller brains are able to tolerate much greater rotational and acceleration forces than animals with larger brains (Gennarelli, 1994; Lighthall and Anderson, 1994). There is also a slender margin between fatal and non-fatal head impacts in animals with smaller brains (Lighthall *et al.*, 1989).

Some of these disadvantages are overcome in our ovine head impact model. Sheep have a relatively large brain weight (mean 120 g) and unlike the almost lissencephalic rodent brains their brains are gyrencephalic with a more complex pattern of gyri and sulci and a relatively large white matter component (Ommaya *et al.*, 1964) more closely resembling the human brain.

In this model an impact is delivered to the unrestrained head resulting in the differential movement of the brain within the skull that generates the shearing forces believed to be important in producing diffuse brain injury (Gennarelli, 1993). This is important because it is the diffuse brain injury which is said to correlate with outcome and this diffuse injury is the most common cause of a persistent vegetative state and severe disability after closed head injury (Graham *et al.*, 1983).

The neuropathological spectrum of lesions seen in this ovine head impact model includes widespread AI, vascular and neuronal damage (Lewis *et al.*, 1996; Van Den Heuvel *et al.*, 1998) resembling that found in human TBI. This ovine model enables us to study the neuropathology of TBI in a closely monitored environment avoiding complicating factors such as hypoxia and ischaemia which are frequently found following human head injury (Graham *et al.*, 1989a). Also, unlike human head injury where the exact forces of impact and the precise survival times are often unknown, in this model the impact force, point of contact and survival time are defined. Perfusion fixation of the brains also reduces artefactual complications caused by poor fixation which are commonly seen in human post-mortem brain tissue.

The ovine head impact model has also been adapted to study the responses of the immature brain to TBI. Very few models, largely confined to laboratory rodents and producing focal damage (Adelson *et al.*, 1996; Biagas *et al.*, 1996; Grundl *et al.*, 1994), have examined TBI in the immature brain. Therefore, we developed a lamb head injury model in which temporal impact to the unconstrained lamb head produces widespread AI and vascular damage (Finnie *et al.*, 1999).

2.2- CHARACTERISTICS OF THE OVINE HEAD IMPACT MODEL

2.2.1- Physiological monitoring

The animals are initially anaesthetized with intravenous administration of 10% thiopentone sodium (Pharmtech, NSW) at a concentration of 14 mg/kg and maintained and ventilated for their variable survival times with isofluorane and a mixture of oxygen (O_2) and nitrogen (N_2).

Mean arterial blood pressure (MABP) is measured from a fluid filled arterial catheter inserted into the left internal carotid artery. Right atrial pressure is measured by a catheter in the right jugular vein and connected to a pressure tip transducer. Heart rate (HR) is measured by a pulse oximeter.

To measure intracranial pressure (ICP) a transducer is positioned at a depth of 5 mm in the cerebral cortex. A small scalp incision is made 1.5 cm lateral to the midline between the cornual process and ears. A 2.7 mm hole is then drilled through the bone and the dura mater

is incised. A hollow adjustable clamping screw is positioned into the skull through which a fibre optic pressure tip transducer (Camino Model 110-4b, Camino Corporation) is passed until it is positioned correctly. Once this is inserted the clamp is tightened which secures the monitor in place.

Cerebral blood flow (CBF) is measured continuously by a Doppler venous outflow method (Upton *et al.*, 1994). This involves undertaking aseptic surgery 1 week prior to the impact by performing a 19 mm craniectomy in the midline, 1 mm behind the fronto-parietal suture line. A 20 MHz Doppler crystal is then placed over the superior sagittal sinus and the bone flap is replaced and secured using a titanium plate and screws. Global cerebral blood flow is recorded in terms of kHz Doppler shift.

A catheter is inserted into the jugular vein for intravenous fluid infusion of 0.9% Sodium chloride (Baxter, Viaflex). This is administered for the duration of the experiment to maintain right atrial pressure at 5 to 10 mmHg. The animals are ventilated with a mixture of O_2 and N_2 titrated against regular blood samples which are analyzed by a Ciba Corning blood gas analyzer. Core body temperature is monitored continuously and maintained at 38.5 ± 0.7 °C by a thermal blanket. It is vital to measure the physiological responses of each animal so as to ensure that the animals do not receive any insult such as hypo/hypercapnia or hypoxia.

All of the above data is recorded continuously by a computerized data acquisition system. In order to assess the physiological response of each animal mean pre-injury values for MABP, ICP, HR and CBF are obtained over a 30 minute interval prior to impact. The grouped data

for each variable is tested for significant departure from baseline by paired t-test analysis. Mean group data post-impact is then taken at specific time points within the first minute and up to the first hour post-injury.

2.2.2- Mechanics of head impact

Sheep are placed in the sphinx position with the head resting on a support to allow free rotational and lateral movement following impact. A humane stunner (model MKL, Karl Schermer and Co., Karlsruhe, Germany) is used to deliver the impact to the left temporal region of the unrestrained head. This mechanical stunning device is approved for euthanasia of domestic livestock (Andrews *et al.*, 1993) since impact after correct placement against the head produces immediate loss of consciousness. Limited forensic experience suggests that the resulting brain damage in humans can be severe (Kirkpatrick, 1988).

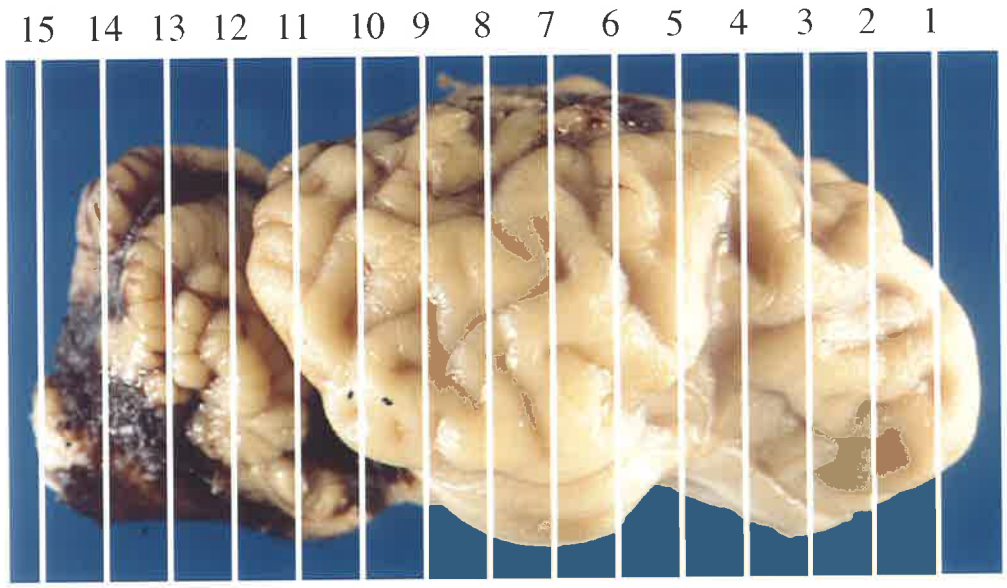
The humane stunner propels a mushroom-shaped captive bolt from the muzzle of the stunner by the discharge of a blank cartridge inserted in the chamber behind the proximal end of the bolt. This captive bolt is aligned with the left temporal fossa beneath the left cornu and to ensure that it is placed correctly the left horn bud is used as an external landmark so that the impact is delivered to the same anatomical region. Varying the charge used can control the magnitude of the impact. The charges range in strength from a No. 11 charge to a No. 21 charge (Schermer and Co., Karlsruhe, Germany). Preliminary studies demonstrated that a No. 17 charge produced the desired pathological and physiological responses and therefore, this charge was used in all of the adult ovine studies.

The head injury protocol was modified in the lamb head-impact model. The same physiological monitoring was undertaken as in the adult sheep, but due to thinness of the skull a lesser charge (No. 11) was used to deliver the head impact. The CBF was also not measured due to technical problems.

2.2.3- Neuropathological assessment

Following the set survival period animals are injected with 10,000 units of heparin sodium (Delta West, Bentley, Australia) into the right jugular vein. This is circulated in the bloodstream for 5 mins before perfusion fixation is commenced. A 2 mm incision is then made along each of the carotid arteries and jugular veins which are then cannulated with tubing while the animal is still under anaesthesia and ventilating. Ten litres of 4% paraformaldehyde is introduced through the carotid arteries under a constant pressure of 120 mmHg. The jugular lines act as the drainage system. This perfusion takes approximately 15 mins. The brains are then carefully removed using a craniotome and immersion fixed in 4% paraformaldehyde for 5 to 7 days. The brains are routinely photographed and weighed. Each brain undergoes full standard neuropathological assessment and 5 mm coronal slices are cut from the cerebral hemispheres and standard horizontal cross sections are taken of the brainstem and attached cerebellum (Figure 2.2.1). On average there are approximately 10 coronal slices of the cerebral hemispheres and 7 of the cerebellum and brainstem.

Figure 2.2.1: Neuropathological assessment. Right lateral view of an impacted sheep brain with the 5 mm coronal slices shown *(white lines)* from N1 (frontal) to N15 (cerebellum). Examples of coronal sections at levels N3 (frontal lobes), N7 (cerebral hemispheres at level of thalamus and N13& 14 (brainstem and cerebellum) are shown.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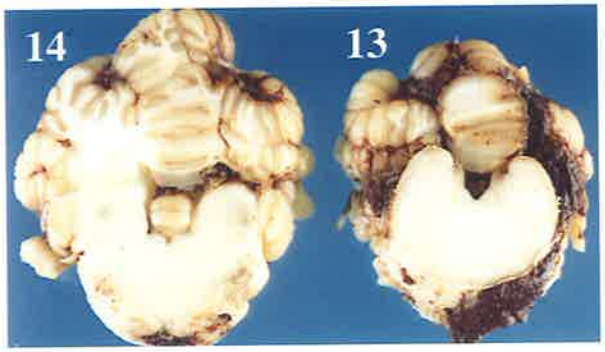
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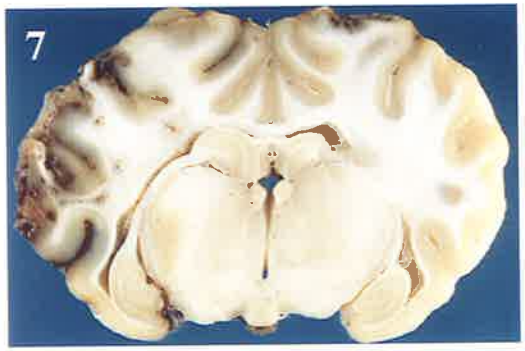
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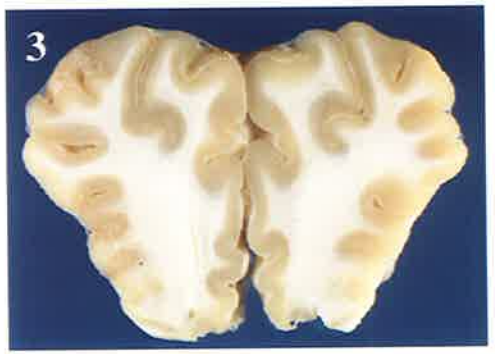
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2.2.4- Ethical considerations

All of the experimental studies were performed within the guidelines established by the National Health and Medical Research Council (NH&MRC) and were approved by the Animals Ethics Committees of the Institute of Medical and Veterinary Science (IMVS) and the University of Adelaide.

2.2.5- Spectrum of physiological and neuropathological changes as a result of impact

Physiological results from the ovine and lamb head impact models demonstrate that there is a response of greater than 20% shift from pre-injury values for all monitored parameters and the peak response of all parameters occurs within the first minute after impact. Statistically significant changes from baseline values are observed for all parameters within the first minute post-impact. Hyperaemia often occurs post-injury in the adult sheep with a 100% increase in CBF from pre-injury levels. (Figure 2.2.2).

Macroscopically, both the adult and lamb impact models produce similar focal and diffuse neuropathological abnormalities common to human TBI cases including coup and contrecoup contusions and bilateral subarachnoid haemorrhage (SAH). Using APP as a marker of AI, widespread AI has been found in both models (Lewis *et al.*, 1996; Finnie *et al*, 1999).

Figure 2.2.2: Typical physiological response to injury within the first 60 minutes following impact in impacted sheep. Baseline (pre-injury and control) levels are represented by a broken line and the grouped mean physiological data ± standard error (SEM) are represented by the solid line. Statistically significant shifts from baseline are observed in all parameters examined. Physiological responses return towards baseline within the first hour after impact. s, seconds; m, minutes.

2.3-CONCLUSIONS

Due to the numerous problems associated with the interpretation of the brain lesions in human TBI, experimental models have been developed to provide closely monitored and reproducible injuries. The ovine head impact model was developed in an attempt to overcome the limitations of using small laboratory rodents and results have shown that both the adult and lamb head impact models produce axonal and vascular damage resembling that found in man after TBI (Lewis *et al.*, 1996; Van Den Heuvel *et al.*, 1998; Finnie *et al.*, 1999).

CHAPTER 3

Early changes in brain APP expression in an ovine head impact model

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3.1- INTRODUCTION

Amyloid precursor protein (APP) is a membrane spanning glycoprotein of nerve cells which is transported by fast axoplasmic transport (Kang *et al.*, 1987; Koo *et al.*, 1990) and has been shown to accumulate within damaged axons following cytoskeletal disruption (Otsuka *et al.*, 1991) possibly due to inhibition of the normal axoplasmic flow of APP (Shigematsu and McGeer, 1992b). APP is therefore a sensitive and early marker of axonal injury (AI) and is found accumulated in damaged axons as early as 1.75 hrs after human head injury (Blumbergs *et al.*, 1995) and 1 hr in the experimental head impact model used in this study (Lewis *et al.*, 1996).

During the course of studies into traumatic AI, using APP immunostaining as a marker, it was found that the neuronal cell bodies (NCBs) were more frequently stained positive with the anti-APP monoclonal antibody Mab 22C11 than the axons. These APP immunoreactive NCBs were not only more widely distributed than the damaged axons but they also developed at an earlier stage. It therefore became the focus of these investigations to study the neuronal cell body (NCB) response to head injury with the hypothesis that the NCBs may provide a more sensitive indicator of neural injury following traumatic brain injury (TBI).

It is the aim of this study to systematically assess the NCB APP immunoreactivity response using Mab 22C11 in an ovine head impact model.

3.2- MATERIALS AND METHODS

3.2.1- Study group

Twelve 2 year old Merino ewes were used in this study. Ten animals were impacted using the methods described in detail in Chapter 2, Section 2.2 and a further 2 animals were used as non-impact controls. Each of the animals survived for 2 hours.

3.2.2- Immunocytochemistry for APP

3.2.2.1- Antibody used

The primary antibody used was a cloned monoclonal antibody 22C11 (Mab 22C11) (a kind gift from Professor Colin Masters). Mab 22C11's epitope region has been assigned to APP $_{66\text{-}81}$ in the extracellular Cys-containing domain (Hilbich *et al.*, 1993). The N terminal directed Mab 22C11 recognizes the rapidly transported glycoproteins 110, 120 and 140 kDa which are full length transmembrane forms of APP. It also recognizes a 100 kDa rapidly transported protein which may be the secreted form of APP cleaved between residues Lys $_{612}$ and Leu $_{613}$ of APP-695 and 2 unknown proteins of 108 and 125 kDa (Moya *et al.*, 1994). Mab 22C11 also recognizes the APLPs 1 and 2 (Wasco *et al.*, 1993; Slunt *et al.*, 1994).

3.2.2.2- Immunocytochemical detection of APP antigen

Prior to immunostaining, sections were microwaved in citrate buffer pH 6.0, blocked for 30 mins in 0.5% H_2O_2 in methanol and finally blocked for 30 mins in Tris buffered saline containing 10% normal horse serum (NHS, pH 7.45). These blocking steps inhibit the non-specific binding with endogenous peroxidase. All antibodies were diluted in NHS. APP antigen was detected in brain tissue sections using the peroxidase-anti-peroxidase

method and all reactions were carried out at room temperature. Sections were reacted overnight with primary antibody Mab 22C11 diluted 1:3000, after which Mab 22C11 was detected using horse anti-mouse immunoglobulin (1:500; 45 mins), followed by mouse peroxidase-anti-peroxidase conjugate (1:250; 45 mins) (Vector ABC kit, Jomar diagnostics). Between steps sections were washed for 15 mins in double distilled water (DDW). Finally, bound antibody was detected by reacting slides with 3,3'-diaminobenzidine (DAB, Sigma) (0.5mg/ml, containing 0.1% H_2O_2) for 7 mins. Slides were then immediately rinsed in water, counterstained with haematoxylin, dehydrated in alcohol and mounted in Depex (Ajax Chemicals). Positive staining appeared as orange/brown staining in NCB cytoplasm and damaged axons. The presence of a nucleus and nucleolus were used as the criteria for identifying neurons.

APP positive and negative control brain tissue with the primary antibody excluded accompanied each staining procedure.

3.2.2.3- Semi-quantitation of neuronal injury

Microscopic assessment of neuronal injury was performed using a semi-quantitative grid system, a modification of our previously described sector scoring method (Lewis *et al.*, 1996). This novel semi-quantitative method provides a means of topographically assessing the NCB and axonal response throughout the entire brain (Van Den Heuvel *et al.*, 1998). A transparent graticule comprising 4 mm x 4 mm grid squares, each with a unique reference number was placed over the section (Figure 3.2.1B). A central and peripheral reference point are marked on the glass slide. These are matched with corresponding reference points on the transparent graticule allowing independent evaluation of brain sections. Any grid square containing 1 or more APP reactive NCBs was scored positive. A schematic diagram was made of each section which recorded the number of positive and negative grids (Figure 3.2.1C). NCBs with APP reactive

granularity covering at least 50% of the surface area of their cytoplasm were recorded as positive. Figure 3.2.2 shows the spectrum of APP immunostaining in NCBs. The total number of positive grids was then summed and the percentage of grids containing APP positive NCBs calculated. The percentage of grid squares containing APP positive axons was calculated in a similar manner and also recorded onto the schematic diagram.

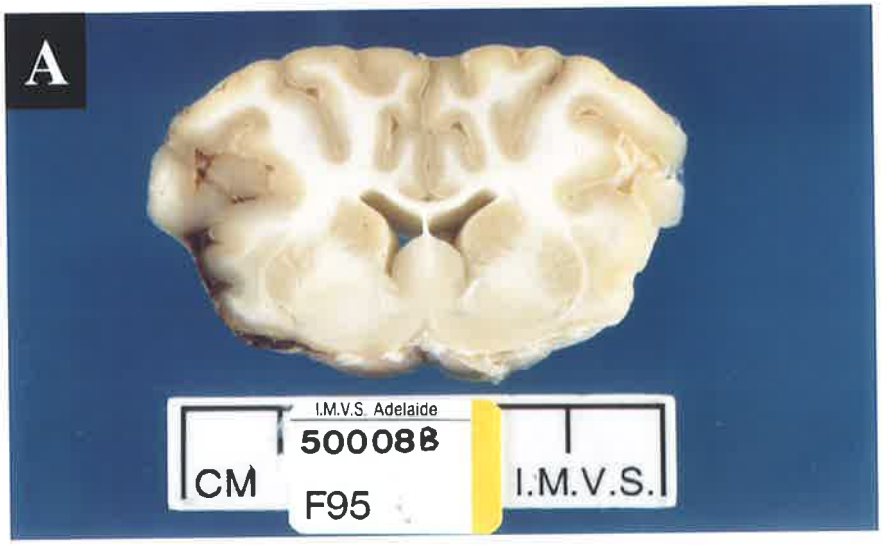
On average there were 10 coronal slices of the cerebral hemispheres and 7 slices of the cerebellum and brainstem. In total there were approximately 1100 grid squares per case representing the total surface area of the sheep brain slices.

3.2.3- Statistical analysis

Repeated measures analysis was used to examine the relationship between survival time, skull fracture, contusions and subarachnoid haemorrhage (SAH) and the number of grids with APP positive NCBs and axons. P values of < 0.01 were statistically significant. As a function of the repeated measures analysis, Z tests were also carried out and Z values of ≥ 2.5 were considered to be statistically significant. The statistical program used was Program 5V from the BMDP Statistical Software Package (W. Dixon, 1993, UCLA).

A kappa test was also used on a random selection of cases to determine intra and inter-observer variability where κ is the proportion of agreement after chance is removed from consideration.

Figure 3.2.1: Semi-quantitation of neuronal and axonal injury. (A) coronal (5 mm) section of a perfusion-fixed impacted sheep case showing recent traumatic subarachnoid haemorrhage over the left (impacted) hemisphere. (B) shows a H&E histological section (5 μm) at the same level overlying the transparent graticule used to semi-quantitate the APP immunocytochemical response. Diagrammatic representation of positive grid squares with APP immunoreactive neuronal cell bodies (*red*) and positive grid squares with APP immunoreactive axons (*black*).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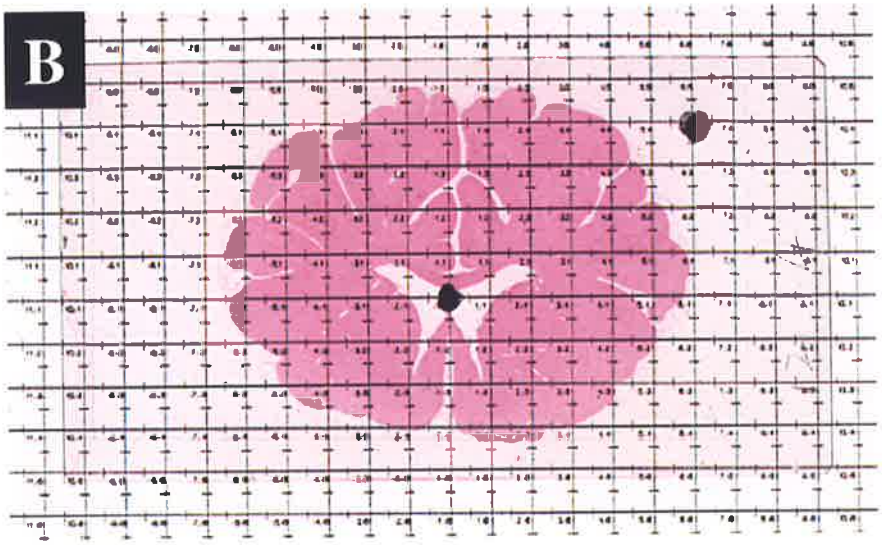
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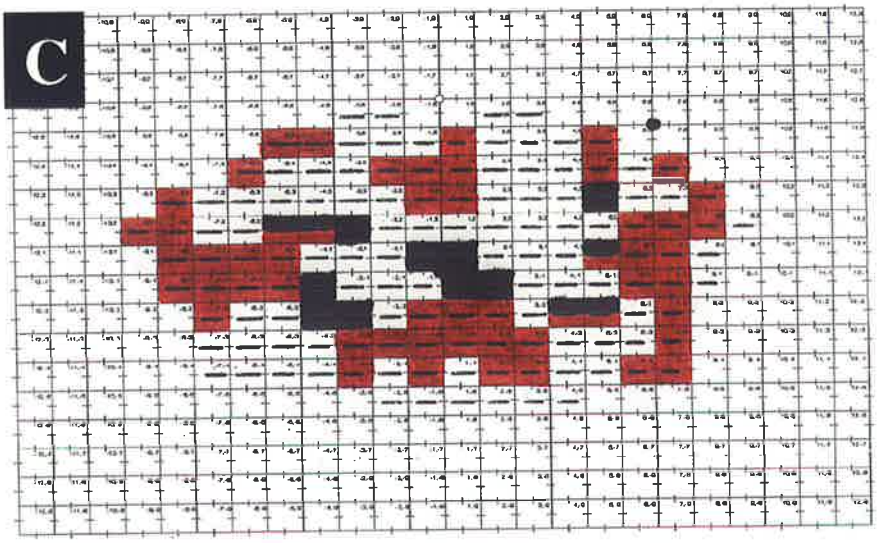
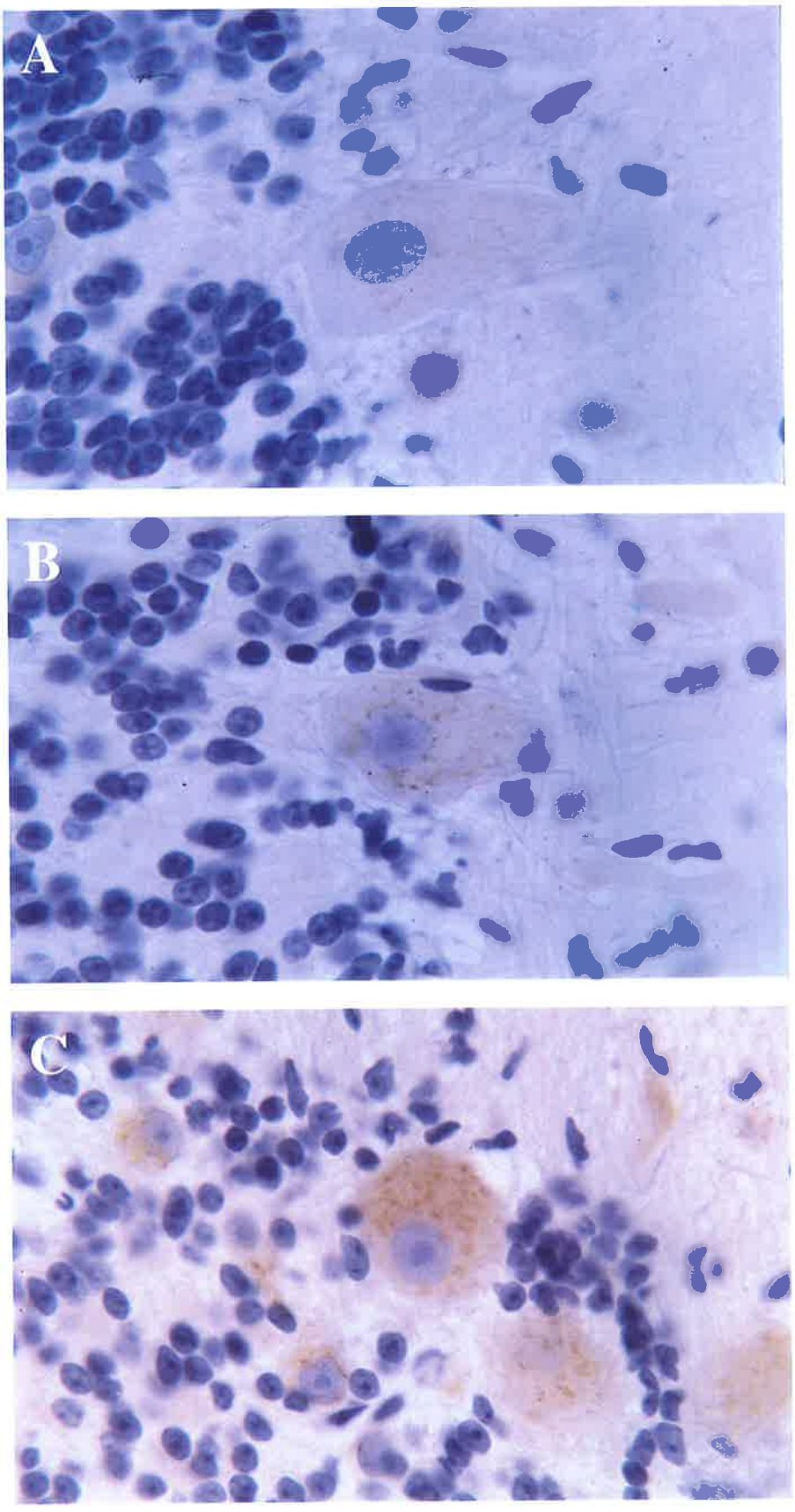

C

Figure 3.2.2: Classification of immunoreactive APP neuronal cell bodies as stained with Mab 22C11. (A) shows a neuronal cell body without any detectable APP staining in the cytoplasm. Undetectable staining of neuronal cell bodies was seen in negative control tissue with the primary antibody deleted and in non-impacted controls. (B) shows a neuronal cell body with few cytoplasmic APP reactive granules, but not covering 50% of the surface area of the cytoplasm. (C) shows an unequivocal APP positive neuronal cell body with intense APP cytoplasmic granularity/ positivity (*orange-brown staining*).(x 600).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3.3- RESULTS

3.3.1- Macroscopic assessment

The mean brain mass was 123 grams (range 111-140 grams). Non depressed linear fractures were evident in 5 of the 10 impacted sheep, 2 had depressed fractures and in 3 there was no evidence of skull fracture. SAH was observed in all animals over the ipsilateral hemisphere and in 8 animals over the contralateral hemisphere. There were focal impact contusions in 9 animals and 6 had contralateral contusions. A summary of pathological data is shown below in Table 3.3.1.

Sheep ID	Survival time (mins.)	Skull fracture	Contusions		Subarachnoid haemorrhage	
			Right	Left	Right	Left
1	120	-	-	-	-	+
2	120	-	+	+	+	+
3	120	+	+	+	+	+
4	120	+	+	+	+	+
5	120	+	+	+	+	+
6	120	+	+	+	+	+
7	120	+	-	+	-	+
8	120	+	-	+	+	+
9	120	+	+	+	+	+
10	120	-	-	-	+	+
Control 1	120	-	-	-	-	-
Control 2	120	-	-	-	-	-

Table 3.3.1: Summary of macroscopic lesions of the 10 impacted cases and 2 non-impact controls. (-, Absent; +, Present)

3.3.2- APP antigen response to TBI

3.3.2.1- Neuronal cell body response

APP immunoreactive NCBs were found in the cerebral hemispheres, cerebellum and brainstem distant to the site of focal injury in all 10 impacted animals. The percentage of brain area with APP positive NCBs ranged from 53 to 85% (Table 3.3.2). The density of APP positive NCBs showed regional differences with greater than 50 positive NCBs observed in some grids and less than 5 randomly scattered APP positive NCBs in others.

Two hrs following injury APP immunoreactive NCBs were seen in 46 to 88% (mean 64%) of grids covering the left impacted hemisphere and 38 to 82% (mean 54%) in the contralateral hemisphere. APP positive NCBs were often found in the vicinity of APP positive axons, but NCB immunoreactivity was more widespread than axonal immunoreactivity (Figure 3.3.1 and Figure 3.3.2A).

There was also a heterogeneous cellular response with some NCBs showing APP immunoreactivity and neighbouring NCBs showing undetectable levels of APP. In the 9 sheep with contusions, loss of neuronal immunoreactivity in the central region of contusions was evident in neurons showing necrotic change whereas NCBs at the contusion margins showed positive immunostaining. There were striking increases in the percentage brain area with APP positive NCBs in the cerebral hemispheres (Figure 3.3.3A), central grey matter (Figure 3.3.2A), brainstem (47 to 95%, mean 74%) and cerebellum (38 to 93%, mean 72%) (Figure 3.3.3B).

Control brains showed very small numbers of weakly APP positive NCBs (7 and 6%) which were statistically less than the impacted brains ($P < 0.01$). There was a statistically

significant increase in the proportion of grids with APP positive NCBs in impacted animals compared to controls. Z values of 10, 11.25, 16.5 and 15.5 were obtained after comparing the left hemisphere, right hemisphere, cerebellum and brainstem respectively (refer Appendix B i).

There was a 97% proportion of agreement between 2 individual observers following semi-quantitation of APP positive NCBs (refer Appendix B iii) and a 93% agreement between 2 individual counts by 1 observer (refer Appendix B iv).

3.3.2.2- Axonal response

APP positive axons were present in 9 impacted animals, the number of positive grids in these cases ranging from 1 to 21%, with immunoreactive axons most prominent in the impacted left temporal hemisphere in cases with contusions. The presence of a linear or depressed skull fracture statistically increased the proportion of positive axons (Z=11.75). No axonal APP immunostaining was identified in the control brains (refer Appendix B i).

	Total APP Immunostaining		Distribution of neuronal cell body APP			
Sheep ID	**Neuronal cell bodies mean (%) grids**	**Axons Mean (%) grids**	**Cerebral hemispheres Right (%) grids**	**Left (%) grids**	**Cerebellum (%) grids**	**Brainstem (%) grids**
1	58	1	53	63	53	47
2	60	5	60	69	38	54
3	70	2	59	75	69	93
4	68	3	58	66	82	86
5	53	0	40	46	60	64
6	70	6	55	76	83	74
7	57	21	54	63	79	65
8	54	4	38	46	71	86
9	58	2	42	46	91	95
10	85	13	82	88	93	75
Control 1	7	0	8	5	0.5	0
Control 2	6	0	8	4	0.5	8

Table 3.3.2: Summary of microscopic pathological data showing the distribution of APP positive neuronal cell bodies and axons in impacted and non-impacted sheep.

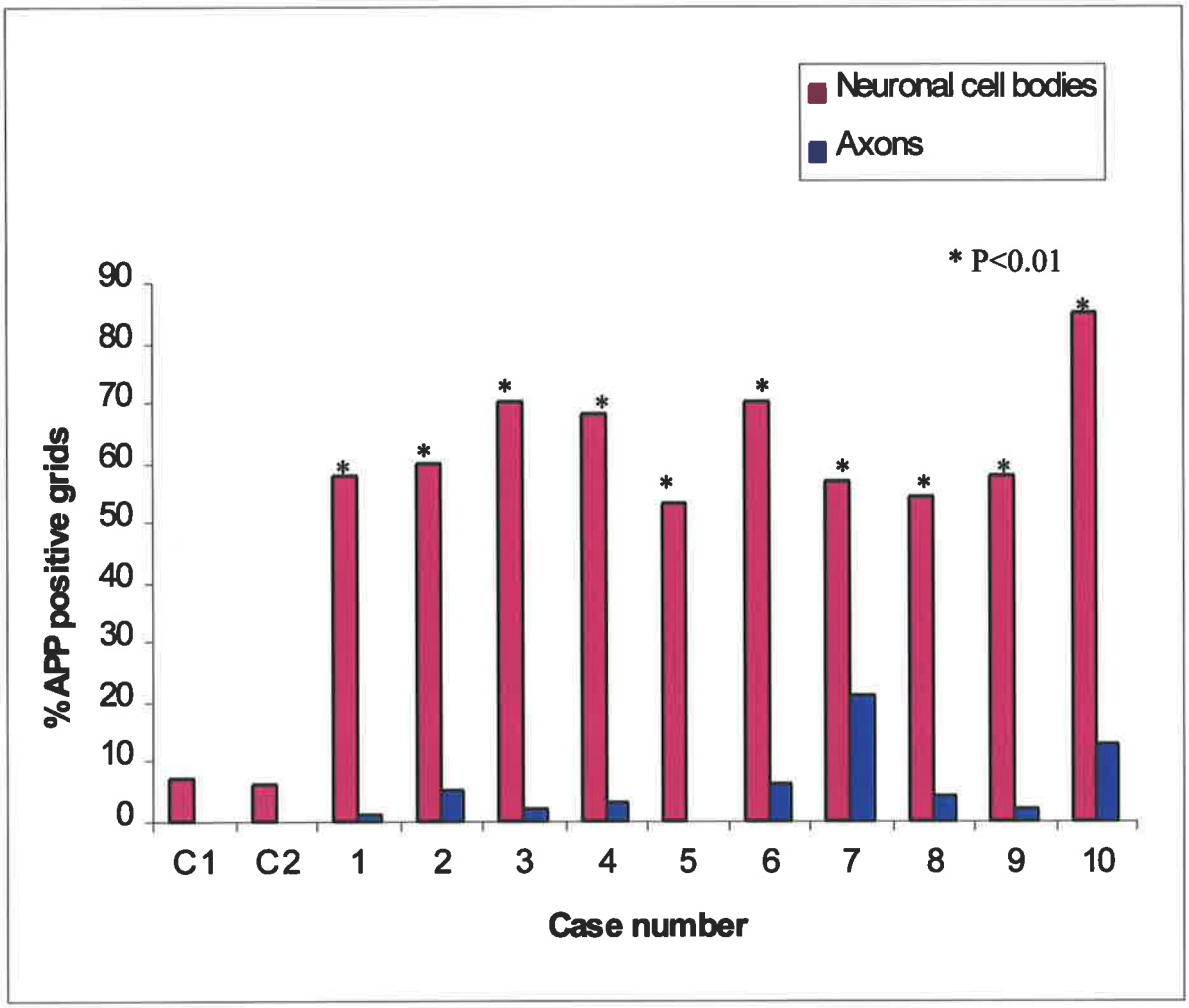
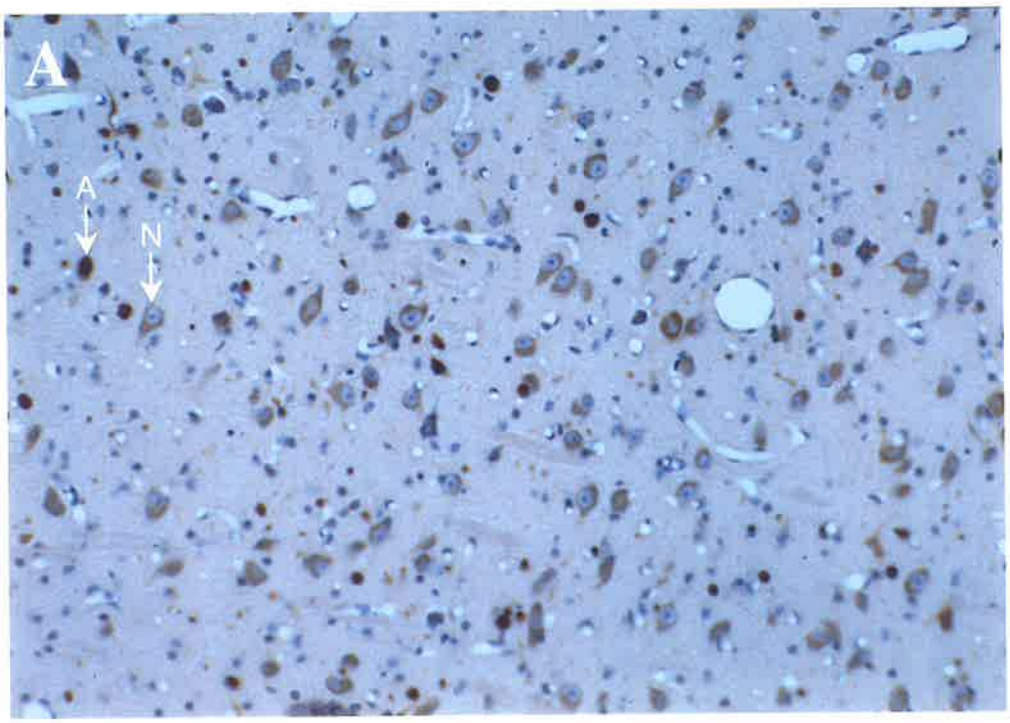


Figure 3.3.1: Histogram showing the percentage brain area with APP immunoreactive neuronal cell bodies and axons in 10 impacted animals and 2 control (non-impacted) animals. C1 and C2 are control animals.

Figure 3.3.2: APP immunostaining in central grey matter. APP immunostaining *(orange/brown staining)* of neuronal cell bodies *(N)* with scattered APP immunoreactive axons *(A)* in an impacted case (A) compared to undetectable staining in a control (non-impacted) case (B). (x200).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A
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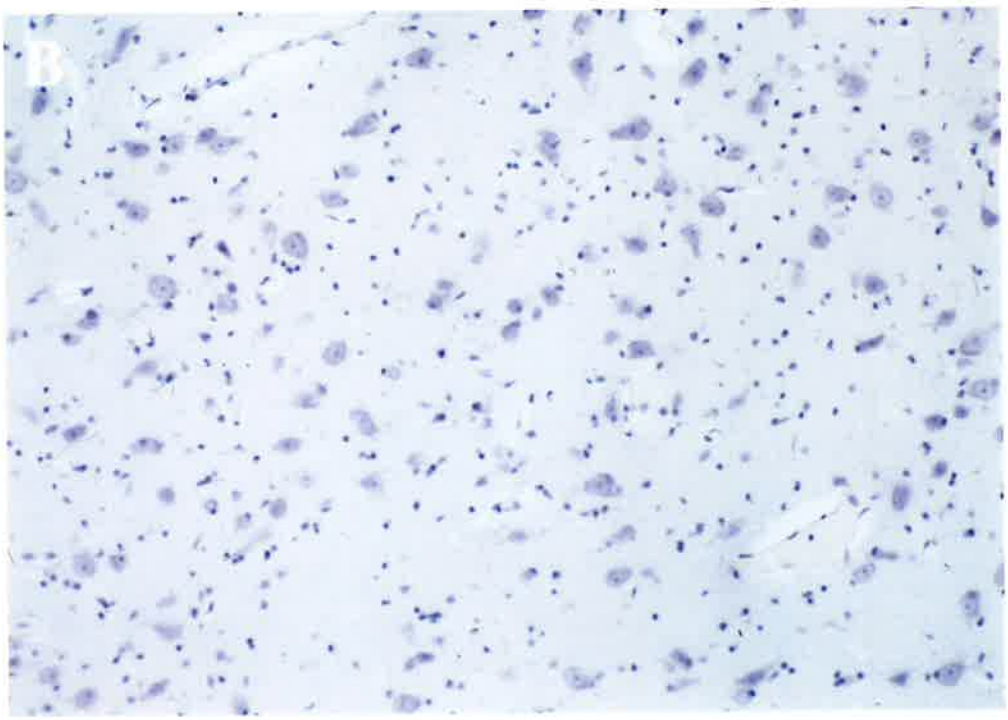
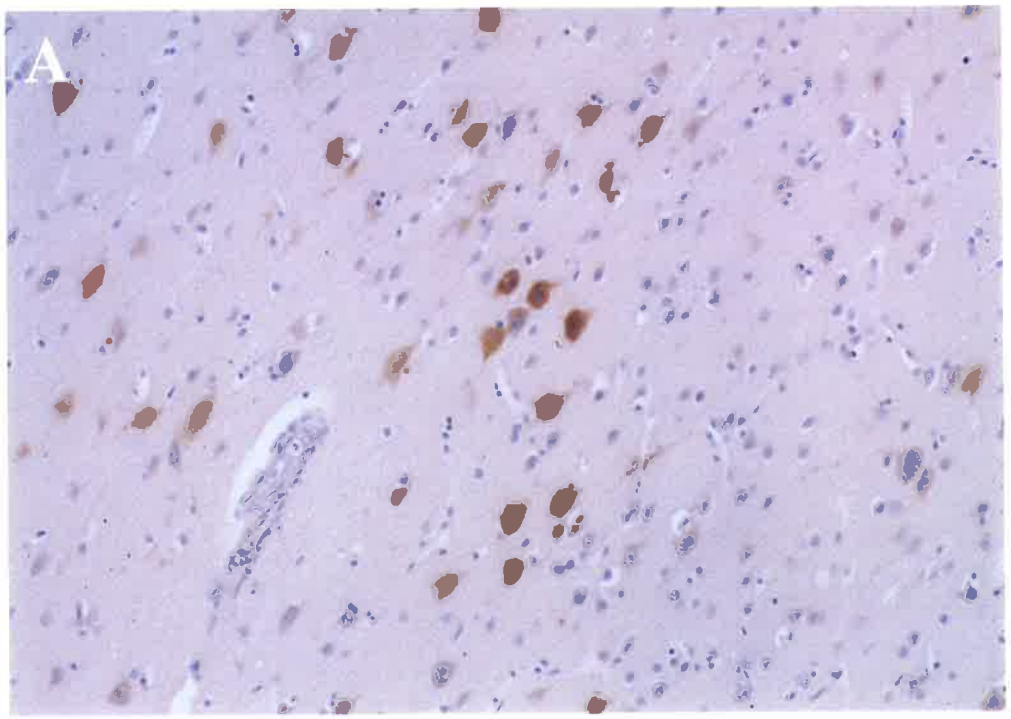

B

Figure 3.3.3: Diffuse APP immunoreactivity within the cerebellum and left (impacted) hemisphere 2 hrs post-injury. (A) APP positive neuronal cell bodies in the left temporal cortex *(orange/brown staining).* APP immunoreactivity in cerebellar Purkinje cells (B). (x200).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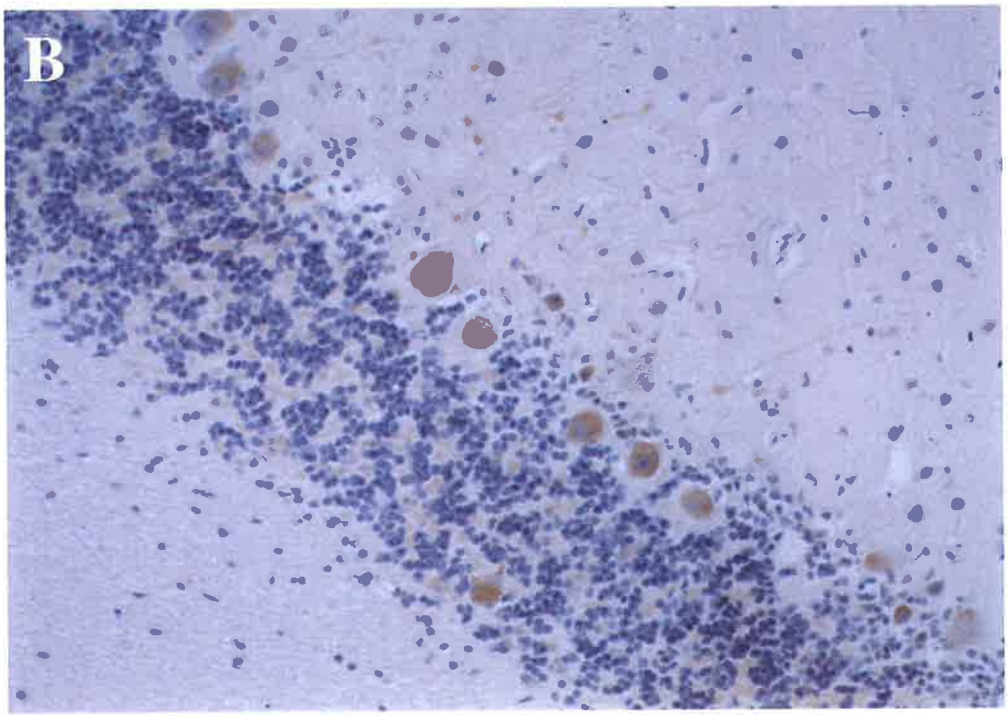
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3.4- DISCUSSION

APP immunostaining with Mab 22C11 detected damaged axons and APP immunoreactive NCBs following a focal head impact. In support of the hypothesis, NCB immunostaining was more widespread than axonal staining. APP immunoreactive NCBs were prominent in the left impacted hemisphere and also involved the contralateral hemisphere, brainstem and cerebellum.

As described in detail in Chapter 1, Section 1.2.6, APP has been used extensively as a sensitive marker of AI in human studies (Gentleman *et al.*, 1993b, 1995, 1999; Sherriff *et al.*, 1994a, 1994b; Blumbergs *et al.*, 1994,1995; McKenzie *et al.*, 1996; Abou-Hamden *et al.*, 1997) and in experimental studies (Kawarabayashi *et al.*, 1991; Otsuka *et al.*, 1991; Nakamura *et al.*, 1992; Shigematsu and McGeer, 1992a, 1992b; Lewen *et al.*, 1995, 1996; Lewis *et al.*, 1996; Pierce *et al.*, 1996; Finnie *et al.*, 1999) but the few studies of APP immunoreactivity in NCBs have produced conflicting results.

As also described in detail in Chapter 1, Section 1.2.7 there have been few human studies which have demonstrated APP positive NCBs following fatal head injury (Gentleman *et al.*, 1993b; McKenzie *et al.*, 1994; Roberts *et al.*, 1994). Experimentally, head impact and fluid percussion models have also demonstrated APP immunoreactive NCBs using different antibodies (Lewen *et al.*, 1995, 1996; Pierce *et al.*, 1996; Bramlett *et al.*, 1997) as have head injury models utilizing intraneural injections of neurotoxins (Kawarabayashi *et al.*, 1991; Nakamura *et al.*, 1992; Shigematsu and McGeer, 1992a, 1992b; Hajimohammadreza *et al.*, 1994; Lin *et al.*, 1998).

Unlike these experimental studies, this is the first detailed topographical study using a large brain (i.e. sheep) to demonstrate an increase in APP expression 2 hrs after trauma. The particular isoforms of APP that are responsible for this increase are not yet known as the antibody used in this study is unable to distinguish between the different isoforms of APP and also detects the APP-like-proteins (APLP's) (see Section 3.2.2.1). Further studies with more specific antibodies are required to determine if there are changes in particular isoforms.

The significance of the NCB APP immunoreactivity is not yet known but it may simply reflect accumulation within NCBs following cytoskeletal disruption as has been proposed for axons (Otsuka *et al.*, 1991) or an increased synthesis of APP molecules (upregulation of APP synthesis). As APP has roles in synaptic function (Schubert *et al.*, 1991) or formation (Moya *et al.*, 1994; Roch *et al.*, 1994; Morimoto *et al.*, 1998), cell-cell (Beher *et al.*, 1996) or cell-matrix adhesion (Beyreuther *et al.*, 1996) and neurite outgrowth (Breen *et al.*, 1991; Milward *et al.*, 1992; Small *et al.*, 1994; Allinquant *et al.*, 1995) it is possible that increased APP may be required for these processes in TBI.

The widespread APP NCB immunoreactivity compared to axonal APP immunoreactivity suggests that the increased APP immunostaining is due to increased synthesis of APP. In-situ hybridization (ISH) to determine whether APP mRNA is upregulated following TBI would confirm the hypothesis that the increased NCB APP immunoreactivity is due to increased synthesis of APP and is the subject of the next chapter.

CHAPTER 4

Upregulation of APP and its mRNA in response to TBI

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4.1- INTRODUCTION

In the previous chapter it was demonstrated that APP antigen expression was increased in neuronal cell bodies (NCBs) 2 hrs after traumatic brain injury (TBI) and that neuronal cell body (NCB) immunoreactivity was more widespread than axonal immunoreactivity following a head impact. These data suggest that detection of NCB APP may be a more sensitive marker of neural injury. Although it has been shown experimentally in vivo that APP is expressed in NCBs after various traumatic insults, such as weight drop (Lewen *et al.*, 1996), fluid percussion (Pierce *et al.*, 1996; Bramlett *et al.*, 1997) and following intraneural injections of neurotoxins (Kawarabayashi *et al.*, 1991; Nakamura *et al.*, 1992; Shigematsu and McGeer, 1992a, 1992b; Hajimohammadreza *et al.*, 1994; Lin *et al.*, 1998) detailed studies of the possible reasons for this immunoreactivity are lacking. In studies described in Chapter 3, apparent APP expression after TBI increased possibly due to (i) accumulation of APP in neuronal cell bodies caused by inhibition of normal axoplasmic transport system; (ii) alteration in the availability of APP epitopes detectable by the antibody used, or (iii) increased transcription of APP messenger RNA (mRNA).

We hypothesized that the apparent increase in APP within NCBs in response to injury, as determined immunocytochemically, is due to increased APP mRNA expression. This hypothesis was addressed by comparing the expression of APP mRNA by in-situ hybridization (ISH) with APP antigen expression (immunocytochemically) over a period of time.

4.2- MATERIALS AND METHODS

4.2.1- Study group

Thirty four 2-year-old Merino ewes comprising 25 impacted animals and 9 non-impacted controls were perfusion fixed with 4% paraformaldehyde (Chapter 2, Section 2.2.3) 15, 30, 45, 60 and 120 mins after impact (5 impacted animals at each survival time). Brains from control (non-impacted) sheep (9 in total) were similarly fixed at corresponding time intervals. As we were unable to obtain approval from our Ethics Committee to undertake long-term survival studies we elected to study the temporal course of APP alterations in the first 2 hrs after injury.

4.2.2- Immunocytochemistry for APP

Methods for the detection of APP antigen using Mab 22C11 have been discussed previously in Chapter 3, Section 3.2.2. In addition 2 impacted sheep cases (1 x 1 hr survivor and 1 x 2 hr survivor) were stained with a rabbit polyclonal antibody specific for the APP-KPI containing isoforms of APP (Ab 93/28), kindly provided by Dr. Janetta Culvenor (University of Melbourne). This antibody is directed at the KPI domain and detects only APP-770 and APP-751 and was used at a dilution of 1:200. Prior to incubation with Ab 93/28 for 16 hrs at 4°C, sections were microwaved in citrate buffer (pH 6.0), blocked for 30 mins in 0.5% H_2O_2 in methanol and subsequently blocked for 30 mins in 10% NHS (pH 7.45) at room temperature. Binding of Ab 93/28 was detected using horse anti-rabbit immunoglobulin (1:500, 30 mins; room temperature) followed by a rabbit peroxidase-anti-peroxidase conjugate (1:250, 30 mins; room temperature) (Jomar

Diagnostics). APP positive and negative control brain tissue with the primary antibody excluded accompanied each staining procedure.

Semi-quantitation was only undertaken for cases stained with Mab 22C11, using the methods described in detail in Chapter 3.2.2.3. Due to the small number of sections stained with Ab 93/28 we did not semi-quantitate these.

4.2.3- In-situ hybridization (ISH)

4.2.3.1- Choice of probes

Riboprobes (RNA) were used for the following reasons: (i) being single-stranded, the chance of self annealing during the hybridization reaction is very low, which effectively increases probe concentration and enhances sensitivity (Angerer *et al.*, 1987) and (ii) when applied for the detection of RNA, opposite sense transcripts can be used on sequential samples as a negative control for non specific probe binding (Gowans *et al.*, 1989). Undetectable hybridization by the sense probe was a means of ensuring that the detectable hybridization by the anti-sense probe was specific.

4.2.3.2- Choice of indicator molecule

Riboprobes were labelled with digoxigenin (DIG), a steroid hapten. The DIG system offers several advantages over conventional radioactive indicator molecules such as ^{35}S, ^{32}P and ^{125}I which are commonly used in ISH. DIG is non-radioactive and therefore safer than radioactive labels. Significantly, the same probe stock is stable for at least one year and can be used repeatedly, thus increasing the degree of reproducibility between experiments. Turnover time of experiments is greatly reduced because newly synthesized probes are not required for each ISH and the development time is typically measured in

hours rather than days or weeks. In addition, colour development can be monitored without stopping the reaction and signal is much more localized compared with emulsion autoradiography.

4.2.3.3- Preparation of coverslips and slides

Glass slides and coverslips were immersed in acid wash solution for 16 hrs, washed in 5 changes of double distilled water (DDW) and air dried. Coverslips were siliconized by dipping into 1% aqueous Prosil-28 (PCR Inc, Gainesville, Florida) for 5 seconds, rinsed twice in DDW, air-dried at 80°C for 2 hrs and heat-sterilized. Slides were dipped in 2% aminopropyltriethoxysilane (APES)/98% ethanol for 20 seconds, rinsed 3 times in 100% ethanol, 3 times in DDW, air-dried and stored for up to 3 months before use. APES coated slides were activated by immersion in 10% glutaraldehyde in PBS for 30 mins (Maples, 1985) immediately before use. Brain sections (5 μm) were cut from paraffin blocks, floated on a 50°C water bath, collected onto activated APES coated slides, dried overnight at 37°C and stored until required.

4.2.3.4- Preparation of DIG-labelled riboprobes

(a)- *Transcription of riboprobes PV-Cap-1 and PGem-Cap-1*

PGem-Cap-1 constructed by cloning a 2.45 kb Sal I fragment (containing cDNA from a human Alzheimer cortex library) into PGem-blue was a kind gift from Dr. Dane Liston via Sonny Bou, Calgary. PV-Cap-1 was constructed by subcloning the 2.4 kb *Xba I-Hind III* fragment of PGem-Cap-1 into PGem-4Z (Promega, Madison, WI). Transcription of PGem-Cap-1 and PV-Cap-1 from the T7 promoter generated sense and anti-sense APP mRNA riboprobes respectively (Figure 4.2.1).

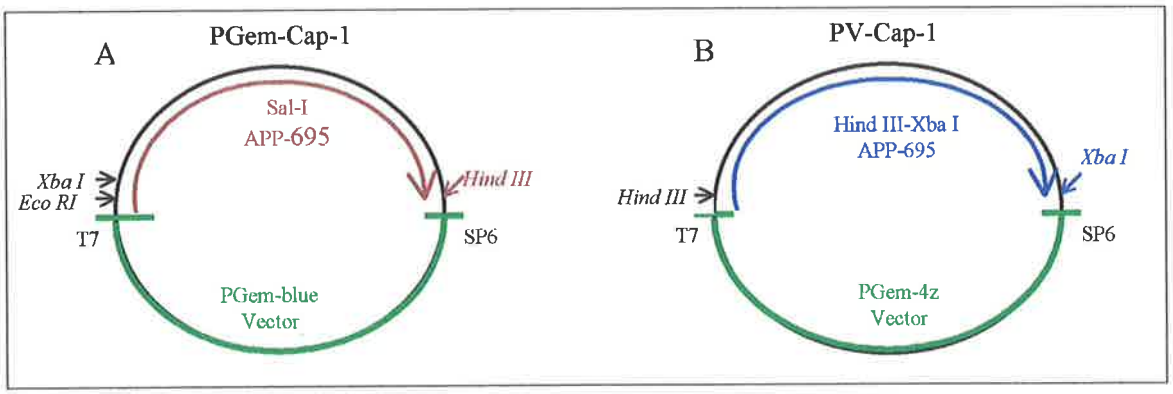


Figure 4.2.1: Diagrams showing the plasmids used for the generation of sense and anti-sense probes for ISH. (A) Shows the Cap-1 plasmid where a 2450 bp *Sal-I* APP-695 fragment was cloned into PGem-blue by Sonny Bou (Calgary). Linearization of this plasmid with *Hind III* gives a template for transcription of sense riboprobes using T7 RNA polymerase. (B) Shows PV-Cap-1 where the *Xba I – Hind III* fragment (2400 bp) was excized from PGem-blue Cap-1 plasmid and subcloned into the *Xba I – Hind III* cut PGem-4Z. Linearization with *Xba I* gives template for transcription of anti-sense riboprobes using T7 RNA polymerase.

Plasmids were linearized by restriction enzyme digestion and linearization was confirmed by electrophoresis on a 1% TAE agarose gel. Uncut templates were removed during gel purification. The transcription reaction was carried out using a modification of the procedure described by Promega (Arthur *et al.*, 1993): Mixtures contained:

1 X Transcription buffer (Promega)

5 mM dithiothreitol (DTT) (Promega)

20 units RNAsin (ribonuclease inhibitor, Promega)

0.5 mM rATP (Promega)

0.5 mM rCTP (Promega)

0.5 mM rGTP (Promega)

0.5 mM rUTP (Bresatec)

250 μM DIG-11-rUTP (DIG-UTP; Boehringer Mannheim)

1 μg linearized template plasmid DNA

15 units T7 RNA polymerase (Promega)

CSL water to give final reaction volume of 25 μl

The reaction mixture was incubated at 37°C for 1 hr and 15 mins. To digest away the DNA template, 1 unit of RNAse-free DNAse (Promega) was added and samples were incubated at 37°C for a further 15 mins. The reaction was stopped with 1 µl 500mM EDTA pH 8.0.

To precipitate RNA transcripts, 20 µl 4 M LiCl and 20 µl 10 mg/ml sheared salmon sperm DNA were added, the mixture was brought to a final volume of 100 µl with CSL water and finally 250 µl of 100% ethanol (-20°C) was added. The mixture was stored at -20°C overnight before centrifugation at 12000 x g for 20 mins. The pellet was washed 3 times in 70% ethanol, once in 100% ethanol, dried under vacuum and redissolved in 100 µl of ultrapure CSL water. Probes were stored at -70°C until required.

(b) Probe construction, primer synthesis and amplification of sequence by polymerase chain reaction (PCR) for the APP-KPI probe

A probe specific for the KPI containing APP isoforms (APP-KPI) was also used. APP-KPI DNA (a kind gift from Robert Cappai, University Of Melbourne) was linearized by restriction enzyme digestion using *Eco R1*. The linearized fragment was purified from agarose gel fragments using a BRESAclean® kit (Bresatac) and spectrophotometrically quantified at 260 nm wavelength, prior to use.

Apposing oligonucleotide primer pairs were designed to amplify the APP-KPI fragment. The forward primer was 5' ACC ACA GEG TCT TGT GAA GAG 3' which extended from bases 835 to 855 and the reverse primer was 5' GGT GTC TCG AGA TAC TTG TC 3' extending from bases 901-920 of the APP-695 sequence (Kang *et al.*, 1987). Primer positions determined to maximize the conditions for strong hybridization and

specificity were obtained from Dr Roberto Cappai (University of Melbourne, personal communication). Oligonucleotide primers used were synthesized using a DNA-RNA synthesiser, model 394 (Perkin Elmer Applied Biosystems). APP-KPI was PCR amplified from total linear APP-KPI DNA using one cycle of initial denaturation (90 seconds at 94°C), followed by 30 cycles of denaturation (30 seconds at 94°C), annealing (30 seconds at 50°C), and extension (150 seconds at 72°C). For this purpose, a Gene Amp PCR system 2400 (Perkin Elmer Applied Biosystems) machine was used. The PCR reaction mixture contained 200 µM each of dATP, dCTP, dTTP and dGTP, 2.5 mM $MgCl_2$, 1.0x amplification buffer [50 mM KCl, 10 mM Tris.HCl, pH 8.3 and 0.01% (w/v) gelatine], 40 pmole of each synthetic oligonucleotide primer, 200 ng of total linear DNA as template and 2.5 units of *taq* DNA polymerase (Promega) in a total volume of 100 µl. Double stranded PCR products were linearized by ethidium bromide staining of 1% agarose gels and the APP-KPI band extracted and cleaned and riboprobes were transcribed using the insert as described above in Section 4.2.3.4*(a)*.

(c) Calculation of melting temperature (Tm_{50})

For ISH experiments the temperature at which 50% of double stranded RNA hybrids will dissociate in liquid into single stranded molecules (TM_{50}) is defined by the equation:

$$Tm_{50}(RNA/RNA) = 79.8 + 18.5\log[Na^+] - (0.35 \times \% \text{ formamide}) + 0.584 \times (\%G+C) + 0.0012 \times (\%G+C)^2$$ (Sambrook *et al.*, 1989)

4.2.3.5- Preparation of tissue sections for ISH

The method used was as described by Pereira *et al* (1994). Paraffin sections (5 µm) were dewaxed in two changes of xylene for 40 mins and rehydrated gradually through graded

ethanol/water mixtures over a period of 1 hr. Finally, slides were immersed in 1 x PBS. Sections were fixed in 4% paraformaldehyde in PBS for 20 mins at 4°C, then washed twice for 5 mins in 1 x PBS. Proteinase K digestion of tissue sections improves the access of probes but over-digestion results in poor morphology and loss of target sequences (Angerer *et al.*, 1987; Gowans *et al.*, 1989). With respect to sheep brain sections it was found that digestion with 30 ug/ml proteinase K (Boehringer Mannheim) in 20 mM Tris HCl pH 7.5, 2 mM $CaCl_2$ with shaking at 37°C for 15 mins, provided strong signal and good preservation of tissue morphology. Sections were subsequently washed once for 1 min in 1 x PBS, refixed in 4% paraformaldehyde for 15 mins at 4°C, washed twice for 5 mins in 1 x PBS, and treated with acetylation solution for 10 mins at room temperature (Hayashi *et al.*, 1978). Sections were subsequently washed twice for 5 mins in PBS before application of the hybridization solution.

4.2.3.6- Hybridization

Hybridization solution (25 µl) was applied to sections and covered with a 24 mm x 50 mm siliconized coverslip, ensuring that no air bubbles were trapped underneath the coverslips. Coverslips were sealed with rubber cement (Super Vulkarn, Maruni Industries) and slides were incubated at 45°C (Tm_{50} -30°C) for a minimum of 16 hrs. All experiments included sections of non-impacted sheep neocortex which acted as negative controls. Further, in each experiment duplicate sections were hybridized with sense and anti-sense probes.

4.2.3.7- Washing

Rubber cement was removed using fine forceps and coverslips gently removed by immersion of slides in 2 x SSC, 10 mM Tris-HCl, pH 7.5. To remove unbound probe, slides were washed in 2L of 2 x SSC, 10 mM Tris-HCl, pH 7.5 for 1/2 hr and 2 changes of 2L of 0.1 x SSC 10 mM Tris-HCl, pH 7.5 for a minimum of 1/2 hr with gentle shaking. High stringency washing was performed at 53°C in 30% deionized formamide in 0.1 x SSC, 10 mM Tris-HCl, pH 7.5 (Tm_{50} -15°C) for 15 mins with gentle shaking. Formamide concentration was chosen to give washing temperatures compatible with good tissue preservation. Finally, slides were washed at room temperature in 0.1 x SSC, 10 mM Tris-HCl, pH 7.5 for 15 mins before colour development.

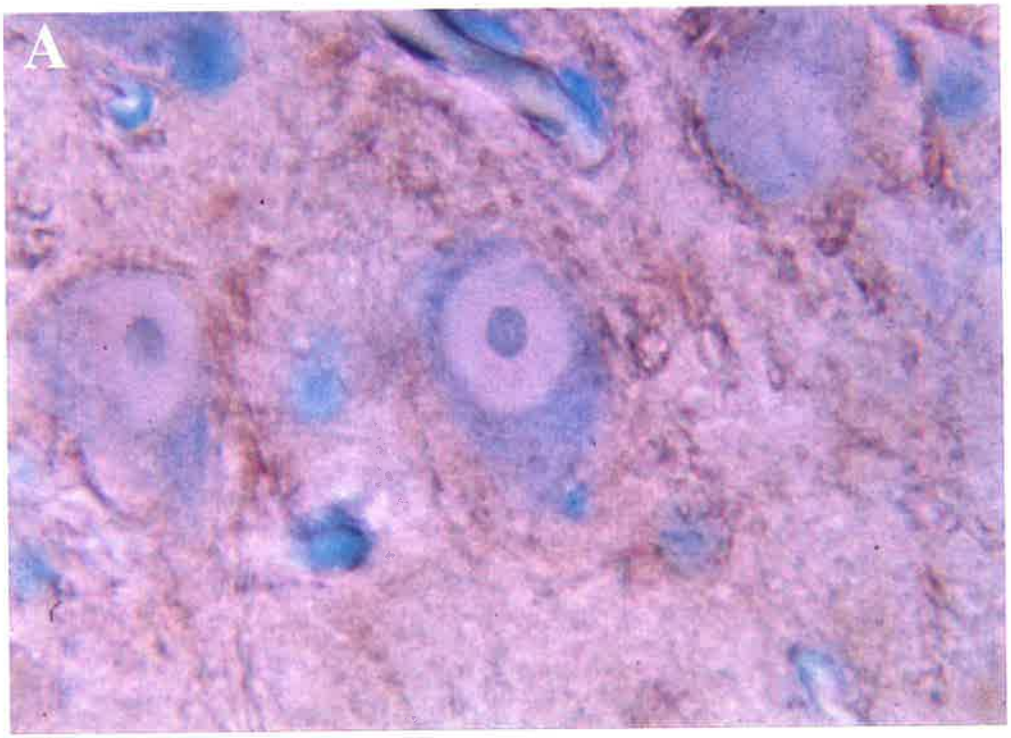
4.2.3.8- Colour development

Bound DIG-labelled probe was detected by colourimetric reaction, using a method described by Boehringer Mannheim. Slides were transferred to coplin jars and washed 5 mins in DIG buffer 1 and blocked in DIG buffer 2 for 30 mins with gentle shaking. Anti-DIG-Fab fragments conjugated to alkaline phosphatase (0.75 units/µl) were diluted 1/500 in 1:1 mixture of DIG buffer 1 and DIG buffer 2 before 120 µl was applied to individual tissue sections and incubated in a prewarmed humidified box at 37°C for 30 mins. Slides were subsequently washed twice in DIG buffer 1 (15 mins) and finally in DIG buffer 3 for 5 mins. Slides were immersed in freshly prepared colour substrate solution containing 135 µl NBT solution and 105 µl BCIP solution in 30 ml DIG buffer 3. Colour development was examined intermittently and allowed to proceed until full colour had developed, which was usually 3 hrs. Positive probe hybridization for APP mRNA appeared as strong brown/black staining in neuronal cell soma nuclei and cytoplasm (see

Figure 4.2.2B). Colour development was stopped by washing in DDW. Slides were either counterstained with haematoxylin or left unstained.

We were unable to use the semi-quantitative grid system for ISH in an analogous way to the immunocytochemistry due to logistical reasons. Therefore, sampling from representative areas of left and right frontal and temporal lobes, central grey matter, cerebellum and brainstem serial to those used for APP immunocytochemistry, was considered adequate to study the APP mRNA response. ISH was undertaken on 15 of the injured animals (3 from each time interval) and all of the non-injured controls.

Figure 4.2.2: In-situ hybridization for APP mRNA using a digoxygenin-labelled APP riboprobe (APP PV-Cap-1) (A) neuronal cell body apparently unstained as seen in brain tissue from control (non-injured) sheep. In contrast, intense staining *(brown/black)* for APP mRNA was demonstrated in a neuronal cell body of injured sheep after 1 hr (B). (x1000). See also Figure 4.3.4 for low power illustration.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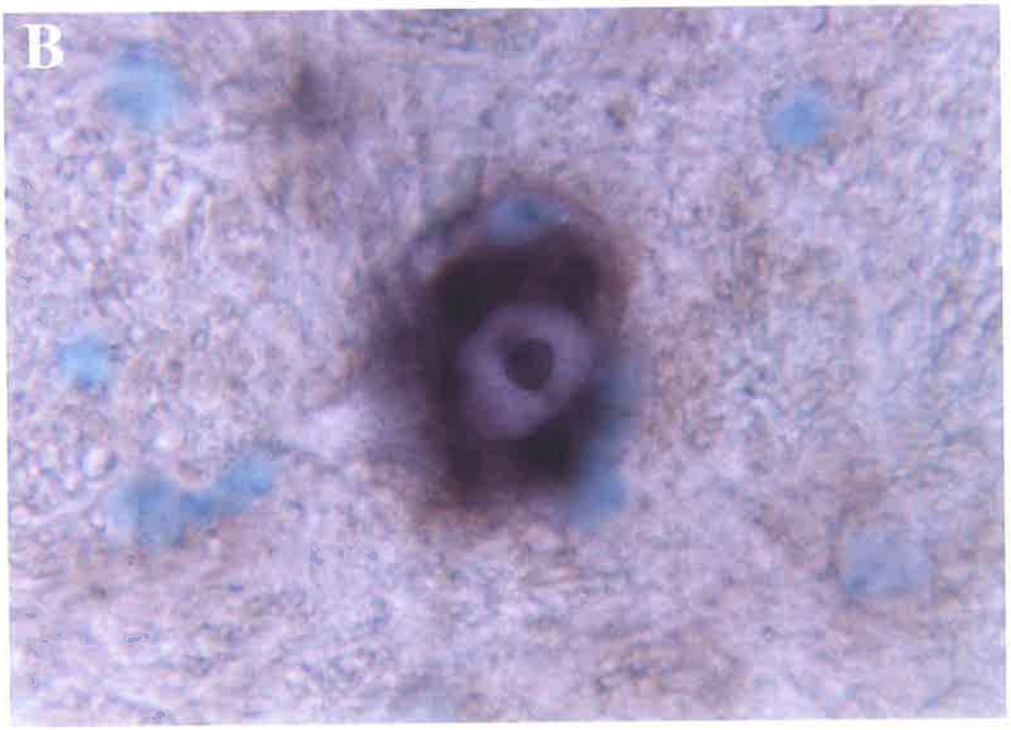

B

4.3- RESULTS

4.3.1- Physiological data

All animals survived the impact and underwent physiological monitoring as described in Chapter 2, Section 2.2.1 for the designated survival times. There were no episodes of hypoxia or sustained hypo/hypercapnia.

4.3.2- Neuropathological data

The mean brain mass of the impacted and control sheep was 118 grams (range 90-135 grams). Thirteen of the 25 impacted sheep had skull fractures (6 depressed and 7 linear fractures). Fifteen sheep showed traumatic subarachnoid haemorrhage (SAH) over the impacted hemisphere, 10 had SAH in the contralateral hemisphere and 9 had bilateral SAH. Nine sheep had temporal lobe contusions beneath the impact site, 6 had contrecoup contusions and 2 sheep had coup and contrecoup contusions. No traumatic damage was observed in the control (non-impacted) sheep. Pathological data are summarized in Table 4.3.1.

Sheep ID	Survival time (mins.)	Skull fracture	Contusions		Subarachnoid haemorrhage	
			Right	Left	Right	Left
1	15	-	+	-	-	-
2	15	-	+	-	+	+
3	15	+	+	+	+	+
4	15	+	+	+	-	+
5	15	+	-	+	-	+
6	30	-	+	-	+	-
7	30	-	-	-	-	-
8	30	+	-	+	+	+
9	30	+	-	-	-	-
10	30	+	-	+	+	+
11	45	-	-	-	-	-
12	45	-	-	-	-	-
13	45	+	-	-	-	-
14	45	+	-	+	-	+
15	45	+	-	+	+	+
16	60	-	-	-	+	+
17	60	-	-	-	-	-
18	60	-	-	+	-	+
19	60	+	-	+	-	+
20	60	-	-	-	-	+
21	120	-	-	-	+	+
22	120	+	-	-	+	+
23	120	+	-	-	-	-
24	120	+	+	-	+	+
25	120	-	-	-	-	-
Control	15	-	-	-	-	-
Control	15	-	-	-	-	-
Control	30	-	-	-	-	-
Control	30	-	-	-	-	-
Control	45	-	-	-	-	-
Control	60	-	-	-	-	-
Control	60	-	-	-	-	-
Control	120	-	-	-	-	-
Control	120	-	-	-	-	-

Table 4.3.1: Summary of macroscopic lesions of each of the 25 impacted sheep and 9 non-impacted sheep which were perfusion fixed with survival times from 15 mins to 120 mins. (-, Absent; +, Present).

4.3.3- APP immunoreactivity

4.3.3.1- Neuronal cell body response

(a) Mab 22C11

Fifteen mins after injury sheep brain sections showed only control levels of APP positive NCBs. Brain area with APP positive NCBs at this time ranged from 5 to 12% (mean 7.5%), increasing to 17 to 22% (mean 17.4%) and 19 to 45% (mean 32.4%) at 30 and 45 mins after impact respectively. At 1 and 2 hrs after impact the number of grids with APP positive NCBs had increased to 21 to 51% (mean 37.2%) and 53 to 57.5% (mean 54.5%) respectively (Table 4.3.2 and Figure 4.3.1). Further, 2 hrs after impact, APP specific immunostaining of NCBs was apparent in cerebral hemispheres, cerebellum (Figure 4.3.2A) and brainstem distant to the site of focal injury. The distribution of APP positive NCBs became more widespread with time and involved brain regions distant to the site of impact. Fifteen mins after impact an average of 8.5% of cerebellar grids had APP positive NCBs, increasing to 60% by 1 and 2 hrs.

Widespread APP immunostaining of NCBs was also noted in impacted brains that were macroscopically normal and did not show any abnormality on H&E staining (n = 8).

There was no detectable APP immunoreactivity in the central region of contusions while NCBs at the margins showed positive immunostaining.

Control brains showed very small numbers of weakly APP positive NCBs ranging from 2 to 14% (mean 7%).

Repeated measures analysis (described in Chapter 3, Section 3.2.3) demonstrated a statistically significant affect of survival time on the proportion of grids with APP immunoreactive NCBs ($P < 0.01$). Specifically, there was a significant increase in the percentage brain area with APP immunoreactive NCBs compared to controls from 45 mins to 2 hrs. No statistically significant increase was observed at 15 or 30 mins post-impact.

(b) Ab 93/28

Despite positive staining by Ab 93/28 in control brain tissue, qualitative assessment of the NCB and astrocytic response in the 2 cases examined 1 and 2 hrs after injury demonstrated only sparse expression of the APP-KPI isoforms.

4.3.3.2- Axonal immunostaining

Semi-quantitative analysis of the APP immunoreactive axons showed that no APP immunoreactive axons in any of the control (non-impacted) brains. In contrast, APP immunoreactive axons were found in all of the impacted sheep brains. APP positive axons were seen scattered throughout the cerebral hemispheres, but were predominantly localized within tiny bundles in the thalamus from 15 mins to 2 hrs (Figures 4.3.3A and 4.3.3B). However, in the 2 hr survivors APP positive axons were more dispersed throughout the left impacted hemisphere and often at the margins of contusions.

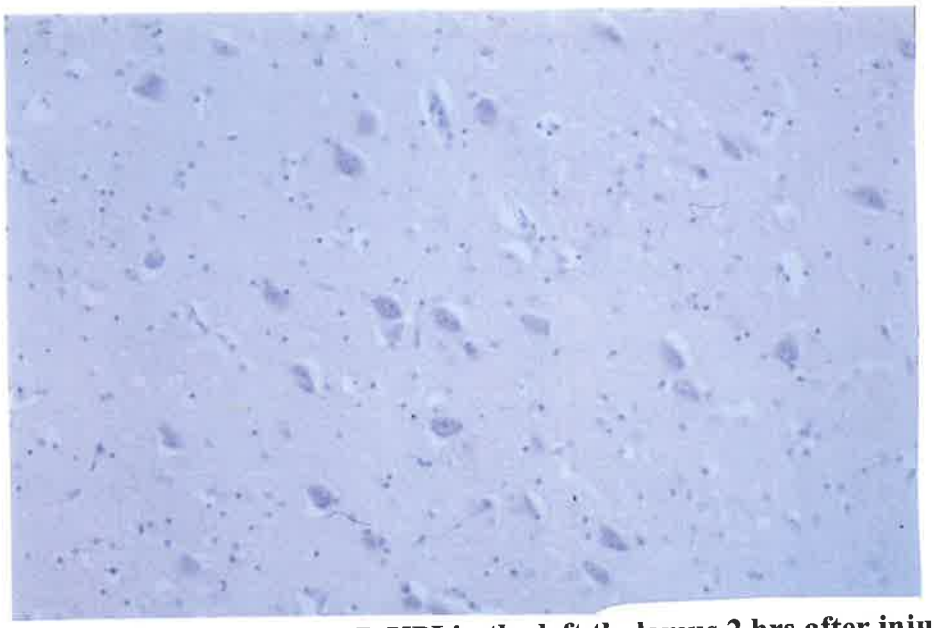


Figure 4.3.2C: Detection of APP-KPI in the left thalamus 2 hrs after injury. APP containing KPI isoforms were undetectable in neuronal cell bodies within the left thalamus 2 hrs after left temporal impact.

	Total APP Immunostaining		Distribution of neuronal cell body APP			
Sheep Survival time	**Neuronal cell bodies mean (%) grids**	**Axons Mean (%) grids**	**Cerebral hemispheres Right (%) grids**	**Left (%) grids**	**Cerebellum (%) grids**	**Brainstem (%) grids**
15 mins n=5	7.5 ± 3.6	4 ± 3.1	10 ± 1.4	9 ± 2.8	8.5 ± 7.9	4 ± 1.4
30 mins n=5	17.4 ± 2.7	3 ± 1.4	26.5 ± 10	36 ± 2.8	9.5 ± 3.5	9.5 ± 5
45 mins n=5	32.4 ± 11.2	2.5 ± 0.3	34 ± 2.1	27 ± 13.5	41.5 ± 19.4	18.5 ± 9.1
60 mins n=5	37.2 ± 14.2	4.5 ± 2.1	34 ± 19.4	39 ± 17.1	60 ± 18.4	45.5 ± 17.7
120 mins n=5	54.5 ± 2.5	6 ± 3.5	57.2 ± 4.5	59 ± 3.7	60 ± 27.5	44 ± 16.3
controls n=9	7 ± 3.5	0	6 ± 3.6	4 ± 2.1	12 ± 6.2	13 ± 11.5

Table 4.3.2: Summary of microscopic pathological data from perfusion fixed sheep showing the distribution of APP positive neuronal cell bodies and APP positive axons in impacted and non-impacted sheep using Mab 22C11. APP immunocytochemical assessment shows the mean percentage grids with APP positive neuronal cell bodies and axons ± the standard error of the mean (SEM) at each of the time intervals.

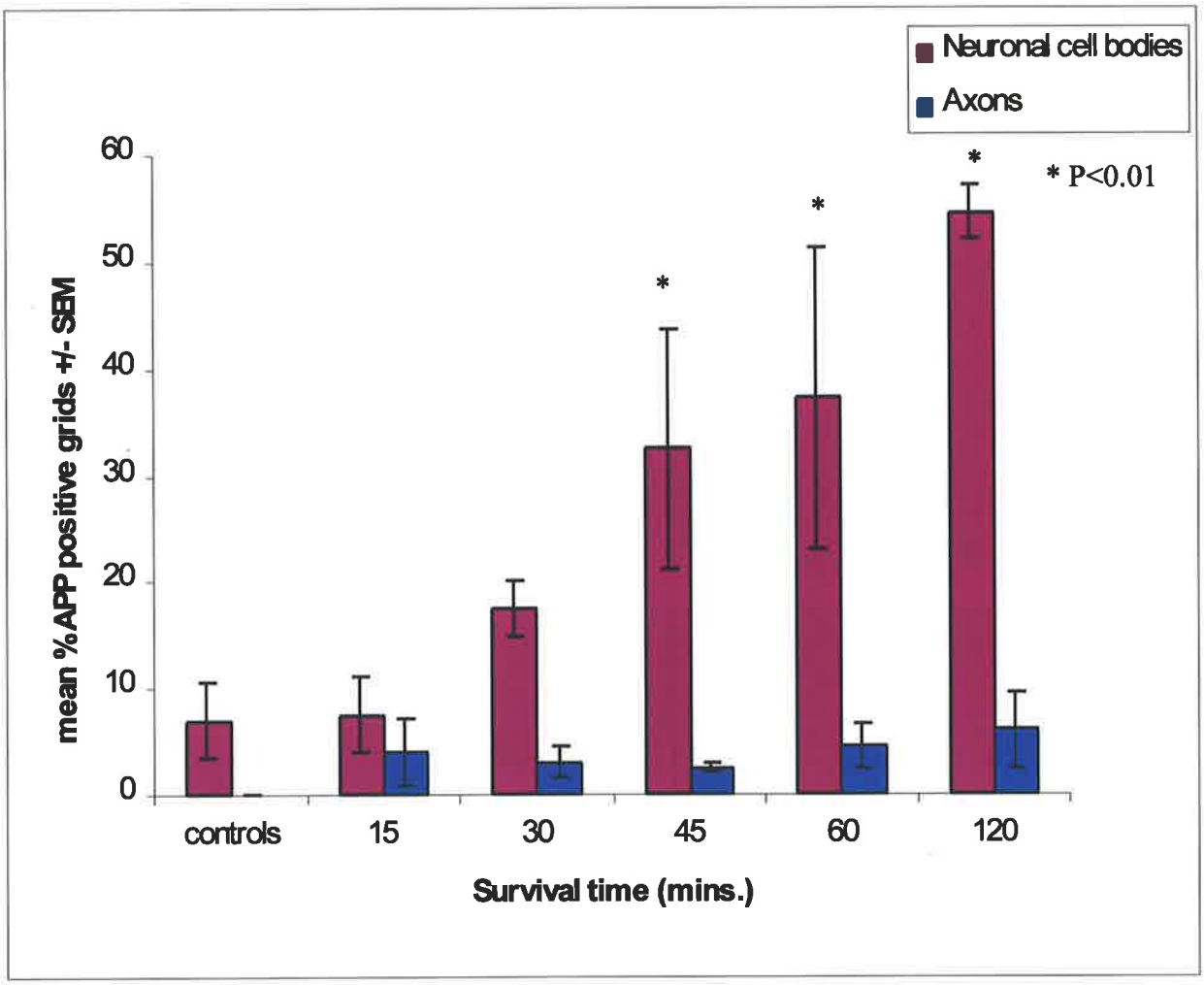
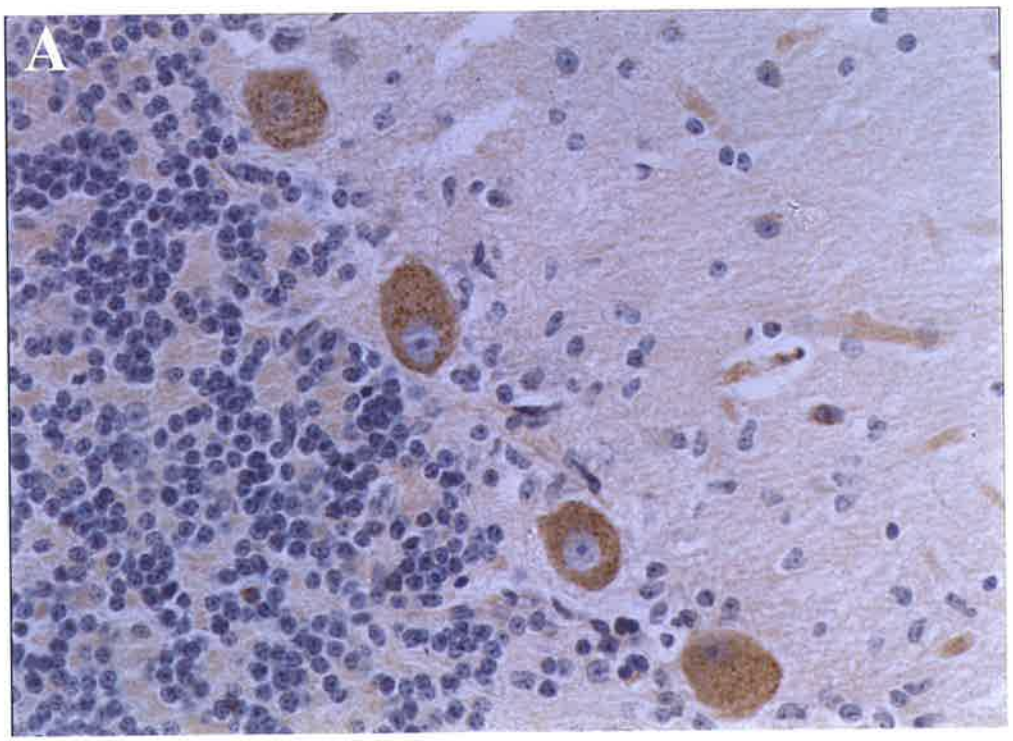


Figure 4.3.1: Histogram showing the mean percentage brain area ± SEM with APP immunoreactive neuronal cell bodies and axons over time from 15 mins to 120 mins using Mab 22C11.

Figure 4.3.2: Comparison of APP antigen and mRNA in the cerebellum 2 hrs after TBI. (A) typical staining *(orange/brown)* in cytoplasm of cerebellar Purkinje cells, showing intense APP immunoreactivity. (B) Purkinje cells in a serial section of the same sheep similarly show strong hybridization *(black/brown staining)* for APP mRNA using PV-Cap-1. (x400).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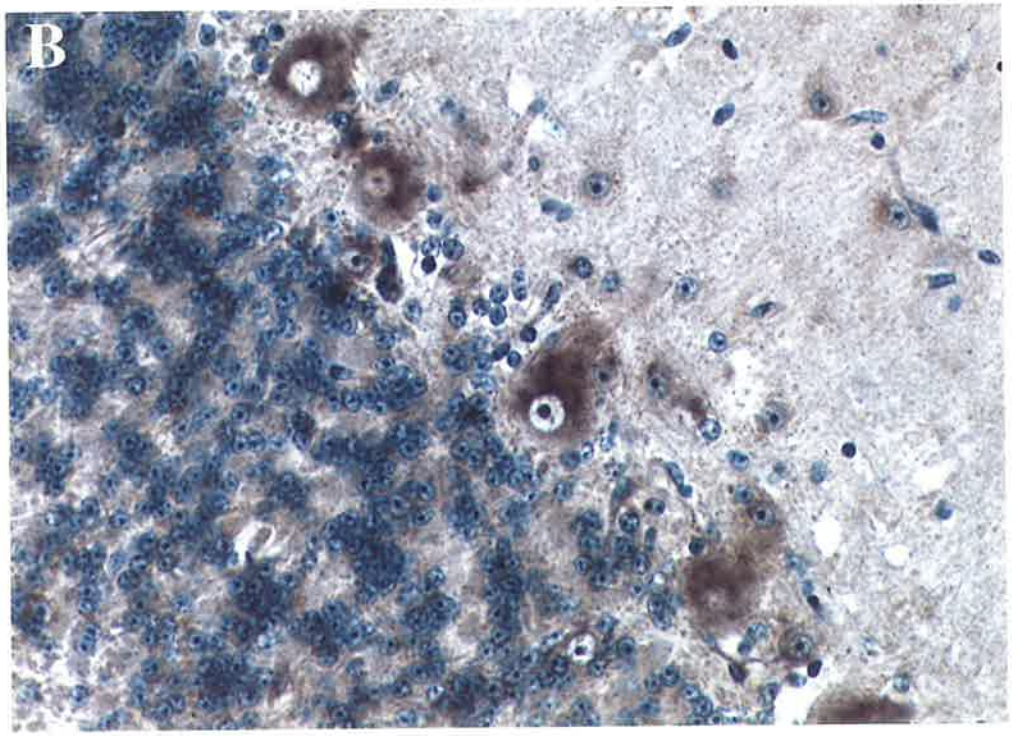
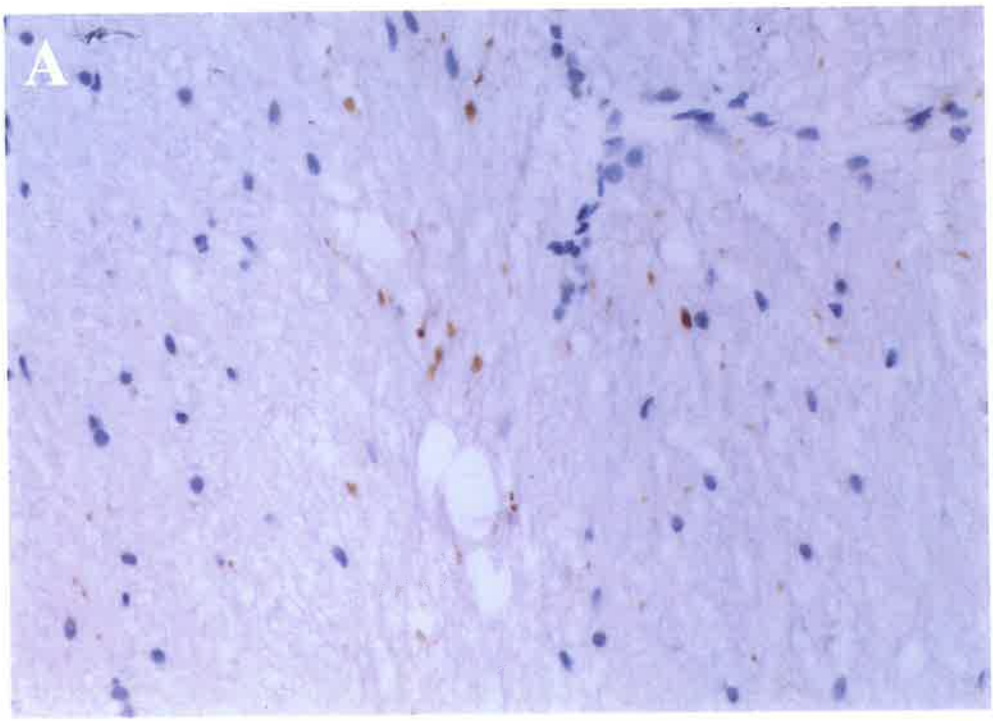

B

Figure 4.3.3: APP immunostaining of axons in injured sheep with time. typical staining *(orange/brown)* pattern seen in axons within the central grey matter 30 mins (A) and 2 hrs (B) after impact using Mab 22C11. (x400).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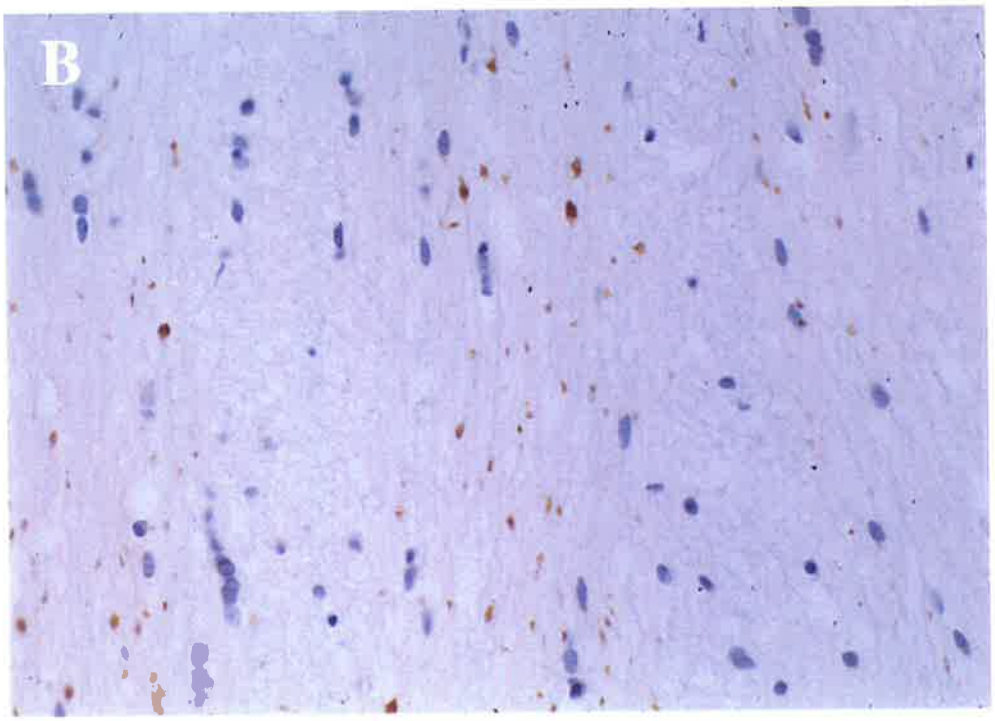

B

4.3.4- Neuronal APP mRNA expression

4.3.4.1- PV-Cap-1 probe

In all brain regions sampled, APP mRNA was not detected in non-impact control sheep (Figure 4.3.4A and Figure 4.3.5A) or in brain tissue removed 15 mins after injury (Figure 4.3.5B). Weak hybridization was detected in the cytoplasm and nuclei of NCBs in the impacted hemisphere, central grey matter and left cerebellum (Figure 4.3.5C) 30 mins after impact, with hybridization in these areas being more intense and widely distributed by 45 mins (Figure 4.3.5D). At this time, there was also weak but detectable hybridization in the contralateral hemisphere and brainstem (Table 4.3.3).

By 1 and 2 hrs after impact, staining for APP mRNA was intense and confluent (Figure 4.3.4B, Figure 4.3.5E and Figure 4.3.5F) with hybridization appearing to be greater in the impacted hemisphere. Intense hybridization was also seen in cerebellar Purkinje cells (Figure 4.3.2B) distant from the impact site, similar to APP immunoreactivity (Figure 4.3.2A). At these times, hybridization was also detected in some astrocytes scattered throughout the brain.

Hybridization was not detected in necrotic NCBs (4.3.6A) within contusions but viable neurons at the periphery of contusions showed strong hybridization for APP mRNA (4.3.6B). APP mRNA upregulation was demonstrated in injured sheep brains that did not show any macroscopic evidence of damage. In all sheep sections examined, APP mRNA expression was not detected in axons.

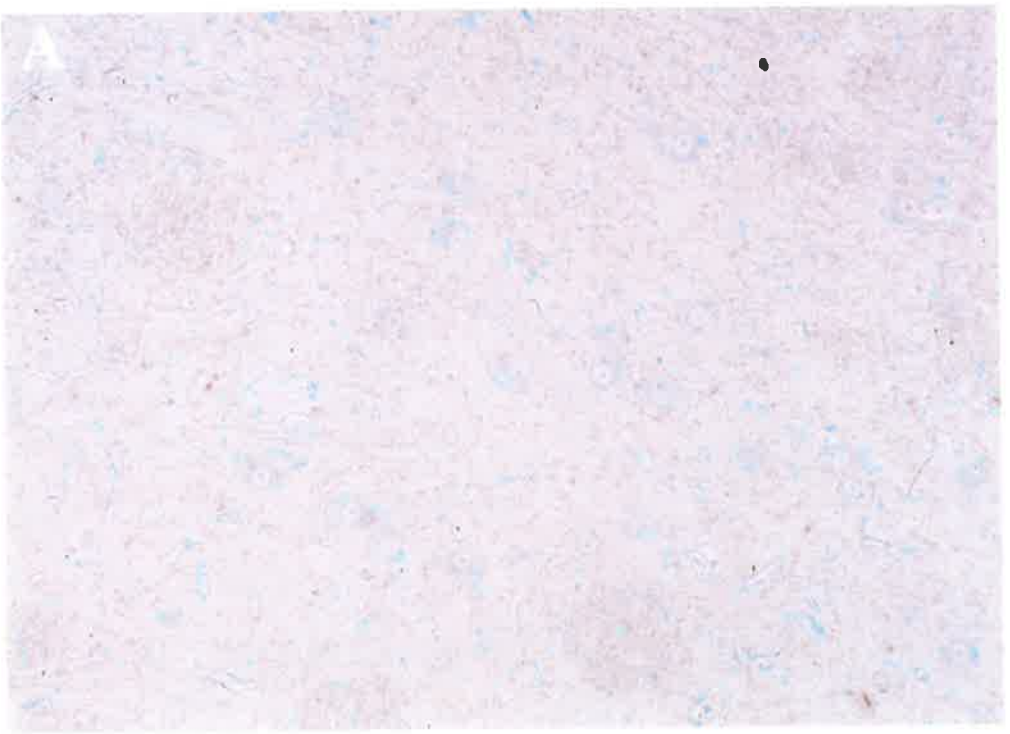
Brain Region	Central Grey Matter	Left Hemisphere	Right Hemisphere	Cerebellum	Brainstem
Survival Time					
Controls	-	-	-	-	-
15 mins	-	-	-	-	-
30 mins	+	+	-	+	-
45 mins	++	++	-	++	+
60 mins	+++	++	+	+++	++
120 mins	+++	+++	++	+++	+++

Table 4.3.3: Summary of subjective in-situ hybridization assessment of APP mRNA using PV-Cap-1 riboprobe over time in non-impact controls and injured sheep.
(-, non-detectable levels of APP mRNA; +, pale, but detectable staining for APP mRNA within select neurons; ++, darker staining in many neurons; +++, very intense, homogeneous staining.)

4.3.4.2- APP-KPI probe

The APP-KPI probe was unable to detect APP-KPI mRNA within NCBs or glia indicating that the level of transcription of these isoforms is below the limits of detection system used in this study.

Figure 4.3.4: Detection of APP mRNA in central grey matter using PV-Cap-1. APP mRNA was undetectable in neuronal cell bodies of control (non-impacted) sheep (A). In contrast intense and widespread staining *(brown/black)* for APP mRNA was demonstrated in neuronal cell bodies 1 hr after injury (B). (x200).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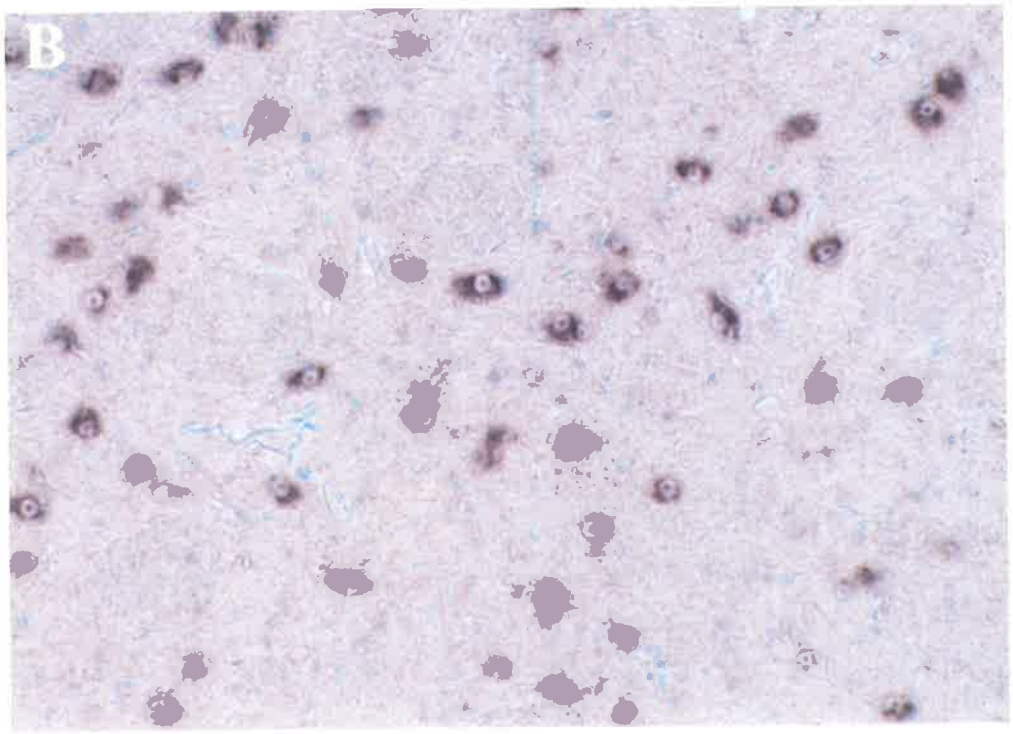
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Figure 4.3.5: Changes in APP mRNA expression in neuronal cell bodies over time using PV-Cap-1. (A) typical neuronal cell body from an uninjured sheep with undetectable hybridization. (B) APP mRNA was also undetectable in impacted sheep 15 mins after injury. (C) 30 mins after impact, staining *(brown/black)* for APP mRNA was weak, but detectable, progressively increasing in intensity and distribution 45 mins (D), 60 mins (E) and 120 mins (F) after impact. (A), (C)-(E) x1000; (B) and (F) x600.

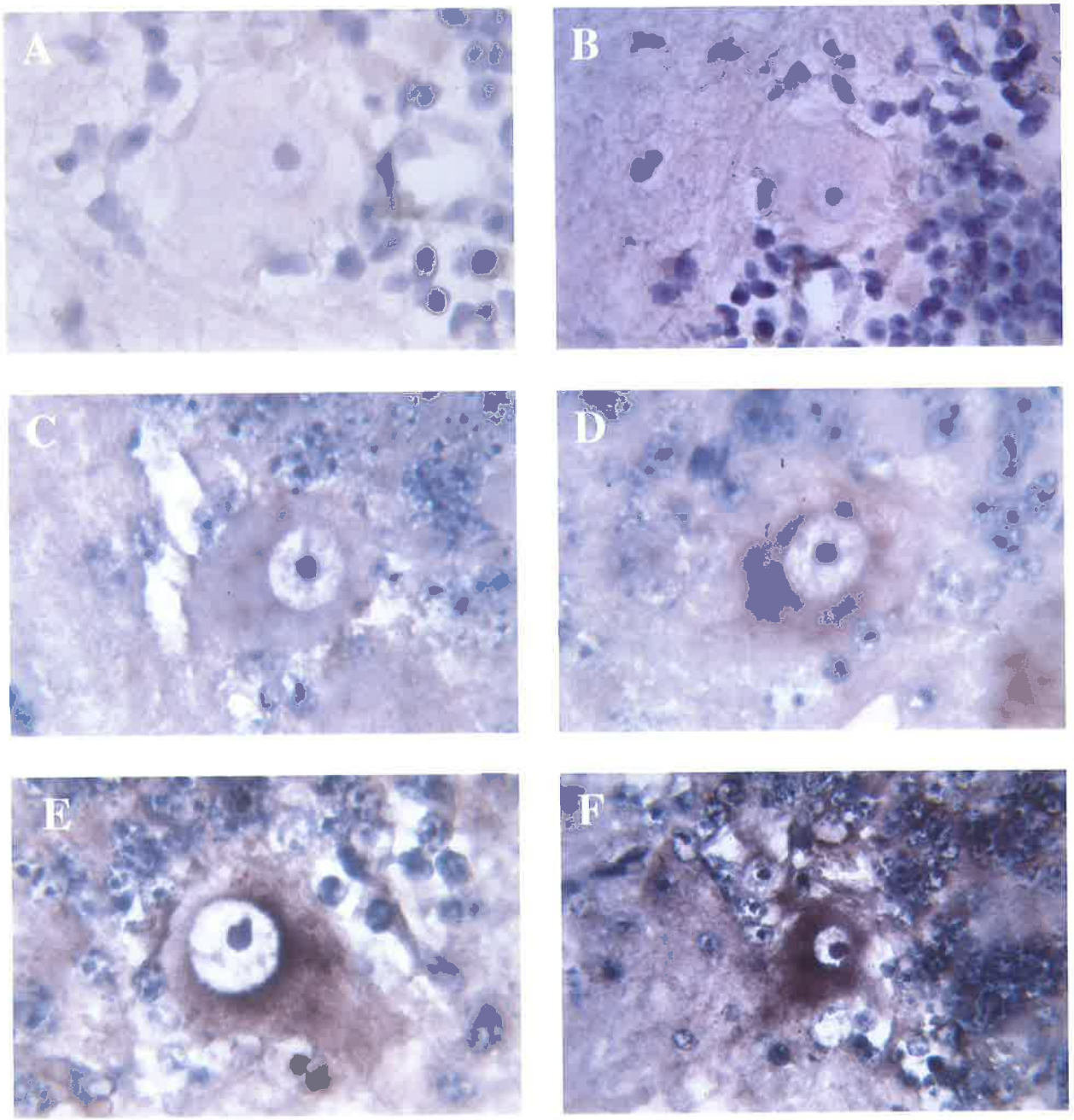
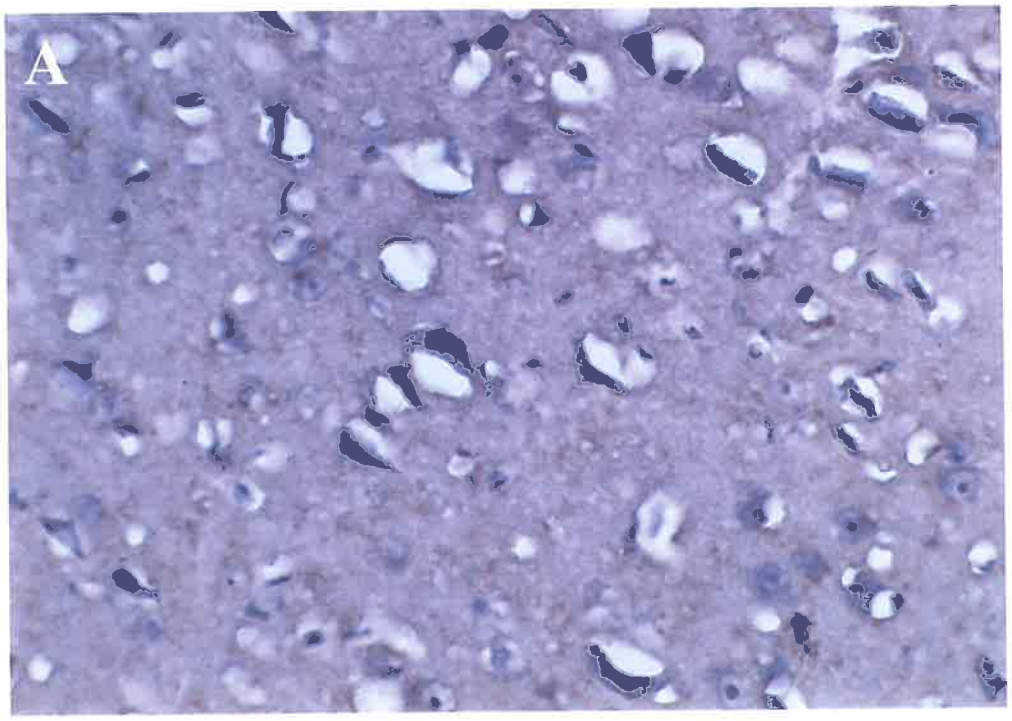

A
B
C
D
E
F

Figure 4.3.6: Detection of APP mRNA in and around contusions 2 hrs after TBI using PV-Cap-1. APP mRNA was not detectable in necrotic neurons within contusions (A) compared to intense hybridization *(brown/black staining)* in neuronal cell bodies around the periphery of contusions (B). (x400).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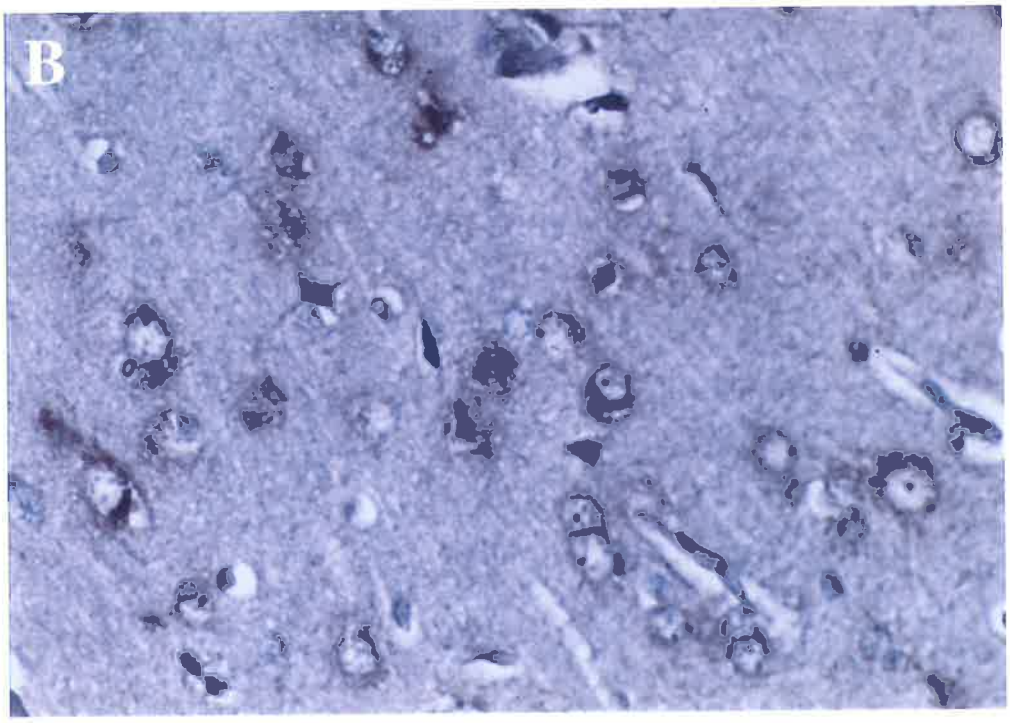
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4.4- DISCUSSION

Consistent with previous observations (see Chapter 3), widespread NCB APP expression was observed in cerebral hemispheres, brainstem and cerebellum distant to the site of focal left temporal impact in all experimental animals. APP immunoreactivity within NCBs was detected 30 mins after impact and the percentage of grids containing APP immunoreactive NCBs progressively increased with time (up to 57.5% of grids at 120 *mins*). Further, the distribution of APP immunoreactivity in NCBs was significantly greater than that in axons as shown in the previous chapter.

The significance of APP upregulation in NCBs in response to trauma is not known. It has been suggested that induction of APP 751/770 in the brain is a normal "acute phase" response to neuronal stress (Gentleman *et al.*, 1993a; Roberts *et al.*, 1991, 1994; Ciallella *et al.,* 1994) similar to the induction of heat shock proteins (Lowe and Mayer, 1990). Early increase of NCB APP may have neuroprotective functions (Mattson *et al.*, 1993; Goodman and Mattson, 1994; Smith-Swintosky *et al.*, 1994; Dewji *et al.*, 1995). However, in the studies described here, it is not known whether APP expression increases early to perform some neuroprotective function. A possibility is that APP upregulation has no specific role in TBI as alterations in APP expression could result from a number of factors common to local or diffuse areas of tissue damage such as oxidative stress and local inflammatory cytokines (Ciallella *et al.*, 1994).

It is known that APP expression is influenced by many genes and proteins including immediate early genes (Raghupathi *et al.*, 1995; Salbaum *et al.*, 1988), interleukin-1 (Goldgaber *et al.*, 1989; Donnelly *et al.*, 1990; Griffin *et al.*, 1998; Grilli *et al.*, 1996; Yang *et al.*, 1998; Panegyres and Hughes, 1998) and heat shock proteins (Abe *et al.*,

1991a; Ciallella *et al.*, 1994; Dewji *et al.*, 1995; Dewji and Do, 1996), which are acutely upregulated following TBI. All of these factors are capable of increasing APP mRNA transcription in vitro by binding to consensus sequences of the APP promoter (Trejo *et al.*, 1994). We hypothesized that increased APP immunoreactivity within NCBs following TBI is due to upregulation of APP mRNA possibly as an adaptive response to TBI and accordingly periodically examined the expression of APP mRNA between 15 mins and 2 hrs after injury.

In this study, detectable levels of hybridization were evident as early as 30 mins post impact, increasing in intensity and distribution by 1 and 2 hrs, consistent with upregulation of APP mRNA. This is the first time that in-situ detection of APP mRNA expression as early as 30 mins post-impact in vivo has been reported.

Scott *et al* (1991) assessed the affect of sciatic nerve axotomy on APP mRNA in the dorsal root ganglia, using a human APP cDNA probe which hybridized to all known isoforms of APP and a APP-KPI probe specific for KPI isoforms of APP. They showed a 3 to 4 fold increase in all APP mRNA at 4 days following axotomy. Similarly, in a rat cerebral ischaemia model, Abe *et al* (1991b), using northern blot analysis with specific APP probes for different APP isoforms showed induction of APP mRNA encoding for the KPI domain in cerebral cortex from day 1 to a maximum at 4 days. In contrast, in studies described here, KPI isoforms of APP (APP-751 and APP-770) were undetectable by immunocytochemistry and ISH in controls and impacted animals. However, Mab 22C11 and the PV-Cap-1 APP riboprobe were able to detect upregulation of antigen and mRNA respectively in response to injury, suggesting that APP-695 is the predominant isoform affected by TBI.

The striking correlation between the distribution of APP immunoreactive NCBs and those expressing APP mRNA indicates that increased APP mRNA transcription is the predominant cause of increased APP immunoreactivity. However, these data do not indicate that APP mRNA transcription is the sole cause of increased APP immunoreactivity within the NCBs following TBI, with accumulation of APP, caused by disruption of normal axoplasmic transport and/or alteration in the availability of APP epitopes, remaining as possible contributors to increased detectability of APP.

Finally, ISH is not only subjective but its sensitivity does not allow detection of low-level transcription. Northern blot hybridization has been shown to be able to detect mRNA not detectable by ISH (Pereira *et al.*, 1994). Further, availability now of software to analyze northern blot band intensities, allows accurate quantitation of differences between mRNA samples from different individuals, and would be a powerful tool for assessing APP mRNA transcription in different parts of the brain, and determining whether severity of injury affects expression.

CHAPTER 5

Quantitation of APP mRNA

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5.1- INTRODUCTION

Results described in previous chapters using in situ hybridization (ISH) and APP immunocytochemistry have shown that there is a rapid increase in APP antigen and mRNA within neuronal cell bodies (NCBs) following trauma in an ovine head impact model. In order to quantitate this APP mRNA response northern blot analysis was undertaken.

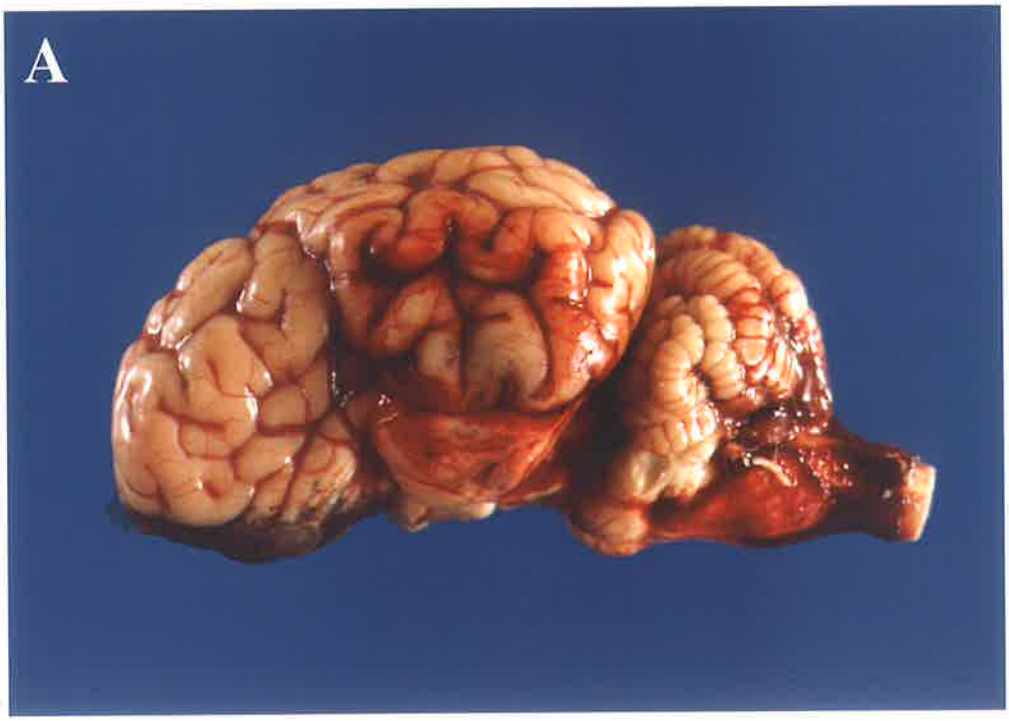
Northern blot analysis has been used in the past to study the normal distribution of APP and its isoforms in brain tissue (Kang *et al.*, 1987; LeBlanc *et al.*, 1991; Konig *et al.*, 1992; Sprecher *et al.*, 1993) and to study the APP mRNA response in Alzheimer's disease (AD) (Clark *et al.*, 1989). It has also been used to assess the APP mRNA response to physiological stimuli in vitro including heat shock (Abe *et al.*, 1991a; Ciallella *et al.*, 1994) interleukin-1 (il-1) (Forloni *et al.*, 1992) and cAMP (Bourbonniere *et al.*, 1997; Shekarbi *et al.*, 1997). No analogous in vivo studies have assessed APP mRNA expression in response to traumatic brain injury (TBI). There have only been 2 previous studies in rats which have quantitated the APP mRNA response to different traumatic insults (Abe *et al.*, 1991b; Scott *et al.*, 1991). As described in Chapter 4 most experimental TBI studies, which have observed alterations in APP after trauma, have primarily focused on APP antigen using APP immunocytochemistry as a marker of injury. Northern analysis provides a means of accurately assessing APP mRNA expression in response to injury. The aim of this study was to quantify APP mRNA levels over time following TBI using non-impacted animals for comparison.

5.2- MATERIALS AND METHODS

5.2.1- Study group

Fourteen sheep, comprising 8 impacted and 6 non-impacted animals underwent the same experimental procedures as described in chapter 2 (section 2.2). However, after 1 (4 sheep) and 2 hrs (4 sheep) the sheep were not perfusion fixed but were sacrificed with a lethal dose of sodium pentobarbitone (Virbac, NSW, Australia) (325mg/ml at 0.5ml/kg concentration) and their brains were removed and immediately sliced into 5 mm coronal sections and snap frozen in dry ice and stored at -70°C until required for RNA extraction and northern blot analysis. Two control animals survived for 1 hr and a further 4 control animals survived for 2 hrs (Figure 5.2.1 shows an illustration of a fresh brain specimen from an impacted sheep).

Figure 5.2.1: An example of an impacted, non-perfusion fixed sheep brain for snap freezing for northern analysis is shown in photo (A). Recent subarachnoid haemorrhage is present over the left hemisphere extending basally to involve the cerebellum and brainstem. Higher magnification of a coronal 5 mm section of the left temporal lobe of the same case is seen in (B), demonstrating a focal impact contusion.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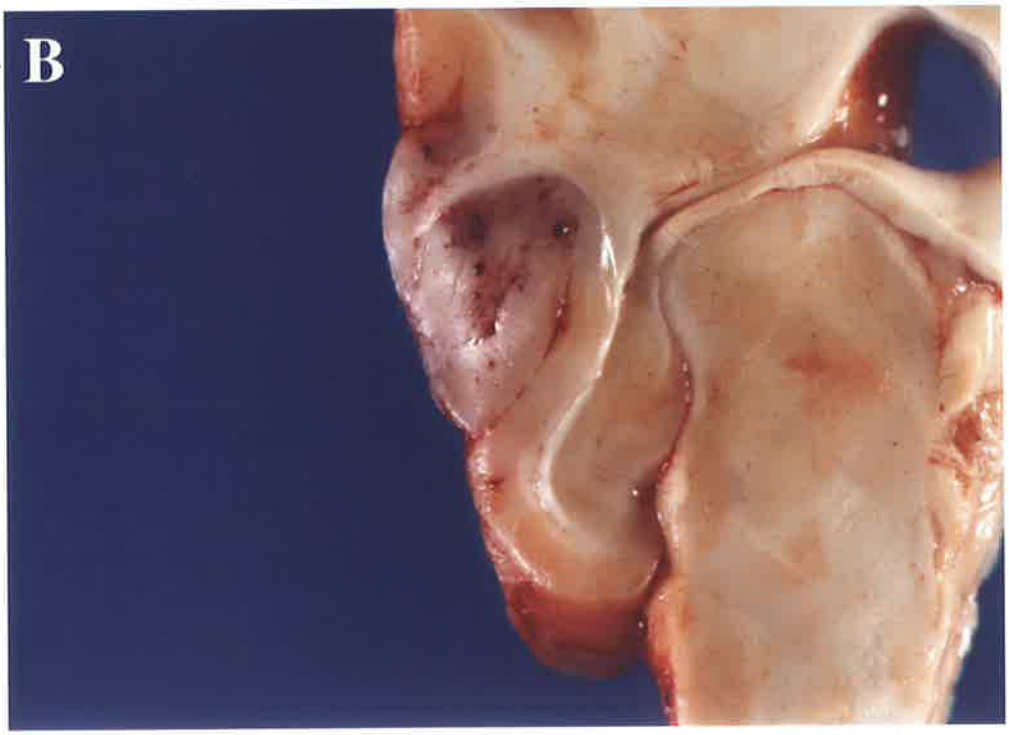

B

5.2.2- Northern blot hybridization

5.2.2.1- RNA extraction from tissue

To minimize the risk of RNA degradation, all glassware and materials were heat sterilized and treated with DEPC water. Nuclease free filtered pipette tips (ART, Molecular Bio-Products) were used throughout.

RNA was extracted from 5 cubic mm of frozen brain tissue using a modification of the single step acid guanidine isothiocyanate method (Chomczynski and Sacci, 1987). Briefly, tissue was homogenized in guanidine acetate buffer (GAB), a half volume of cold 100% ethanol was added and the homogenate was incubated for 16 hrs at -20°C. Samples were microcentrifuged at 12000 ×g for 20 mins, the supernatant discarded and the pellet resuspended by vigorous homogenization in approximately 500 µl Urea-SDS buffer. Samples were extracted twice by phenol/chloroform (1:1) followed by the addition of a 1/10 volume of 3M sodium acetate (pH 5.5) and 3 volumes of cold 100% ethanol. RNA was precipitated for 16 hrs at -20°C, pelleted at 12000 ×g for 20 mins and washed 3 times with 70% ethanol and once in 100% ethanol and resuspended in CSL water (50 µl). The amount and purity of total RNA was assessed by spectrophotometry. Samples were stored at -70°C until required for northern blotting.

5.2.2.2- Electrophoresis and northern blot transfer

RNA samples (10µg) were denatured by incubation at 70°C for 5 mins in 50% formamide, 2M formaldehyde and 1xMOPS buffer and quenched on ice. Following the addition of a 1/10 volume of RNA loading buffer, samples were loaded into a 1% agarose gel containing 2.2M formaldehyde and electrophoresed for 4 hrs at 70 volts in 1xMOPS

buffer containing 2.2M formaldehyde. A RNA marker sample (Promega, Madison USA) was included in all gels. The gel was stained with ethidium bromide (10 mg/ml), photographed, washed in 20 x SSC and transferred to Hybond–N membrane (Amersham International) by standard northern blotting (Sambrook *et al.*, 1989). After transfer, RNA was UV cross-linked in an Ultra-Lum UV cross-linker at 0.45 J/cm^2, rinsed briefly in 2 x SSC (to remove excess salt), air dried and stored at 4°C between sheets of Whatman 3MM chromatography paper until required.

5.2.2.3- Preparation of probes for northern blot hybridization

(a) Probe construction of PV-Cap-1-Bam HI and GAPDH

The probes used in this work were as follows: (i) an 800 bp human APP cDNA probe (PV-Cap-1-Bam HI) from 3' coding and non-coding sequences (nucleotides 30-830 of the APP 695 cDNA; numbering according to Kang *et al.*, 1987) (Figure 5.2.2) and (ii) a cDNA probe for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Tso *et al.*, 1985), a cellular housekeeping gene. Inserts were excised from plasmids using restriction endonucleases and separated from vector sequences in 1% agarose gels. The DNA was purified from gel fragments using a BRESAclean® kit (Bresatec) and quantified by gel electrophoresis against known DNA size markers (*Eco*RI digested phage SPPI DNA, Bresatec, Australia).

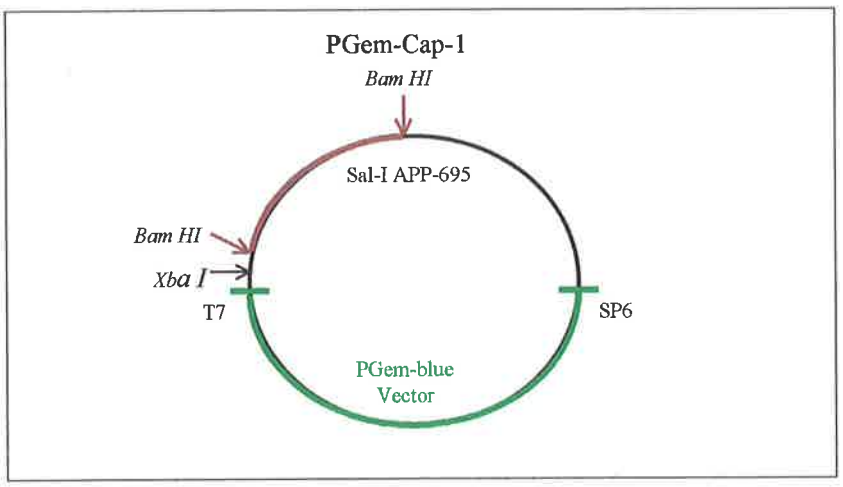


Figure 5.2.2: Diagram showing a representation of the PGem-Cap-1 plasmid where a 2450 bp *Sal-I* APP-695 fragment was cloned into PGem-blue by Sonny Bou (Calgary). The *Bam HI* restriction sites are shown which were used to linearize a Bam HI insert of APP (*red*) used to make the PV-Cap-1-Bam HI probe.

The APP-KPI probe which was described in section 4.2.3.4 (b) for ISH was also used in northern analyses.

(b) Probe labelling

Each probe was labelled with α ^{32}P-ATP according to manufacturers recommendations using a Gigaprime DNA labelling kit (Bresatec, Australia). Unincorporated nucleotides were removed from labelled probe using a G-25 sephadex spin column. Monitoring of probe using a radioactive counter (Beckman LS6800 scintillation counter) before and after column treatment provided an estimate of incorporation efficiency. Where optimal amounts of template were available, incorporation ranged from 60-85% and total activity of probes ranged from $5x10^7$ - 10^8 counts per minute (cpm).

(c) Calculation of Tm_{50}

For northern blot hybridization experiments the Tm_{50} for double-stranded DNA/RNA hybrids is defined as the average of the Tm_{50} values for RNA/RNA and DNA/DNA hybrids:

$Tm_{50}(RNA/DNA) = [Tm_{50}(RNA/RNA) + Tm_{50}(DNA/DNA)]/2$ (Sambrook *et al.*, 1989)

5.2.2.4- Prehybridization and hybridization

All hybridization was done in glass tubes (Coatasil, Ajax Chemicals) using a rolling hybridization incubator (Robbins Scientific). Filters were prehybridized for 4 hrs at 42°C (Tm_{50} -30°C) in 20 ml of prehybridization solution and hybridized overnight at 42°C in 20 ml of hybridization solution containing approximately 5 x 10^7 cpm ^{32}P-labelled denatured probe.

5.2.2.5- Washing and detection of bound probe

All washes were performed using pre-warmed solutions in a rolling hybridization incubator (Robbins Scientific). Filters were initially washed twice for 30 mins at 42°C in 2 x SSC, 0.1% SDS. They were then washed (2 x 30 mins) at high stringency in 0.1 x SSC, 0.1% SDS at 52°C (Tm_{50}-25°C). Damp filters were wrapped in plastic wrap (Vitafilm, Goodyear) and placed in phosphor screens at room temperature for 8 hrs. Phosphor screens were processed using a 400 series PhosphorImager (Molecular Dynamics, Sunnyvale, Ca.), and the relative amount of RNA in bands was determined using ImageQuant software (v 3.3, Molecular Dynamics, Sunnyvale, Ca). In all experiments, probe was stripped off filters and filters were reprobed for GAPDH to allow normalization of APP mRNA ImageQuant values.

5.2.2.6- Stripping nitrocellulose filters

To facilitate reprobing, filters were stripped of bound probe using the following method: A solution containing 0.05 x SSC and 10 mM EDTA pH 7.5 was heated to boiling prior to the addition of SDS to a final concentration of 0.1% (w/v). Filters were immersed in the solution for 15 mins and the procedure repeated with fresh solution before being rinsed briefly in 0.01 x SSC at room temperature. Stripping was confirmed by exposing filters to phosphor screens prior to reprobing.

5.3- RESULTS

5.3.1-Neuropathological data

Of 8 impacted animals, 5 had skull fractures (1 linear and 4 depressed), 5 had temporal lobe contusions beneath the impact site, 4 had SAH in the ipsilateral hemisphere, 2 had contralateral SAH and 7 had basal SAH. No pathological lesions were seen in the non-impact control animals (Table 5.3.1).

Sheep ID	Survival time (mins.)	Skull fracture	Contusions		Subarachnoid haemorrhage		
			Right	Left	Right	Left	Basal
1	60	+	-	-	-	-	+
2	60	-	-	-	-	-	-
3	60	+	-	+	+	+	+
4	60	+	-	+	-	+	+
5	120	+	-	+	+	+	+
6	120	+	-	+	-	+	+
7	120	-	-	-	-	-	+
8	120	-	-	+	-	-	+
Control	60	-	-	-	-	-	-
Control	60	-	-	-	-	-	-
Control	120	-	-	-	-	-	-
Control	120	-	-	-	-	-	-
Control	120	-	-	-	-	-	-
Control	120	-	-	-	-	-	-

Table 5.3.1: Summary of macroscopic lesions of each of the 8 impacted and 6 non-impacted sheep. (-, Absent; +, Present).

5.3.2- Quantitation of APP mRNA in various regions of the brain of impacted sheep

In initial studies RNA was extracted from the thalamus, temporal cortex, frontal cortex, brainstem and cerebellum and analyzed by northern blot hybridization to determine the region of most abundant APP transcription. Normalized ImageQuant values showed the thalamus to contain highest APP mRNA levels, being 2.4 fold times greater than brainstem which had the least abundant APP mRNA (Figure 5.3.1). This result was reproduced in a separate experiment. The size of sheep brains and labour intensive RNA extraction and northern blot procedures meant it was not feasible to investigate all regions of the brain. Therefore, thalamus RNA was used in all subsequent northern blot experiments.

5.3.3- Quantitation of APP mRNA over time

In ISH studies, staining for APP mRNA was shown to be intense and widespread 2 hrs after injury (Chapter 4, Section 4.3.4.1). Therefore, initial attempts to quantify differences in APP mRNA expression between impacted and control animals were made using RNA extracted from brains after a 2 hr survival period. There was a 3 ± 0.1 fold (means of ImageQuant values of 4 impacted sheep versus 4 controls) greater increase in APP mRNA in the injured group compared with controls (Figure 5.3.2). (Refer Appendix C for ImageQuant values).

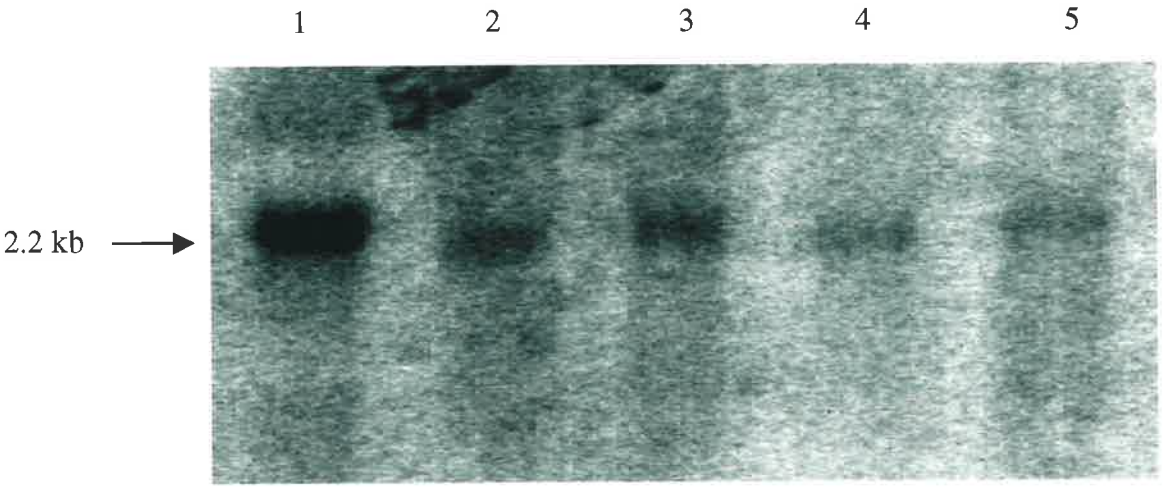
In a separate experiment, to quantify APP mRNA expression over various survival periods there was a 2.2 ± 0.1 (means of ImageQuant values of 4 impacted animals versus

2 controls) fold increase in APP mRNA in the impacted group compared with controls 1 hr after impact, increasing to 3.3 ± 0.3 (means of ImageQuant values from 4 impacted animals versus 4 control animals) fold greater expression in impacted sheep at 2 hrs (Figure 5.3.3A shows a representation of the results from 1 impacted case at each survival time and 1 control case at each survival time).

In the same experiment, tissue from only one sheep was examined at 15 mins and 30 mins after impact. Consistent with previous ISH findings, there was no significant difference in APP mRNA levels between control and impacted sheep (not shown). The PV-Cap-1-Bam HI APP probe is unable to distinguish between the different isoforms of APP. Therefore, each filter was stripped and reprobed with the APP-KPI probe to determine whether APP-KPI containing isoforms were increased in impacted animals. Consistent with ISH results (Chapter 4, Section 4.3.4.2) APP-KPI isoforms were not significantly affected 1 and 2 hrs post-injury (Figure 5.3.3B).

Figure 5.3.1: Northern blot comparing APP mRNA in different locations within the brains of 1 impacted animal 2 hrs after TBI. (1) thalamus, (2) temporal cortex, (3) frontal cortex, (4) brainstem, (5) cerebellum.

Figure 5.3.2: Comparison of APP mRNA in the thalamus of impacted sheep with control sheep 2 hrs after TBI using PV-Cap-1-Bam HI APP probe. Lanes 1-4 are impacted case numbers 5,6,7,8 and lanes 5-8 are controls. M is the RNA marker.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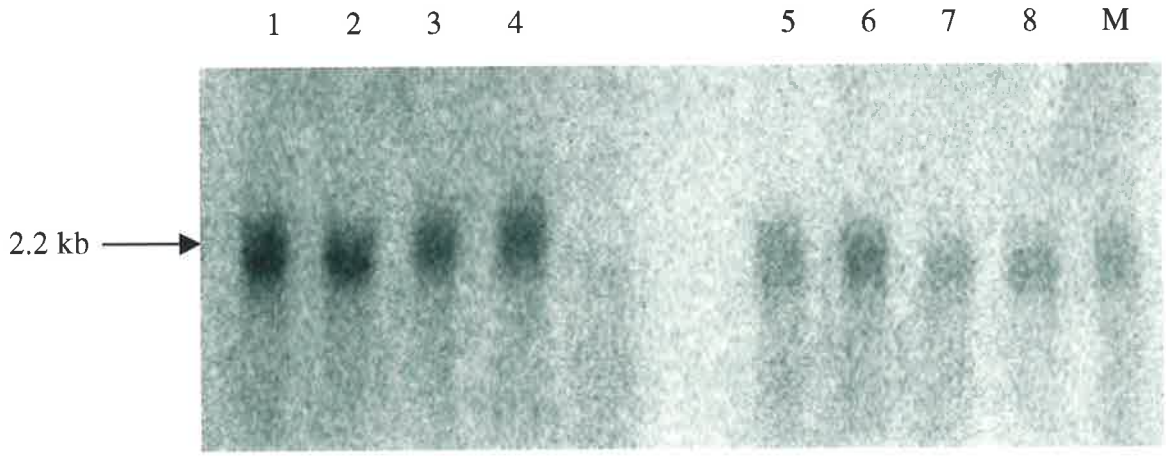
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Figure 5.3.3: Comparison of APP mRNA in the thalamus of impacted sheep with control (non-impacted) sheep over time using PV-Cap-1-Bam HI probe (A) and APP-KPI probe (B). 2 hr impacted sheep (1), 2 hr control sheep (2), 1 hr impacted sheep (3) and a 1 hr control sheep (4).

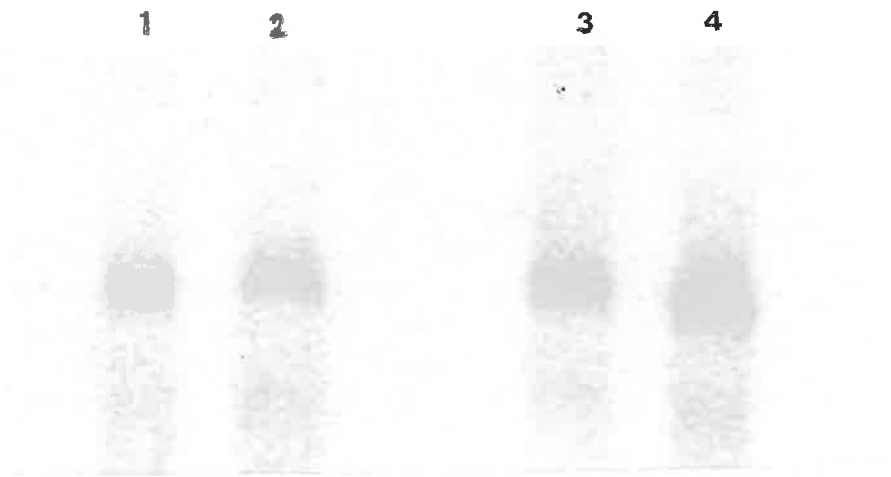
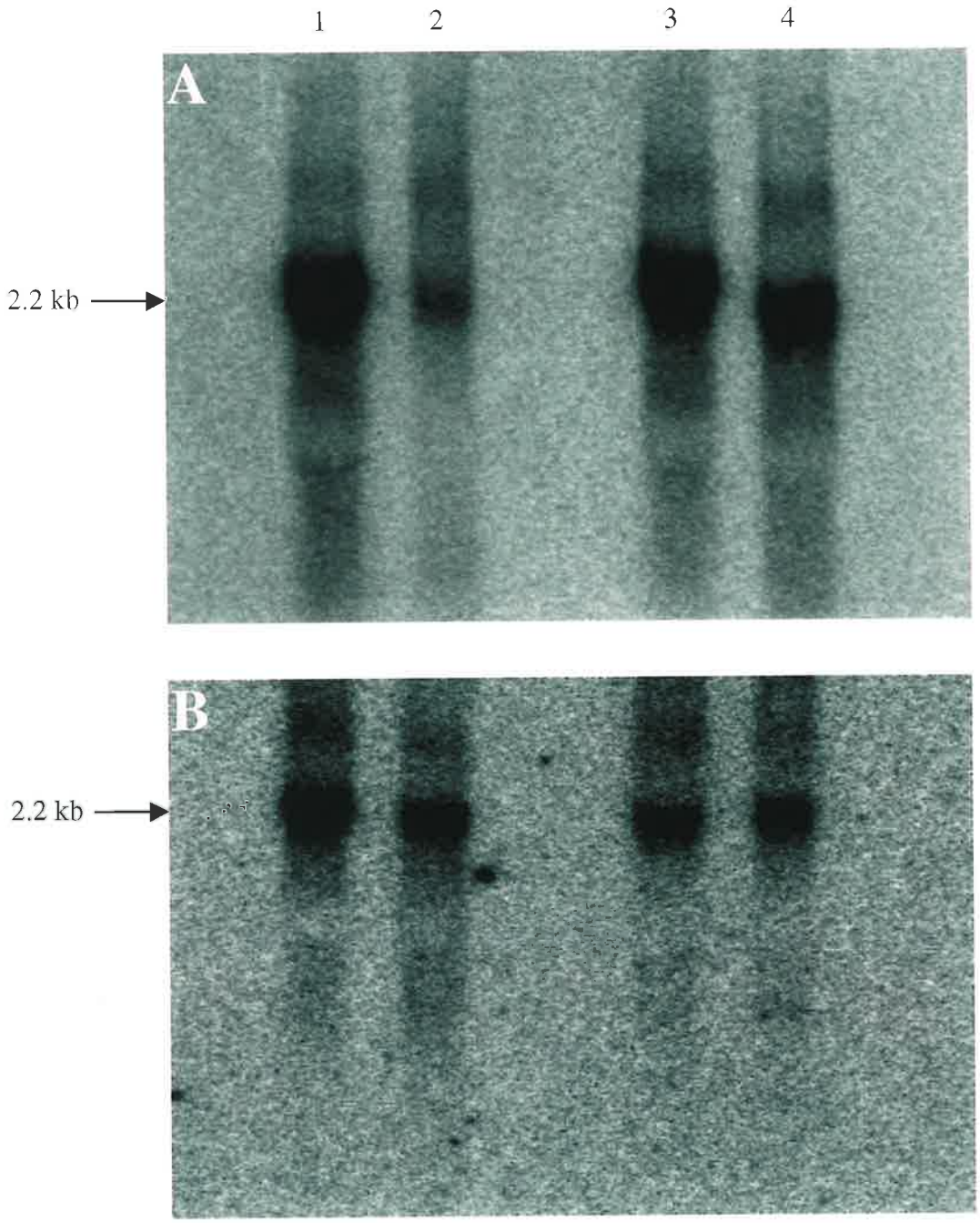


Figure 5.3.3C: Northern blot comparing GAPDH mRNA in the thalamus of impacted sheep and control sheep. 2 hr impacted sheep (1), 2 hr control sheep (2), 1 hr impacted sheep (3) and a 1 hr control sheep (4).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5.4- DISCUSSION

The results described in this chapter confirm ISH findings in Chapter 4. Consistent with ISH results it was shown that APP mRNA is upregulated within the thalamus in all impacted brains compared to controls 1 and 2 hrs after injury, the greatest difference being at 2 hrs.

Northern blot analysis has been used by others to study APP mRNA expression in response to various traumatic stimuli such as axotomy (Scott *et al.,* 1991) and ischaemia (Abe *et al.*, 1991b). In these studies, APP mRNA increases were only examined at 1 day (Abe *et al.*, 1991b). Results described here are consistent with previous observations, however, additionally, this is the first time a rapid rise (as early as 1 hr) in APP mRNA after injury has been shown.

In contrast to studies of Scott *et al.*, (1991) and Abe *et al.*, (1991b) which demonstrated upregulation of APP-KPI isoforms, we were unable to show early alterations in APP-KPI isoforms following injury, the APP-695 isoform being predominantly affected following trauma in this model of TBI.

Further, due to the high sequence homology between APLPs and APP (Slunt *et al.*, 1994), it is possible that the probe used in these studies is also detecting upregulation of APLPs.

A novel finding was the reproducibility of detectability of the amount of APP mRNA

upregulation (refer Appendix C for ImageQuant data) suggesting that transcription of APP mRNA is an " all or none" phenomenon provided the cell is not immediately killed by the insult. It seems that any insult significant to stress the nerve cell but is not severe enough to cause cell death is capable of increasing the synthesis of APP mRNA.

Our results demonstrate that northern blot analysis is a sensitive means of quantifying differences in mRNA levels in response to trauma and would therefore be a useful tool to examine possible changes of APP mRNA as a result of neuroprotective therapeutic interventions.

CHAPTER 6

Upregulation of neuronal APP and APP mRNA following magnesium sulphate ($MgSO_4$) therapy in TBI

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6.1- INTRODUCTION

The study described in this chapter focuses on changes in APP following magnesium sulphate ($MgSO_4$) therapy in the ovine head impact model.

Several experimental studies have reported a decline of magnesium (Mg^{2+}) in the brain following traumatic brain injury (TBI) (McIntosh *et al.*, 1988, 1989; Vink *et al.*, 1987, 1988, 1996; Feldman *et al.*, 1996; Heath and Vink, 1996, 1998a, 1998b) resulting in exacerbation of neurological dysfunction including impaired motor function (McIntosh *et al.*, 1988; Vink *et al.*, 1988, 1996; Heath and Vink, 1996, 1998a, 1998b). Post-traumatic administration of Mg^{2+} salts including $MgSO_4$ and magnesium chloride ($MgCl_2$) have been shown to have neuroprotective effects and improve post-traumatic outcome in a variety of experimental models of TBI (McIntosh *et al.*, 1989; Smith *et al.*, 1993a; Okiyama *et al.*, 1995; Feldman *et al.*, 1996; Heath and Vink, 1997,1998a, 1999a, 1999b), cerebral ischaemia (Izumi *et al.*, 1991), spinal cord ischaemia (Vacanti and Ames, 1984) and hypoxia (Hoffman *et al.*, 1994).

As described previously, neuroprotection may be one of the many functions of APP (Mattson *et al.*, 1993; Goodman and Mattson, 1994; Smith-Swintosky *et al.*, 1994; Dewji *et al.*, 1995) and there is evidence of increased neuronal APP immuno-expression following injury in various other experimental models of TBI. We have shown that there is widespread diffuse neuronal cell body (NCB) expression of APP involving the whole brain after a focal head impact and that this is likely due to increased transcription of neuronal APP mRNA as early as 30 mins after impact. It was also shown in the previous

chapter that northern blot analysis provides a quantitative measure of the APP mRNA response to TBI and it was hypothesized that this would be useful in assessing the effect of putative therapeutic interventions (such as $MgSO_4$) on APP mRNA expression. This hypothesis was addressed by topographically assessing and quantitating the effects of $MgSO_4$ therapy on APP antigen and APP mRNA response 2 hrs after a controlled head impact.

6.2- MATERIALS AND METHODS

6.2.1- Study group

Thirty six 2-year-old Merino ewes were used in this study. All of the animals were anaesthetized, ventilated and maintained using the same protocol described in chapter 2.2.1. Each of the animals used in this study survived for 2 hrs as our previous studies had shown that there was prominent APP mRNA transcription at this time.

Eighteen animals were impacted and a further 18 were used as non-impact controls. The brains from 24 of these animals (12 impacted and 12 controls) were removed and snap frozen in dry ice for northern blot analysis and the brains from the remaining 12 animals (6 impacted and 6 controls) were perfusion fixed for immunocytochemistry and in-situ hybridization (ISH).

6.2.2- Experimental protocol

All animals underwent the same experimental protocol as described in detail in chapter 2, section 2.2. However, therapy in the form of intravenous administration $MgSO_4$ (Magnesate, Parnell Laboratories, NSW, Australia) into the jugular vein at a concentration of 12 mg/kg was given to 9 impacted animals (6 from frozen group and 3 from perfusion fixed group) and 9 control animals (6 from frozen group and 3 from perfusion fixed group). The dosage and methods of administration were similar to that described in the literature by Heath and Vink, (1997, 1998a, 1999a, 1999b).

$MgSO_4$ was administered 30 mins post-impact in the injured group and at the same time after induction of anaesthesia in the control group.

APP immunocytochemistry was undertaken on the perfusion fixed brains as described in chapter 3, section 3.2.2.2, using Mab 22C11. Semi-quantitation of the NCB and axonal response was also undertaken using the methods described in chapter 3, section 3.2.2.3.

ISH was undertaken on serial sections of perfusion fixed brain tissue from the left and right central grey matter, left and right cerebral hemispheres, cerebellum and brainstem, using previously described methods in chapter 4, section 4.2.3. Only the anti-sense probe PV-Cap-1 and the sense probe PGem-Cap-1 were used.

Northern blot hybridization was undertaken on the frozen brain tissue using the same methods described in chapter 5, section 5.2.2 and only PV-Cap-1-Bam HI and GAPDH probes were used. As previously explained, RNA was only extracted from the thalamus due to abundant transcription of APP mRNA in this region.

6.3- RESULTS

6.3.1- Neuropathological data

6.3.1.1- Frozen brains

Of the 6 non-$MgSO_4$ treated impacted cases, 4 had depressed skull fractures. One sheep had a temporal impact contusion beneath the impact site, basal SAH was found in all impacted animals and 1 sheep also had bilateral SAH. Of the 6 $MgSO_4$ treated impacted cases, 2 had depressed skull fractures and 4 had temporal impact contusions beneath the impact site. One of the latter also had a contrecoup contusion. All animals had basal SAH, 3 had ipsilateral SAH and 2 of these also had contralateral haemorrhage. All frozen control (non-impacted) animals had no pathological changes. (Table 6.3.1)

6.3.1.2- Perfusion fixed brains

Two of the non-$MgSO_4$ treated impacted cases had depressed skull fractures, 1 had a contrecoup contusion, 1 had bilateral SAH and 2 had SAH extending over the base of the brain. All $MgSO_4$ treated impacted cases (3) had depressed skull fractures, 2 animals had bilateral SAH extending basally and 1 also had coup and contrecoup contusions. The control animals for this group had no pathological changes. (Table 6.3.1).

Sheep ID	Survival time (mins)	Skull fracture	Contusions		Subarachnoid haemorrhage		
			Right	Left	Right	Left	Basal
FROZEN CASES							
Impact without $MgSO_4$							
1	120	+	-	+	+	+	+
2	120	-	-	-	-	-	+
3	120	-	-	-	-	-	+
4	120	+	-	-	-	-	+
5	120	+	-	-	-	-	+
6	120	+	-	-	-	-	+
Impact with $MgSO_4$							
7	120	-	-	+	+	+	+
8	120	+	+	+	+	+	+
9	120	+	-	+	-	+	+
10	120	-	-	-	-	-	+
11	120	-	-	+	-	-	+
12	120	-	-	-	-	-	+
Controls without $MgSO_4$							
13-18	120	-	-	-	-	-	-
Controls with $MgSO_4$							
19-24	120	-	-	-	-	-	-
PERFUSION FIXED CASES							
Impacts without $MgSO_4$							
25	120	+	-	-	-	-	-
26	120	+	+	-	+	+	+
27	120	-	-	-	-	-	+
Impacts with $MgSO_4$							
28	120	+	-	-	-	-	-
29	120	+	-	-	+	+	+
30	120	+	+	+	+	+	+
Controls without $MgSO_4$							
31-33	120	-	-	-	-	-	-
Controls with $MgSO_4$							
34-36	120	-	-	-	-	-	-

Table 6.3.1: Summary of macroscopic lesions in 24 frozen brain cases with and without $MgSO_4$ treatment and 12 perfusion fixed cases with and without $MgSO_4$ therapy.
(-, Absent; +, Present).

6.3.2- Effect of $MgSO_4$ on APP antigen expression

In $MgSO_4$ treated impacted animals the percentage brain area with APP positive neuronal cell bodies (NCBs) ranged from 67 to 74% (mean 71%) compared to 53 to 57.5% (mean 56%) in untreated animals, suggesting an increase in response to $MgSO_4$ treatment. All impacted cases regardless of $MgSO_4$ treatment had diffuse NCB APP immunoreactivity, not exclusive to the left temporal impacted hemisphere but also apparent in sites as far from the impact site as the frontal lobes, cerebellum and brainstem (Table 6.3.2).

Consistent with previous findings, non-impacted sheep had significantly less percentage brain area with APP positive NCBs than both of the impacted groups ($P<0.01$) (Figure 6.3.1). However, in $MgSO_4$ treated non-impacted (control) animals the percentage brain area with APP positive NCBs ranged from 26 to 29% (mean 27.5), compared with non-$MgSO_4$ treated controls ranging from 4 to 7% (mean 5.5%), again reflecting an increase in response to $MgSO_4$ (as was shown in impacted cases). Non-impacted sheep with $MgSO_4$ therapy showed scattered positive NCBs in both of the cerebral hemispheres, cerebellum and brainstem, but in these regions the percentage brain area with APP positive NCBs was significantly less than that in impacted animals (Table 6.3.2).

Complementary to the results shown in chapters 3 and 4, APP positive axons were detected in all impacted brains, but were of lower magnitude to NCBs (Figure 6.3.1). There was no significant difference between the percentage brain area with APP positive axons in the $MgSO_4$ treated impacted group compared to the untreated impacted group ($P>0.01$). Non-impact controls had no detectable axonal APP positivity.

		Total APP immunostaining		Distribution of neuronal cell body APP			
Sheep ID	**Survival Time (mins)**	**Neuronal cell bodies**	**Axons**	**Cerebral hemispheres**		**Cerebellum**	**Brainstem**
				Right	**Left**		
		(%) grids	**(%) grids**	**(%) grids**	**(%) grids**	**(%) grids**	**(%) grids**
Impact without $MgSO_4$							
25	120	57.5	7	60	48	73	53
26	120	57	4	63	60	74	61
27	120	53	4	56	55.6	44	48
Impact with $MgSO_4$							
28	120	74	6.3	75	75	80	71
29	120	72	13	70	64	80	68
30	120	67	24	55	63	89	96
Controls without $MgSO_4$							
31	120	7	0	8	5	0.5	0
32	120	6	0	8	4	0.5	8
33	120	4	0	5	4.4	2	1
Controls with $MgSO_4$							
34	120	26	0	36	27	7	7
35	120	28	0	27	32	13	9
36	120	29	0	38	34	7	8

Table 6.3.2: Summary of microscopic pathological data from 12 perfusion fixed sheep. APP immunocytochemical results showing the distribution of APP positive neuronal cell bodies and APP positive axons in impacted and non-impacted animals with and without $MgSO_4$ therapy.

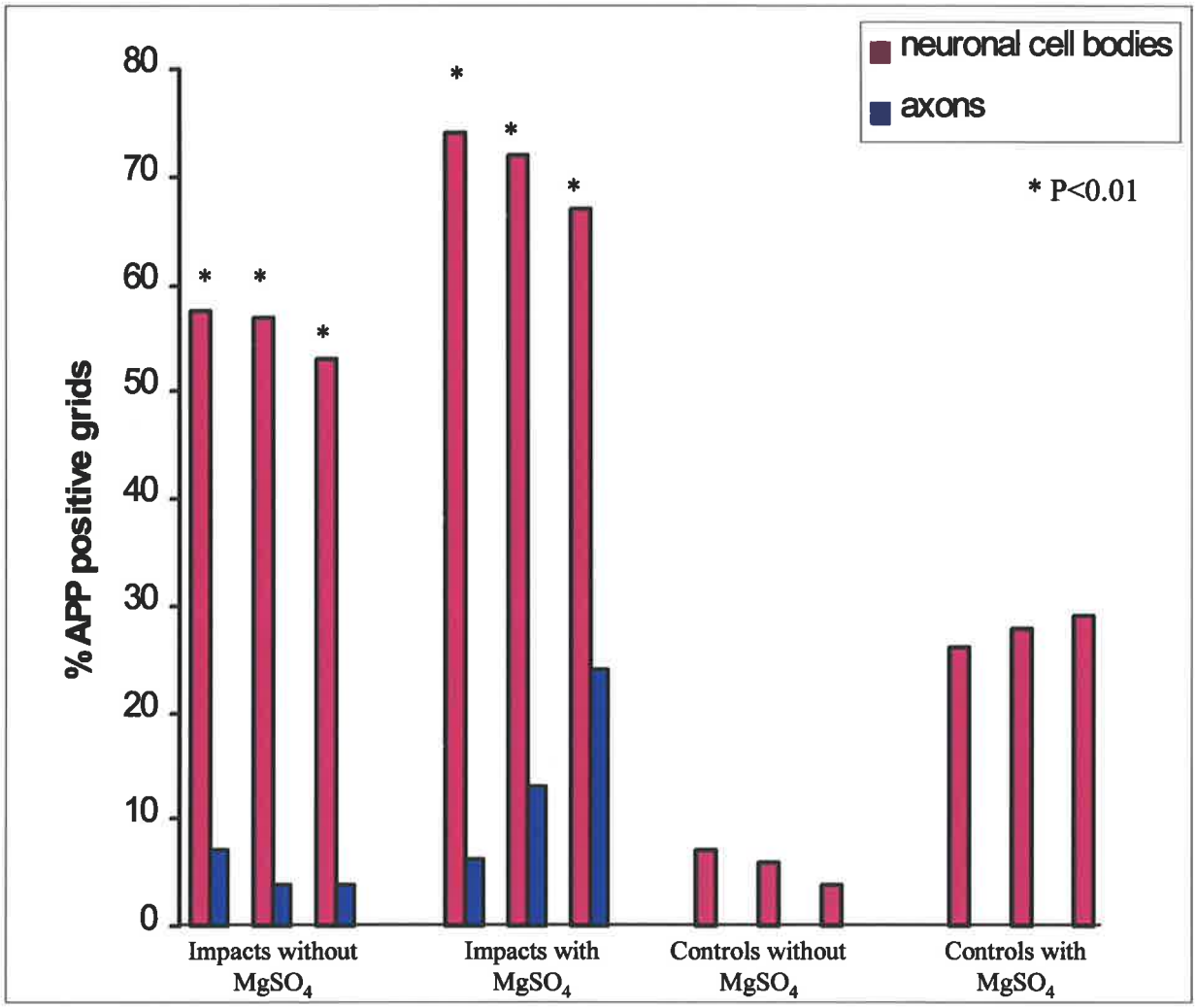


Figure 6.3.1: Histogram showing the percentage brain area with APP positive neuronal cell bodies and axons in impacted animals with and without $MgSO_4$ administration and control animals with and without $MgSO_4$.

6.3.3- Qualitative assessment of APP mRNA expression by ISH

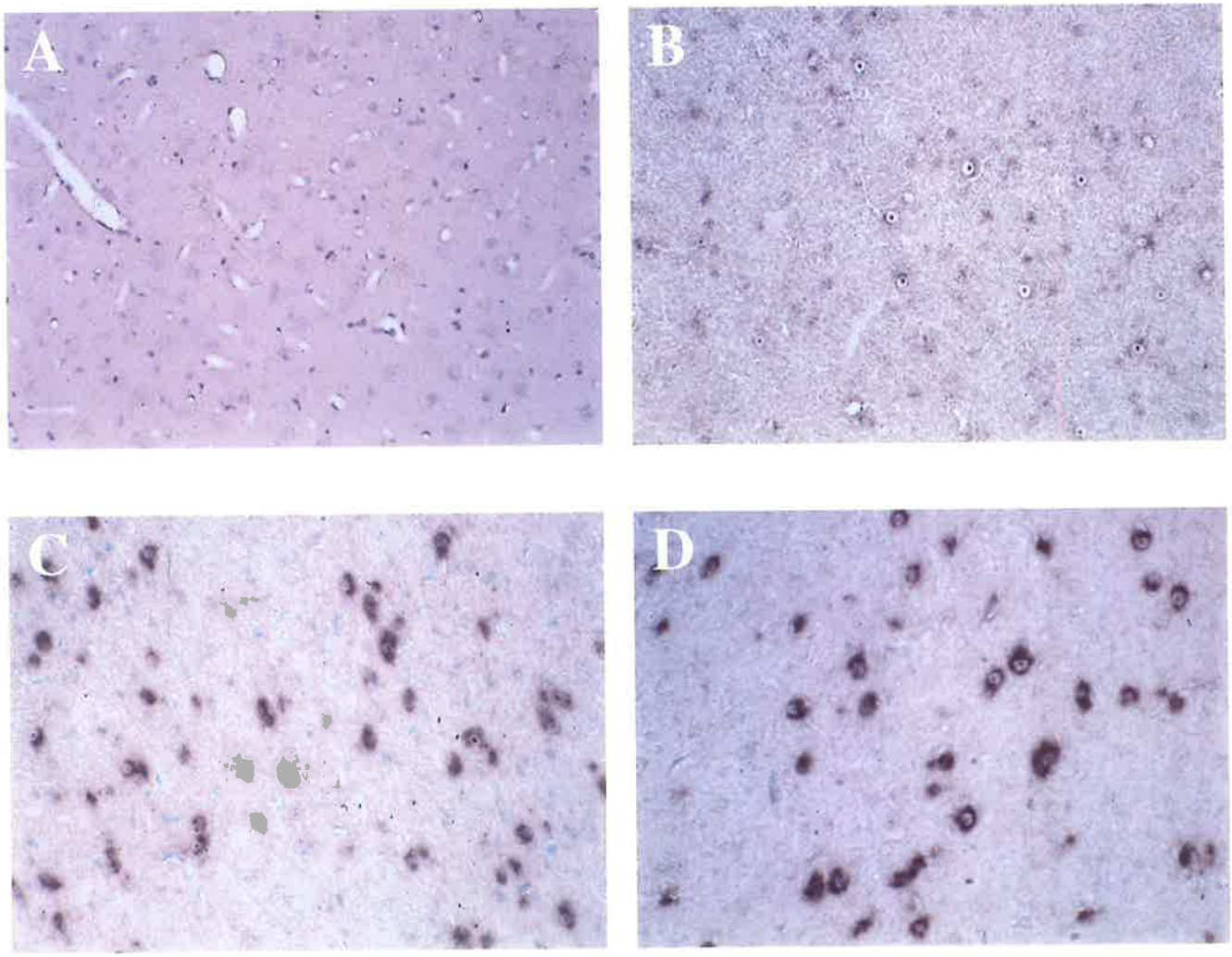
Left and right frontal, temporal lobes and central grey matter, cerebellum and brainstem showed strong staining for APP mRNA in impacted sheep regardless of $MgSO_4$ treatment (Figures 6.3.2C and 6.3.2D). This upregulation was evident in a similar pattern as previously described in Chapter 4 with increased APP mRNA expression seen in NCBs throughout the impacted and contralateral hemispheres, cerebellum and brainstem.

In the untreated control group APP mRNA was undetectable in all regions sampled (Figure 6.3.2A). However, suprisingly, in the $MgSO_4$ treated control (non-impacted) group, APP mRNA was detectable in some NCBs scattered throughout the cerebral hemispheres (6.3.2B) but not, in contrast to the pattern seen in impacted brains, in cerebellar Purkinje cells or in NCBs within the brainstem (Refer Table 6.3.3 below for summary of ISH results).

Brain Region	**Central Grey Matter**	**Left Hemisphere**	**Right Hemisphere**	**Cerebellum**	**Brainstem**
Study group					
Controls without $MgSO_4$	-	-	-	-	-
Controls with $MgSO_4$.	+	+	+	-	-
Impacts without $MgSO_4$	+++	+++	++	+++	++
Impacts with $MgSO_4$	+++	+++	++	+++	++

Table 6.3.3: Summary of in-situ hybridization assessment of APP mRNA within all regions sampled in controls with and without $MgSO_4$ therapy and impacted animals with and without $MgSO_4$ therapy. (-, non-detectable levels of APP mRNA; +, weak, but detectable levels of APP mRNA within select neurons; ++, stronger hybridization occurring in many neurons; +++, very intense, homogeneous hybridization.)

Figure 6.3.2: Effects of $MgSO_4$ treatment on the expression of APP mRNA in the thalamus of impacted (2 hr) and control sheep by in-situ hybridization. APP mRNA was undetectable in controls without $MgSO_4$ treatment (A). Weak *(pale brown/black staining)* but detectable levels of APP mRNA were evident in neuronal cell bodies in controls with treatment (B). Intense and homogeneous APP mRNA hybridization *(brown/black staining)* was evident within the neuronal cell bodies in impacted animals without treatment (C) and with treatment (D). (x200).

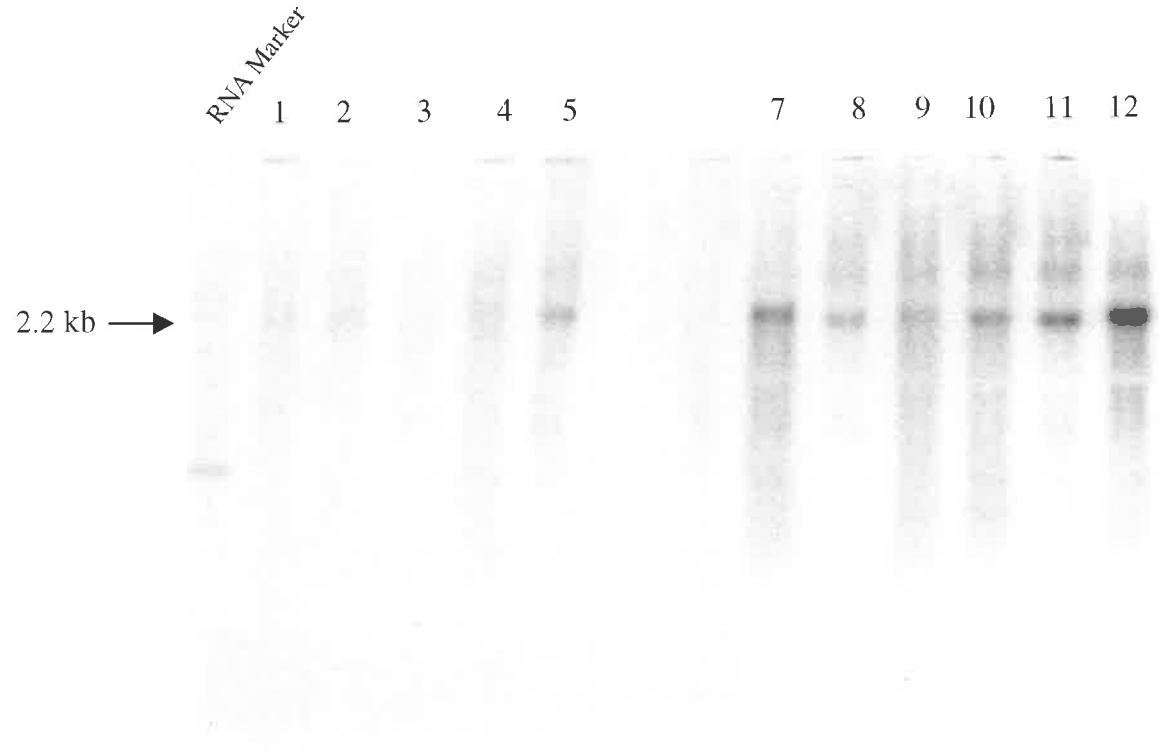

A
B
C
D

6.3.4- Quantitative analysis of the APP mRNA following $MgSO_4$ therapy by northern blot hybridization

Quantitation of APP mRNA in the thalamus of all impacted animals with and without $MgSO_4$ therapy revealed a 2.3 ± 0.2 fold (mean of ImageQuant values from 6 animals in each group) increase in APP mRNA levels in $MgSO_4$ treated impacted animals compared to untreated impacted animals (Figure 6.3.3). An unexpected finding was that APP mRNA expression in $MgSO_4$ treated non-impacted animals was 1.6 ± 0.1 (mean of normalized ImageQuant values from 6 animals in each group) times more than that in untreated control animals (Figure 6.3.4). Comparison of $MgSO_4$ treated groups showed a 2.4 ± 0.3 fold greater APP mRNA expression in impacted than control sheep, which is not significantly different to a comparison of untreated impacted animals with controls (Refer Appendix C for ImageQuant data).

Figure 6.3.3: Northern blot hybridization using PV-Cap-1-Bam HI to quantitate the expression of APP mRNA in the thalamus of impacted sheep (2 hr survival) with (6-11) and without (1-5) $MgSO_4$ treatment.

Figure 6.3.4: Northern blot hybridization using PV-Cap-1-Bam HI to quantitate the expression of APP mRNA in the thalamus of control sheep (2 hr survival) with (sheep no 19-24) and without (sheep no 13-17) $MgSO_4$ treatment.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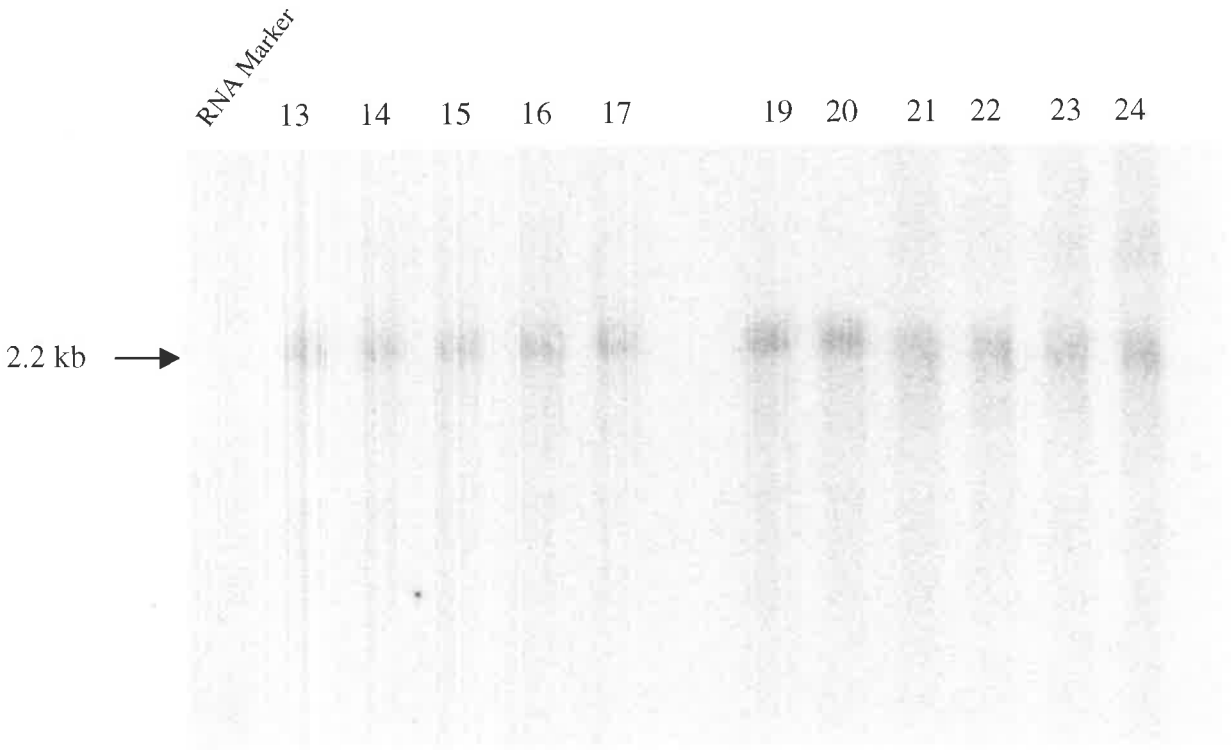
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6.4- DISCUSSION

This study demonstrates for the first time a link between $MgSO_4$ therapy and APP expression. It was shown that in non-injured nerve cells, Mg^{2+} causes upregulation of APP mRNA which further increases in response to TBI. In biological systems Mg^{2+} is a ubiquitous cation which is vital in maintaining and regulating general cellular metabolism and function. Mg^{2+} is essential for normal cell functions such as cellular respiration (Ebel and Gunther, 1980), membrane integrity, transcription of mRNA, protein synthesis (Terasaki and Rubin, 1985), glucose and energy metabolism (Grubbs and Maguire, 1987) and glycolysis (Garfinkel and Garfinkel, 1985) (for review see Ebel and Gunther, 1980). However, the"gating effect" of Mg^{2+} within neurotransmitter-linked ionic channels blocking Ca^{2+} entry into the cell is thought to be its principal mode of neuroprotection (Clark and Roman, 1980; Mayer *et al.,* 1984; Nowak *et al.*, 1984; McIntosh *et al.*, 1989; Zhang *et al.,* 1996). Thus, decreased Mg^{2+} may be deleterious to many or all of these cell functions and bioenergetic processes and contribute to secondary neural injury.

Post traumatic administration of Mg^{2+} salts improves outcome in a variety of experimental models of TBI, ischaemia and neurotoxic convulsive disorders, summarized in the following tables.

Table 6.4.1: Experimental studies assessing the effects of Mg^{2+} on neurological outcome.

Authors	Experimental protocol	Treatment	Results and conclusions
TBI studies			
McIntosh *et al.*, 1989	Male Sprague Dawley rats n = 60. Fluid percussion injury	Intravenous $MgCl_2$ admin. of either a low dose (12.5 μmol/kg) a high dose (125 μmol/kg) 30 mins post-injury.	Small improvement in neurological function 24 hrs after a small dose of $MgCl_2$, but a significant improvement after a high dose. By 1 week both groups had significant improvement.
McDonald *et al.*, 1990	Male Sprague Dawley rats. Stereotaxic injections of NMDA (25 nmol)	Intravenous $MgCl_2$ admin.of a single dose of 2mmol/kg or 4 mmol/kg 15 mins after injury or 6 doses of 1.8 mmol/kg over 6 hrs.	$MgCl_2$ reduced NMDA mediated brain injury.
Smith *et al.*, 1993a	Male Sprague Dawley rats. Fluid percussion injury	Intravenous admin. of 12.5 μmol/kg of $MgCl_2$ 15 mins after injury.	At 42 hrs post-injury there was an increase in memory retention and cognitive function
Heath and Vink, 1998a	Male Sprague Dawley rats. Weight drop	Intramuscular admin. of a single dose of 100μmol/kg $MgSO_4$ or 100 μmol/kg $MgCl_2$, 30 mins post-injury.	1 week after TBI there was an improvement in post-traumatic outcome. $MgSO_4$ may act more rapidly than $MgCl_2$.
Heath and Vink, 1999	Male Sprague Dawley rats. Weight drop	Intramuscular admin of 750 μmol/kg of $MgSO_4$ 30 mins, 8 hrs, 12 hrs or 24 hrs post-injury.	Early treatment with $MgSO_4$ Resulted in a significant improvement in post-traumatic outcome. The critical time for $MgSO_4$ treatment was up to 24 hrs after TBI.
Ischaemia			
Vacanti and Ames, 1984	New Zealand white rabbits n= 51. Spinal cord ischaemia	Intravenous admin. of 5 mmol/kg $MgCl_2$ before the insult and then continual infusion of 2.5 mmol/kg for 30-60 mins after the insult.	$MgCl_2$ therapy combined with hypothermia improved neurological outcome and increased survival 3 fold.
Neurotoxic convulsive disorders			
Richards, 1982	Merino sheep, n=10 Lethal dose of corynetoxin	Intramuscular admin. of 200mg/kg or 600mg/kg of $MgSO_4$ or 200mg/kg intravenous $MgSO_4$at the onset of convulsions.	$MgSO_4$ administration inhibited convulsions for up to 12 hrs.
May and Stewart, 1998	Merino sheep, n=160 Injection of tunicamycin.	Intraperitoneal/ intravenous admin. of 200mg/kg $MgSO_4$ when convulsions commenced	$MgSO_4$ administration increased survival rates possibly by neuromuscular blockade.

Table 6.4.2: Experimental studies assessing the effects of Mg^{2+} treatment on brain tissue.

Authors	Experimental protocol	Treatment	Results and conclusions
TBI studies			
Okiyama *et al.*, 1995	Male Sprague Dawley rats n= 33 Fluid percussion injury	Constant infusion of $MgCl_2$ (300μmol/kg) beginning 15 mins post-injury.	Following removal and weighing of the brains $MgCl_2$ attenuated brain oedema in the left hippocampus.
Feldman *et al.*, 1996	Male Sprague Dawley rats n= 85. Weight drop technique	Subcutaneous admin. of $MgSO_4$ (600mg/kg) 1 hr after injury.	Following removal and weighing of the brains, $MgSO_4$ attenuated brain oedema formation and improved neurological outcome.
Hypoxia/ischaemia			
Izumi *et al.*, 1991	Male Fischer rats, n=61 Left middle cerebral artery occlusion.	Two intraperitoneal injections of $MgCl_2$ 1mmol/kg, 60 mins post-ischaemia.	$MgCl_2$ reduced cortical damage induc by MCA occlusion.
Hoffman *et al.*, 1994	Newborn piglets Cerebral hypoxia	Initial intravenous admin. of 600mg/kg $MgSO_4$ and continuous infusion of 300mg/kg over a 60 min period following hypoxia.	Assessed brain tissue concentrations of Na^+, K^+ and ATPase function which provided an index of brain cell membrane function. $MgSO^4$ preserved NMDA receptor binding properties and maintained cellular homeostasis.
Neurotoxic convulsive disorders			
Wolf *et al.*, 1991	Male Wistar rats Intracerebroventricular injections of kainate causing convulsions	One intravenous admin. of 600mg/kg $MgSO_4$ 1 hr after injection.	Assessment of the lesioned hippocampus showed that $MgSO_4$ protected hippocampal neurons from damage. There was a time and dose dependent protection. $MgSO_4$ decreased convulsions.

Experimental cell culture and in vivo studies have shown that APP transcription is influenced by many proteins and genes which bind to or indirectly affect the APP promoter thus causing increased APP production. These include interleukin-1 (Goldgaber *et al.*, 1989; Donnelly *et al.*, 1990; Forloni *et al.*, 1992; Griffin *et al.*, 1998; Panegyres and Hughes, 1998; Yang *et al.*, 1998), immediate early genes (Raghupathi *et al.*, 1995), cyclic AMP (Bourbonniere *et al.*, 1997; Lee *et al.*, 1997) and heat shock proteins (Ciallella *et al.*, 1994; Dewji *et al.*, 1995; Dewji and Do, 1996).

Many of these genes and proteins which affect APP transcription are known to be acutely upregulated following stress and injury and therefore may stimulate APP mRNA

transcription. APP contains a heat shock element in its promoter sequence and, because its mRNA and protein increase with heat shock, can be classified as a protective heat shock protein (HSP) (Dewji *et al.*, 1995). APP-695 and APP-751 protect hippocampal neurons against excitotoxic, metabolic and oxidative insults, including glucose deprivation-induced injury and glutamate toxicity both in cell culture (Mattson *et al.*, 1993; Goodman and Mattson, 1994) and in vivo (Smith-Swintosky *et al.*, 1994).

As described in detail in chapter 1, the structure of APP resembles cell surface receptors (Kang *et al.*, 1987; Nishimoto *et al.*, 1993; Okamoto *et al.*, 1995). APP also plays a role in synaptic function (Schubert *et al.*, 1991) and formation (Moya *et al.*, 1994; Roch *et al.*, 1994; Morimoto *et al.*, 1998) and as a novel signalling system in neuronal plasticity and survival (Tominaga *et al.*, 1997). The secreted form of APP (sAPP) is localized primarily in the axon and cell body in caveolae-like vesicles suggesting that it may serve as a transmembrane signalling molecule for glycosyl phosphatidylinositol (GPI)-linked glycoproteins (Bouillot *et al.*, 1996). sAPP also modulates NMDA receptor activity in cultured embryonic rat hippocampal neurons implying a role in regulating developmental and synaptic plasticity (Furukawa and Mattson, 1998). The highest levels of APP are found in the second postnatal week in rats indicating a possible function in cell differentiation and synaptic contacts (Loffler and Huber, 1992) and in transgenic mice lacking APP there is evidence that it may play a role in neural plasticity (Zheng *et al.*, 1995).

Since Mg^{2+} may non-specifically affect protein synthesis rates (Terasaki and Rubin, 1985) it may be that APP is just one of many proteins upregulated.

Our studies did not examine neurological function so we have no direct evidence that increased APP induced by $MgSO_4$ is neuroprotective. This remains to be determined by further studies. However, the substantial body of evidence supporting neuroprotective roles for Mg^{2+} and APP suggest that they may both play crucial parts in the complex cascade of protective cellular events which occur following TBI. Exactly how Mg^{2+} and APP interact in these processes and the specificity of this relationship requires further study.

CHAPTER 7

Upregulation of APP and its mRNA in the developing brain: an experimental model of paediatric head injury

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7.1- INTRODUCTION

Traumatic brain injury (TBI) is the major cause of death and disability in children in Western industrialized countries, with motor vehicle accidents involving pedestrians or occupants causing severe brain damage in 10-15% of all child trauma cases (Luerssen *et al.*, 1988; Ward, 1996; Fearnside *et al.*, 1997).

The neuropathology of child head injury has received scant attention (Vowles *et al.*, 1987; Graham *et al.*, 1989b). Adult and immature brains differ physiologically and chemically (Kriel *et al.*, 1989) and respond differently to brain trauma (Dobbing, 1981). The effect of trauma on the orderly development and interactions of neural elements in the immature brain is largely unknown. Experimental TBI in immature brains has been largely confined to the examination of focal damage in laboratory rodents (Grundl *et al.*, 1994; Smith *et al.*, 1995; Adelson *et al.*, 1996; Biagas *et al.*, 1996).

We have modified our adult ovine head injury model to produce a lamb head injury model in which impaction results in widespread axonal injury (AI) (Finnie *et al.*, 1999). We utilized this lamb model to study the neuronal APP response following head impact using an experimental paradigm similar to that used in the adult head impact model. We hypothesized that the neuronal cell body (NCB) and axonal APP response in the developing brain following head impact would be similar to that in the adult sheep brain.

7.2- MATERIALS AND METHODS

7.2.1- Study group

Twelve 4-5 week-old Merino lambs were used in this study. Ten lambs were impacted and 2 were non-impact controls. All animals were anaesthetized and ventilated according to the standard protocol described in Chapter 2. Animals were maintained under anaesthesia for 2 hrs after impact, as were the non-impact control lambs. However, a lesser charge (Schermer No. 11) was used to deliver the left temporal head impact as our pilot studies showed excessive brain damage with the Schermer No. 17 charge due to the thinness of the lamb skulls.

7.2.2- Detection methods used for APP and APP mRNA

APP immunocytochemisty and in-situ hybridization (ISH) were undertaken using the same methods described in Chapter 3, Section 3.2.2.2 and Chapter 4, Section 4.2.3 respectively. Mab 22C11 was used for immunocytochemical detection of APP and PV-Cap-1 was used as the anti-sense probe for ISH. Semi-quantitation of the neuronal APP response was undertaken using the methods described in Section 3.2.2.3.

ISH was performed on samples obtained from left and right frontal, temporal lobes and central grey matter, cerebellum and brainstem serial to those sections taken for APP immunocytochemistry as in our previous adult studies.

7.3- RESULTS

7.3.1- Physiological responses

All 10 impacted animals survived the designated 2 hr survival period with no hypoxic, hypotensive or hypo/hypercapnic episodes.

7.3.2- Neuropathological data

Three lambs had linear (outer table only) skull fractures and 2 had depressed fractures involving both the outer and inner tables. The remaining 6 animals had no skull fractures. Mean brain mass was 77 grams (range 64-88 grams). Eight lambs had impact contusions in the left impacted hemisphere and 2 of these also had contrecoup contusions. Subarachnoid haemorrhage (SAH) was found in all impacted animals of varying extent and severity. Five lambs had SAH in the left impacted hemisphere only, and 5 had bilateral SAH. In 9 impacted animals, there was basal SAH. The 2 control animals were macroscopically normal (Table 7.3.1).

Sheep ID	Skull fracture	Contusions		Subarachnoid haemorrhage		
		Right	Left	Right	Left	Basal
1	-	-	-	+	+	+
2	+	-	+	-	+	+
3	-	-	-	-	+	+
4	+	+	+	+	+	+
5	+	-	+	+	+	+
6	-	-	+	+	+	+
7	-	-	+	+	+	+
8	+	-	+	-	+	+
9	+	+	+	-	+	-
10	-	-	+	+	+	+
Control 1	-	-	-	-	-	-
Control 2	-	-	-	-	-	-

Table 7.3.1: Summary of macroscopic lesions of each of the 10 impacted lambs compared 2 non-impacted lambs (-, Absent; +, Present).

7.3.3- APP immunoreactivity

7.3.3.1- Neuronal cell body response

Two hrs after impact APP immunostaining of neuronal cell bodies (NCBs) was widespread in the cerebral hemispheres, cerebellum and brainstem distant to the site of focal injury in all impacted lambs. Percentage brain area with APP positive NCBs ranged from 26 to 60% (mean 40.4) (Table 7.3.2 and Figure 7.3.1). APP positive NCBs were found as distant from the impact site as the cerebellum, where 50 to 100% (mean 74%) of cerebellar grids scored positive. Similarly, in the brainstem, up to 65.5% of grids contained APP positive NCBs (range 5 to 65.5%, mean 31%).

Widespread staining of NCBs was seen in impacted brains which were otherwise histologically normal.

Statistically there was no effect of skull fracture, contusions or SAH on the NCB response ($P< 0.01$).

There was no detectable APP immunoreactivity in necrotic NCBs in the centre of contusions, but NCBs at the periphery showed positive immunostaining.

Control brains had very small numbers of weakly positive NCBs, ranging from 1.5 to 3% of grids (mean 2.25%) which was significantly less than impacted animals ($P< 0.01$). (refer Appendix B ii for calculation of Z values).

There was a 97% proportion of agreement between 2 observers following semi-quantitation of APP positive NCBs (refer Appendix B iii) and a 93% agreement between 2 counts for 1 observer (refer Appendix B iv).

7.3.3.2- Axonal immunoreactivity

APP positive axons were seen in all impacted lambs with percentage brain area ranging from 3.8 to 28% (mean 12.8%). This axonal response in the lambs is greater than the axonal response in adult sheep at the same survival time (mean 5.7%). Consistent with the results obtained in adult sheep, the percentage brain area with APP immunoreactive NCBs was consistently greater than for brain areas with APP positive axons. (Refer Table 7.3.2 and Figures 7.3.2A and 7.3.2B for photomicrographs of APP positive NCBs and axons.)

	Total APP Immunostaining		**Distribution of neuronal cell body APP**			
Sheep ID	**Neuronal cell bodies mean (%) grids**	**Axons Mean (%) grids**	**Cerebral hemispheres Right (%) grids**	**Left (%) grids**	**Cerebellum (%) grids**	**Brainstem (%) grids**
1	27	10.5	14	29	85	10
2	27	3.8	29	26	50	6
3	32	4.6	16.3	27	52	33
4	53	25.5	21.6	74	88	50
5	38	8.7	17	59	50.4	12
6	26	7	14	32	71.5	5
7	34	11.6	33.7	62	70	36.4
8	50	11.4	33.7	62	70	36.4
9	60	17	23	77	100	65.5
10	57	28	40.5	69.5	96	53.5
Control 1	1.5	0	1.5	0.5	1	0
Control 2	3	0	2.5	2	3	0

Table 7.3.2: Summary of microscopic pathological data showing the distribution of APP immunoreactive neuronal cell bodies and axons in impacted and non-impacted lambs

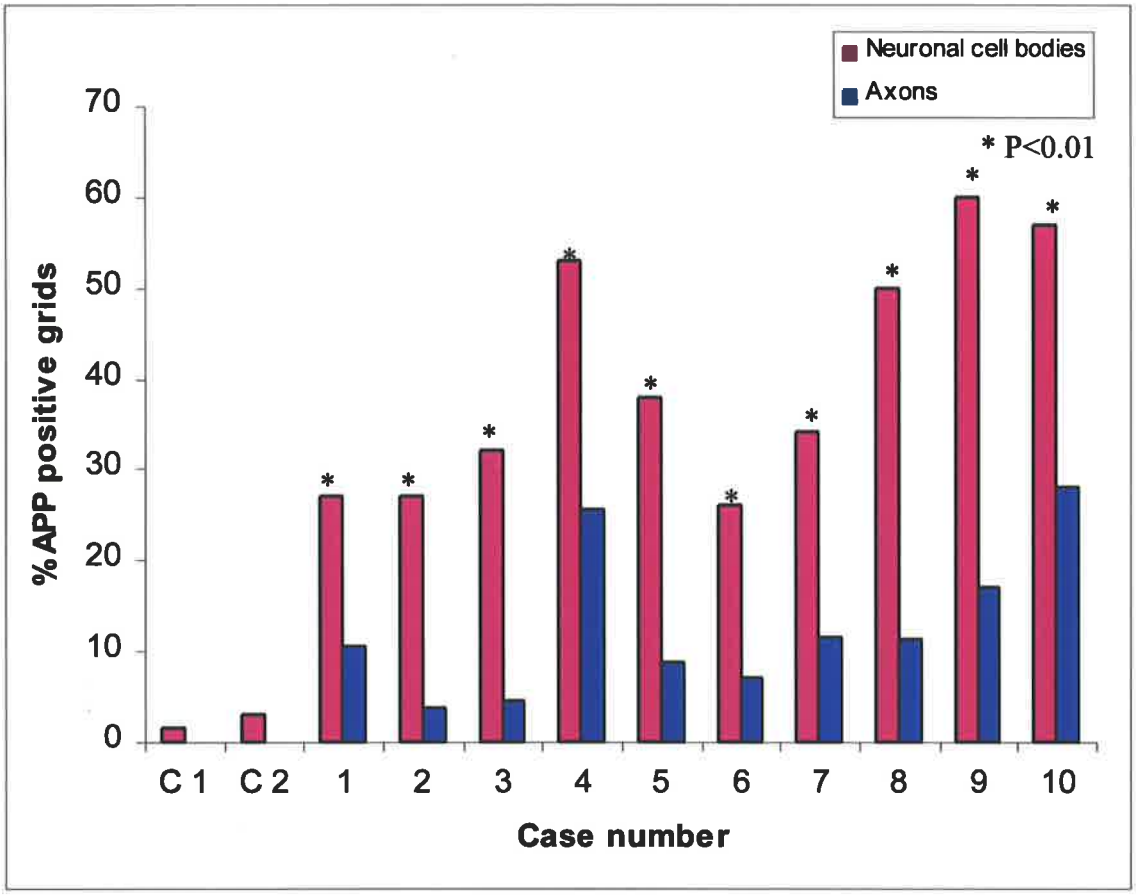
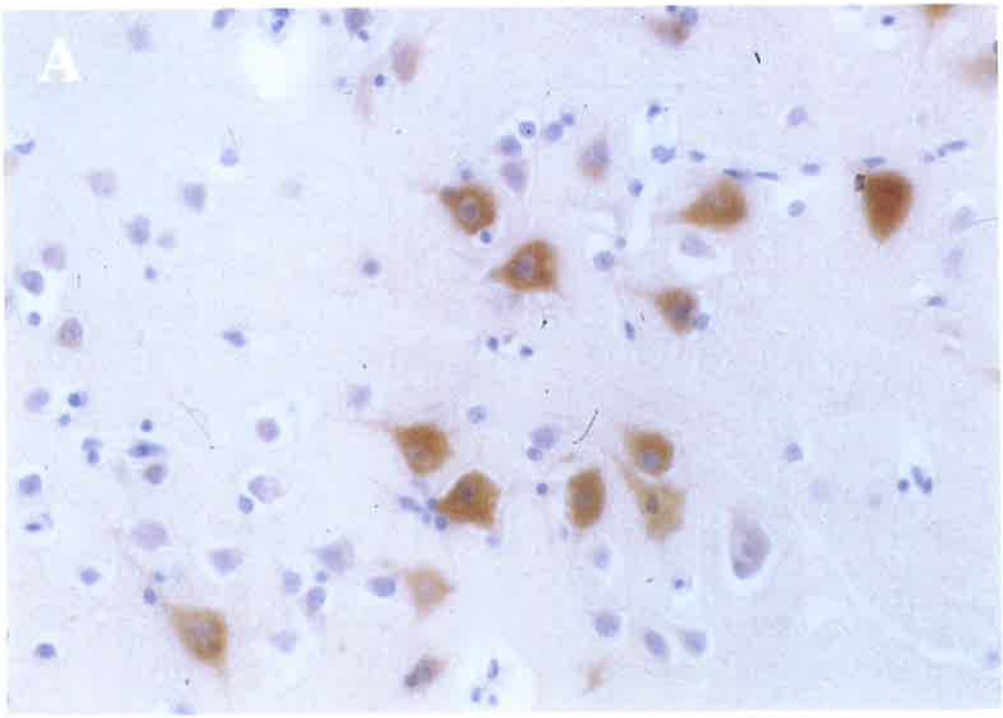


Figure 7.3.1: Histogram showing the percentage brain area with APP immunoreactive neuronal cell bodies and axons in 10 impacted lambs compared to 2 non-impacted controls. C1 and C2 are the control lambs.

Figure 7.3.2: APP immunoreactivity within neuronal cell bodies and axons in head-injured lambs. APP immunoreactive neuronal cell bodies were seen in the thalamus in impacted lambs *(orange/brown staining)* (A). APP immunoreactive axons were also present in the thalamus (B). (x 400).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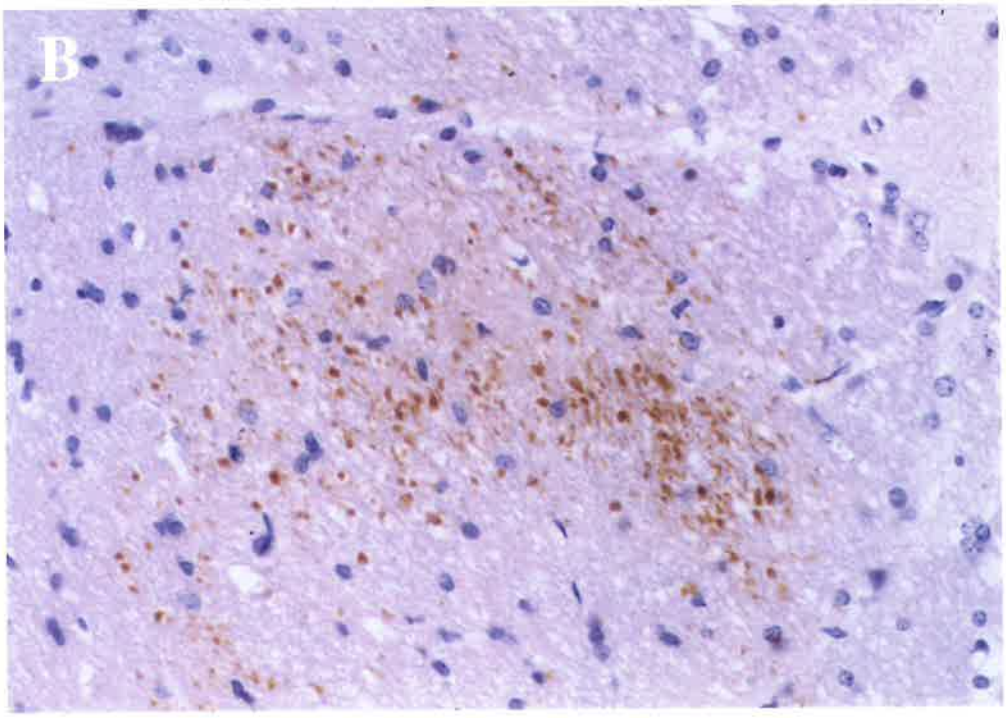
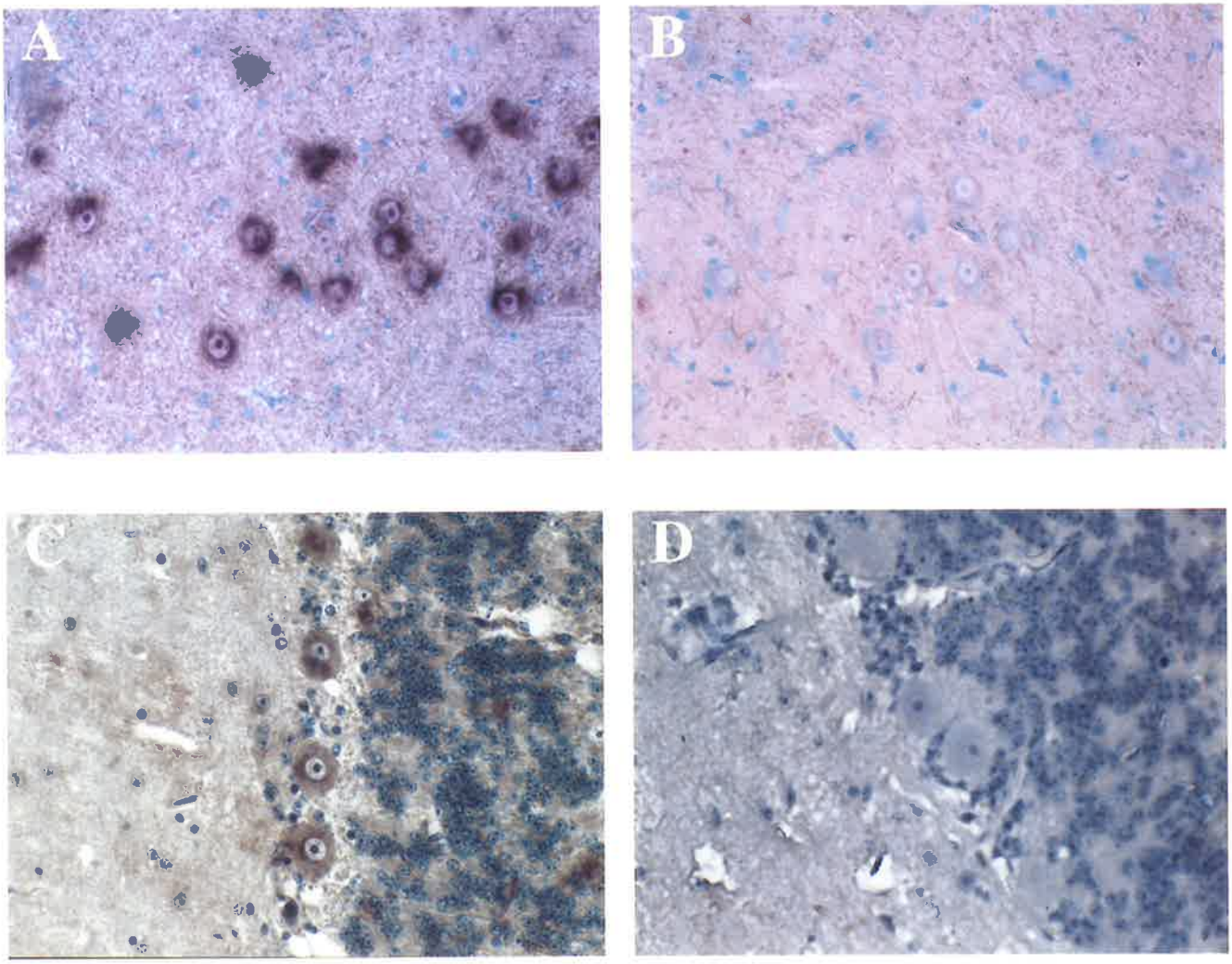

B

7.3.4- Neuronal APP mRNA response

Left and right frontal and temporal lobes, central grey matter, cerebellum and brainstem showed upregulation of APP mRNA within NCBs in all impacted lambs. (Refer Figure 7.3.3A for a representation of APP mRNA staining) in a similar distribution to APP antigen. Intense and confluent staining for APP mRNA was evident in cytoplasm and nuclei of NCBs in all regions examined and also involved regions distant to the impact site such as the cerebellar Purkinje cells (Figure 7.3.3C).

APP mRNA was undetectable in necrotic NCBs within contusions and necrotic Purkinje cells. However, viable NCBs at the periphery of contusions and unaffected Purkinje cells showed strong hybridization. NCB APP mRNA upregulation was also evident in injured lambs that were otherwise histologically normal using conventional H&E staining. In contrast, neuronal APP mRNA was undetectable in brain sections of non-impacted lambs (Figures 7.3.3B and 7.3.3D).

Figure 7.3.3: Upregulation of APP mRNA expression in lambs. Two hrs after impact to the left temporal lobe hybridization for APP mRNA was detected *(brown/black staining)* in the left thalamus (A) compared to undetectable hybridization in the thalamus of non-injured controls (B). Intense hybridization was also seen as distant to the site of impact as the cerebellar Purkinje cells (C) but was not detectable in Purkinje cells of non-injured lambs (D). (x400).


A
B
C
D

7.4- DISCUSSION

This is the first study to demonstrate acute upregulation of APP 2 hrs after TBI in the lamb brain. These results are similar to our previous observations of acute upregulation of APP and its mRNA in the adult ovine head impact model.

Widespread APP expression was seen in NCBs in impacted and contralateral hemispheres, cerebellum and brainstem in both sheep and lambs. In contrast, there were only a small number of weakly APP immunoreactive NCBs and no detectable APP mRNA in tissue from non-impacted lambs. APP mRNA was not detected in controls because the stringent hybridization conditions used are unable to detect low levels of transcription.

As seen in the adult sheep brain, APP positive NCBs were more widespread than APP positive axons. Interestingly, there was a greater axonal APP response in the impacted lambs compared to the adult sheep. The significance of this increase is unknown. It is possible that developing axons are more susceptible to damage or it may simply be due to the fact that the lambs may have received a relatively more severe mechanical injury. This may be on account of the thinness and pliability of the skull due to unfused suture lines and open fontanelles (Bruce *et al.*, 1981).

The significance of this early, sensitive and widespread NCB APP response is not known. However, these findings support our hypothesis that there is neuronal upregulation of APP in lamb brains similar to that seen in adult sheep brains following trauma.

In future studies we would like to quantitate the APP mRNA response by northern blotting to assess whether the magnitude of the response is greater in the lamb brain.

CHAPTER 8

Neuronal cell body and axonal APP immunoreactivity and APP mRNA response in human head injury

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8.1- INTRODUCTION

The APP response to human head injury has been studied in the past by numerous groups. However, the focus of these studies was to use APP as a marker of axonal injury (AI) (Gentleman *et al.*, 1993b, 1995, 1999; Blumbergs *et al.*, 1994, 1995; Sherriff *et al.*, 1994a, 1994b; McKenzie *et al.*, 1994; Graham *et al.*, 1995b; Abou-Hamden *et al.*, 1997). A few of the above studies also demonstrated APP immunoreactivity within neuronal cell bodies (NCBs) but did not involve detailed systematic investigation of this finding.

The aim of this study was to topographically describe the neuronal cell body (NCB) and axonal APP response using whole brain sections in a series of fatal head injury cases of varying survival times. APP immunostaining was used to semi-quantitate the antigen response and in-situ hybridization (ISH) was used to assess the expression of APP mRNA.

8.2- MATERIALS AND METHODS

8.2.1- Description of study cases

Twelve cases of traumatic brain injury (TBI) from various causes (10 males, 2 females, age range 17-88) were selected for this study and 5 cases of non-traumatic controls (2 males, 3 females, age range 39-88) were also assessed (Table 8.2.1). The trauma group was selected from a series of head injury cases who survived long enough to be admitted to the Royal Adelaide Hospital and underwent a full post-mortem examination. Case notes and ambulance reports were used to obtain information regarding the duration, cause of death and clinical course with particular attention to increased intracranial pressure (ICP), hypoxia, hypotension and electrolyte imbalance. The delay between death and post-mortem was 12 hrs in 1 case, 24 hrs in 15 cases and 72 hrs in 1 case (refer Tables 8.2.1 and 8.2.2).

Many of the TBI cases had previously been neuropathologically examined using the sector scoring method (Blumbergs *et al.*, 1995; Abou-Hamden *et al.,* 1997) to examine hypoxic and vascular injury. The percentage of sectors with any ischaemic damage, ranging from "red cell change" to well developed infarction, provided the hypoxic-ischaemic score (HIS) and the percentage of sectors involved with haemorrhagic lesions provided the vascular injury score (VIS) (refer Table 8.3.2).

Case no.	Sex	Age	Circumstances of accident	Cause of death	Post-mortem delay
1	F	24	Pillion passenger on motorcycle, no helmet	Head injury	24 hrs
2	M	18	Motorcyclist versus car	Head injury	24 hrs
3	F	88	Fell backwards while climbing up stairs	Head injury	24 hrs
4	M	86	Fell 0.7 metres onto wooden floor	Head injury	24 hrs
5	M	17	Motorcyclist versus semi trailer	Head injury	24 hrs
6	M	25	Fell onto rocks while rock climbing	Head injury	24 hrs
7	M	17	Motorcyclist versus car	Head injury	72 hrs
8	M	77	Fell 1.9 metres onto tiled floor	Head injury	24 hrs
9	M	18	Roof surfing whilst intoxicated, 60-80 KPH	Head injury	24 hrs
10	M	72	Hit on head by falling 60kg branch	Cardiac arrhythmia	24 hrs
11	M	53	Fell from ledge through tree branches onto concrete	Head injury	24 hrs
12	M	18	Motorvehicle accident (driver)	Head injury	24 hrs

Table 8.2.1 : Description of TBI study cases.

The control sample was also selected from Royal Adelaide Hospital reports and each case underwent full post-mortem examination and neuropathological examination. Cases were selected on the grounds that they had a macroscopically and microscopically normal brain (Table 8.2.2).

Case no.	Sex	Age	Cause of death	Previous medical history	Post-mortem delay
1	M	39	Bronchopneumonia	Alcoholic, diabetes mellitus	24 hrs
2	F	68	Pericardial and intramyocardial haematoma	Ischaemic heart disease, hypertension, stenosis, emphysema	24 hrs
3	M	60	Sepsis	Carcinoma of right pyriform fossa, hypertension, cirrhosis of liver	24 hrs
4	F	42	Pulmonary embolism	Leiomyosarcoma of uterus metastatic to the left lung and lesser omentum	12 hrs
5	F	88	Bronchopneumonia	Bronchitis	24 hrs

Table 8.2.2: Description of control cases

8.2.2- Neuropathological examination

All brains were fixed in 40% formalin for a minimum of 10 days, weighed, photographed and sectioned according to a standard neuropathological protocol (Blumbergs *et al.*, 1995). Cerebral hemispheres were sectioned coronally at 1 cm intervals, with the first cut being at the level of the mamillary bodies. A minimum of 11 slices were obtained and processed as whole sections. A whole sagittal section was also taken from each cerebellar hemisphere and 2 transverse sections taken from each midbrain, pons and medulla.

8.2.3- APP immunocytochemistry

For APP immunocytochemistry, histological sections of 7 μm were cut from each of the double hemispheres, cerebellum and brainstem and each section was incubated with Mab 22C11 using the same methods previously described in Chapter 3.2.2. Sections serial to those used for the immunocytochemical studies were also stained with H&E.

8.2.3.1- Semi-quantitation of NCB and axonal APP response

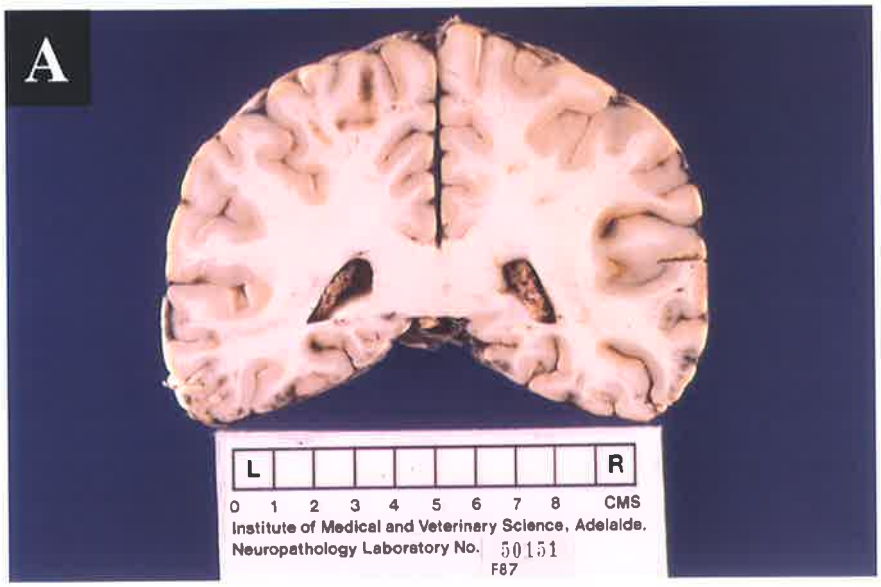
To topographically assess and semi-quantitate the NCB and axonal APP response, we used the same graticule system as in the sheep studies (refer Chapter 3.2.2.3). This graticule system provides a means of topographically assessing the NCB and axonal response in the entire brain. The graticule is comprised of 4 mm x 4 mm grid squares. The semi-quantitative methods and criteria for assessing positive NCBs were undertaken in the same manner as the sheep (refer Figure 8.2.1 for semi-quantitation protocol). In total there were approximately 5000 grid squares per case.

However the sector scoring system was used to semi-quantitate hypoxic-ischaemic and vascular injury (Blumbergs *et al.*, 1995; Abou-Hamden *et al.*, 1997).

8.2.4- In-situ hybridization for APP mRNA

ISH was undertaken on 7 of the TBI cases (survival time range from 2.5 hrs to 7 days) and 3 control cases. 5 μm representative sections of the left and right frontal and temporal lobes, central grey matter, cerebellum and brainstem serial to those used for immunocytochemistry, were taken for ISH analysis. The same ISH protocol as previously described in chapter 4.2.4 was used for the human tissue. PV-Cap-1 was used as the anti-sense probe and pGEM-Cap-1 was used as the sense probe. The only modification to the protocol was that digestion with 15μg/ml proteinase K (Boehringer Mannheim) in 20 mM Tris HCl pH 7.5; 2 mM $CaCl_2$ shaken at 37°C for 15 mins, provided strong signal and good preservation of tissue morphology.

Figure 8.2.1: Semi-quantitation of neuronal cell body and axonal injury using APP immunocytochemistry. (A) shows a coronal (1 cm) section of a human TBI case showing patchy left and right temporal contusions. (B) shows a histological section (7 μm) of the same case stained with H&E overlaid by a transparent graticule used to semi-quantitate the APP immunocytochemical response. Record of positive grids with APP immunoreactive neuronal cell bodies *(red)* and positive grid squares with APP immunoreactive axons *(black)* (C).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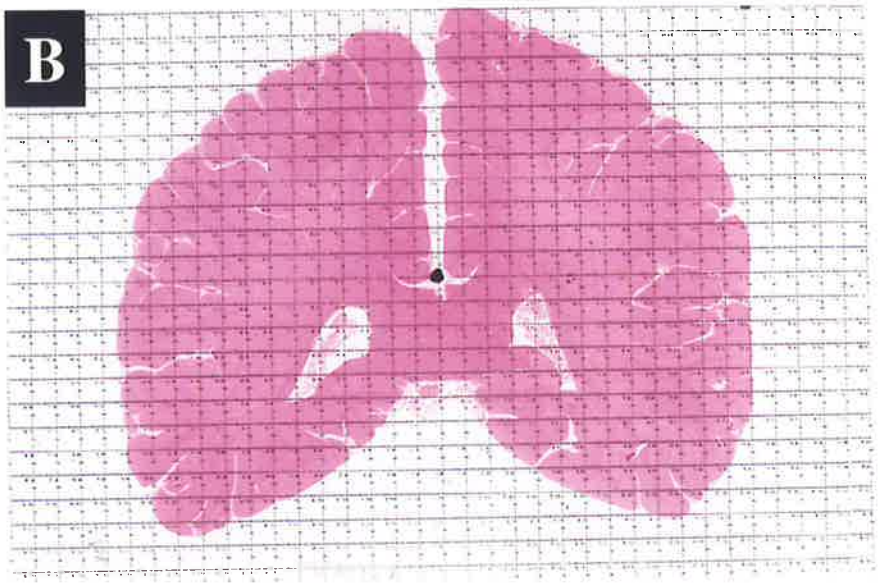
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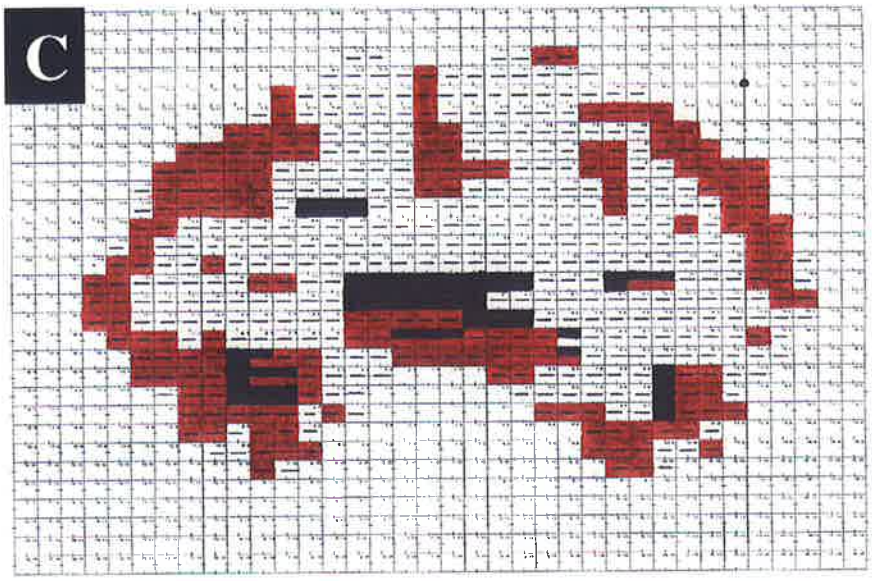

C

8.2.5- βA4 immunocytochemistry

In addition to APP immunostaining, representative sections were taken from the left and right frontal, left and right temporal and central grey matter of 4 TBI cases (age range 17-88 years) and 3 control brains (age range 39-88 years) for βA4 immunocytochemistry. A monoclonal antibody directed to amino acids 8-17 of βA4 (Dako Ltd, UK), was used at a dilution of 1:1000, which has also been used previously by Gentleman *et al.*, (1993b); Roberts *et al.*, (1994). Binding of the antibody was detected using horse anti-mouse immunoglobulin (diluted 1:500), followed by a 1:250 dilution of mouse peroxidase-anti-peroxidase conjugate (all Vector, Jomar Diagnostics). All sections were pretreated with 80% formic acid for 10 mins and sections were then microwaved in citrate buffer (pH 6.0) and blocked for 30 mins in 0.5% H_2O_2 in methanol. Sections were then blocked for 30 mins in 10% NHS (pH 7.45). Reactions were then incubated with the primary antibody for 16 hrs at room temperature. Bound antibody was detected using DAB (refer Chapter 3, Section 3.2.2.2).

8.3- RESULTS

8.3.1- Clinical features

TBI cases ranged in age from 17-88 years and comprised of 2 females and 2 males. The Glasgow Coma Scale (GCS) of these cases, on admission to hospital, ranged from 3 to 14. The duration of loss of consciousness (L.O.C.) ranged from a few mins to 7 days. Survival time ranged from 2.5 hrs to 7 days. Only 2 patients experienced a lucid interval. (refer Table 8.3.1 below for clinical details). The age range of controls was 39-88 years.

Case no.	Age	Sex	GCS	Duration L.O.C.	Lucid Int.	Survival time	Operation	PMHx
1	24	F	3	2.5 hrs	-	2.5 hrs	nil	nil
2	18	M	3	3.5 hrs	-	3.5 hrs	nil	nil
3	88	F	6	4.5 hrs	-	4.5 hrs	nil	HT
4	86	M	4	6.5 hrs	+	7 hrs	nil	HT, CCF, PD Fall> CSDH
5	17	M	3	16 hrs	-	16 hrs	ICP mon	nil
6	25	M	4	2 days	-	2 days	nil	nil
7	17	M	4	2.5 days	-	2.5 days	ICP mon	nil
8	77	M	13	? mins	-	3 days	nil	HT, COAD, Recent MI.
9	18	M	5	3.25 days	-	3.25 days	Craniotomy + ICP mon	nil
10	72	M	14	o	+	4 days	nil	nil
11	53	M	3	6 days	-	6 days	Craniotomy + ICP mon	nil
12	18	M	4	7 days	-	7 days	ICP mon	nil

Table 8.3.1: Clinical details of TBI cases. CCF, Congestive cardiac failure; COAD, Chronic obstructive airways disease; GCS, Glasgow Coma Scale; ICP, Intracranial Pressure; CSDH, Chronic subdural haematoma; HT, Hypertension; ICP mon., ICP monitor; L.O.C., Loss of consciousness; Lucid Int., Lucid interval; MI, Myocardial infarction; PD, Parkinson's disease; PMHx, Past medical history.

8.3.2- Pathological features of the TBI group

Skull fractures and subarachnoid haemorrhages (SAH) were present in all cases irrespective of the initiating injury. Acute subdural haematoma (SDH) was present in 10 of the 12 cases (83%). Contusions and/or lacerations (C/L) were present in 11 of the 12 cases (92%). Gliding contusions (GC) were seen in 9 of the 12 cases (75%) (8 macroscopic and 1 microscopic). Pathological evidence and/or clinical evidence (ICP monitor) of raised ICP (> 20mmHg) was present in 10 of the 12 cases (83%). Haemorrhagic marker lesions of DAI were present in 5 of the 12 cases (42%) (Table 8.3.2)

Case no.	Skull fracture	Macro DAI	EDH	ASDH	TSAH	IVH	ICH	C/L	GC	ICP	Sector scoring results	
											HIS	VIS
1	L. parietal	+	o	+	+	+	+	+	M	N	?	?
2	L. frontal	+	o	+	+	+	+	+	M	↑	?	?
3	R BOS	o	o	+	+	o	+	+	m	↑	22	34
4	R basal	o	o	+	+	o	o	o	o	↑	3	7
5	L BOS + L. occipital	o	o	o	+	+	o	+	M	↑	?	?
6	L occipital + R MCF	o	+	+	+	o	o	+	M	↑	71	49
7	L basal	+	o	+	+	o	o	+	M	↑	44	40
8	R occipital	o	o	o	+	o	o	+	o	N	18	25
9	Multiple	+	o	+	+	+	+	+	M	↑	98	56
10	L occipital + R MCF	o	o	+	+	o	o	+	M	↑	3	4
11	R temporal + R basal	o	o	+	+	o	o	+	o	↑	14	26
12	R frontal + R temporal	+	o	+	+	o	o	+	M	↑	100	45

Table 8.3.2: Neuropathological data of TBI cases. o, Absent; +, Present; ↑, elevated (above 20 mmHg); L, Left; m, Microscopic; M, Macroscopic; N, Normal levels; R, Right; BOS, Base of skull; C/L, Contusion/Laceration; Macro DAI, Macroscopic markers of DAI (haemorrhagic lesions in corpus and/or dorsolateral quadrant of rostral brain stem); MCF, Middle cranial fossa; ICP, Intracranial pressure; HIS, Hypoxic-ischaemic score; VIS, Vascular injury score; GC Gliding contusion; EDH, Extradural haematoma; ASDH, Acute subdural haematoma; TSAH, Traumatic subarachnoid haemorrhage; IVH, Intraventricular haemorrhage; ICH, Intracranial haematoma.

8.3.3- APP immunocytochemical response

Widespread APP positive NCBs were evident in all TBI cases from 2.5 hrs up to 7 days and the percentage brain area with APP positive NCBs ranged from 17 to 69% (mean 43%) (Figure 8.3.1). The greatest NCB response was seen at 2.5 hrs and the second highest percentage brain area with APP immunoreactive NCBs was seen at 7 days (refer Figure 8.3.1). However, because the magnitude of the impact forces is unknown and secondary complicating factors exist in these cases, it is difficult to interpret and statistically analyze these data.

In 10 of the 12 TBI cases, APP immunoreactive NCBs were more diffuse than the APP immunoreactive axons. APP immunoreactive axons were evident in all TBI cases and the percentage brain area ranged from 1 to 50% (mean 23%). As described above, it is difficult to qualify and interpret these findings due to the unknown impact forces. However, the subjective impression was that APP immunoreactive axons and axonal swellings increased in size with duration of survival (Figures 8.3.2A, 8.3.2B and 8.3.2C).

In contrast to the impacted group, non-traumatic controls showed minimal APP immunoreactivity within NCBs (from 6 to 10.5%) and no AI (Figures 8.3.1 and 8.3.3A).

These results demonstrate that the discrepancy between the greater degree of AI in the human cases compared to the sheep cases is likely to be a survival time phenomenon.

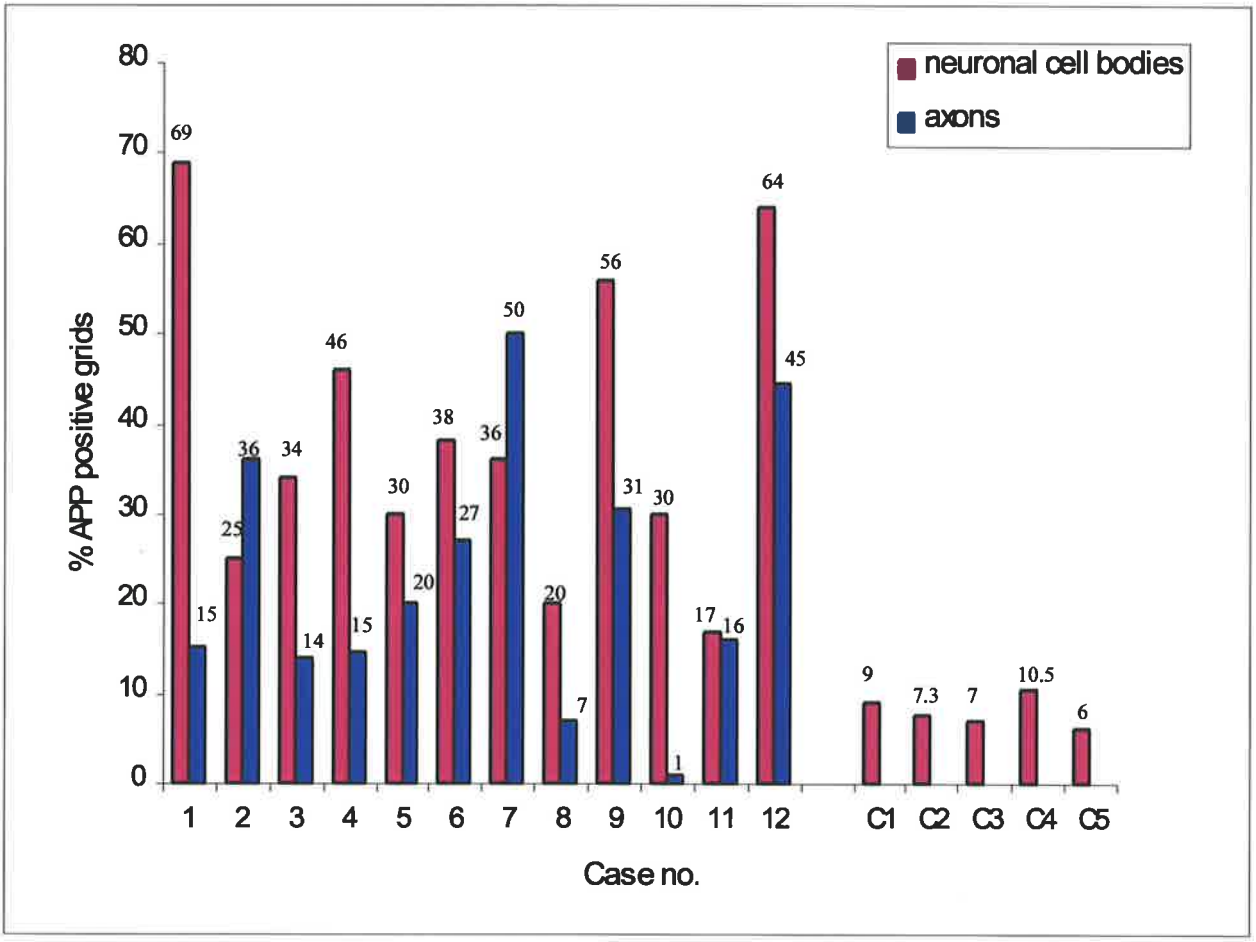
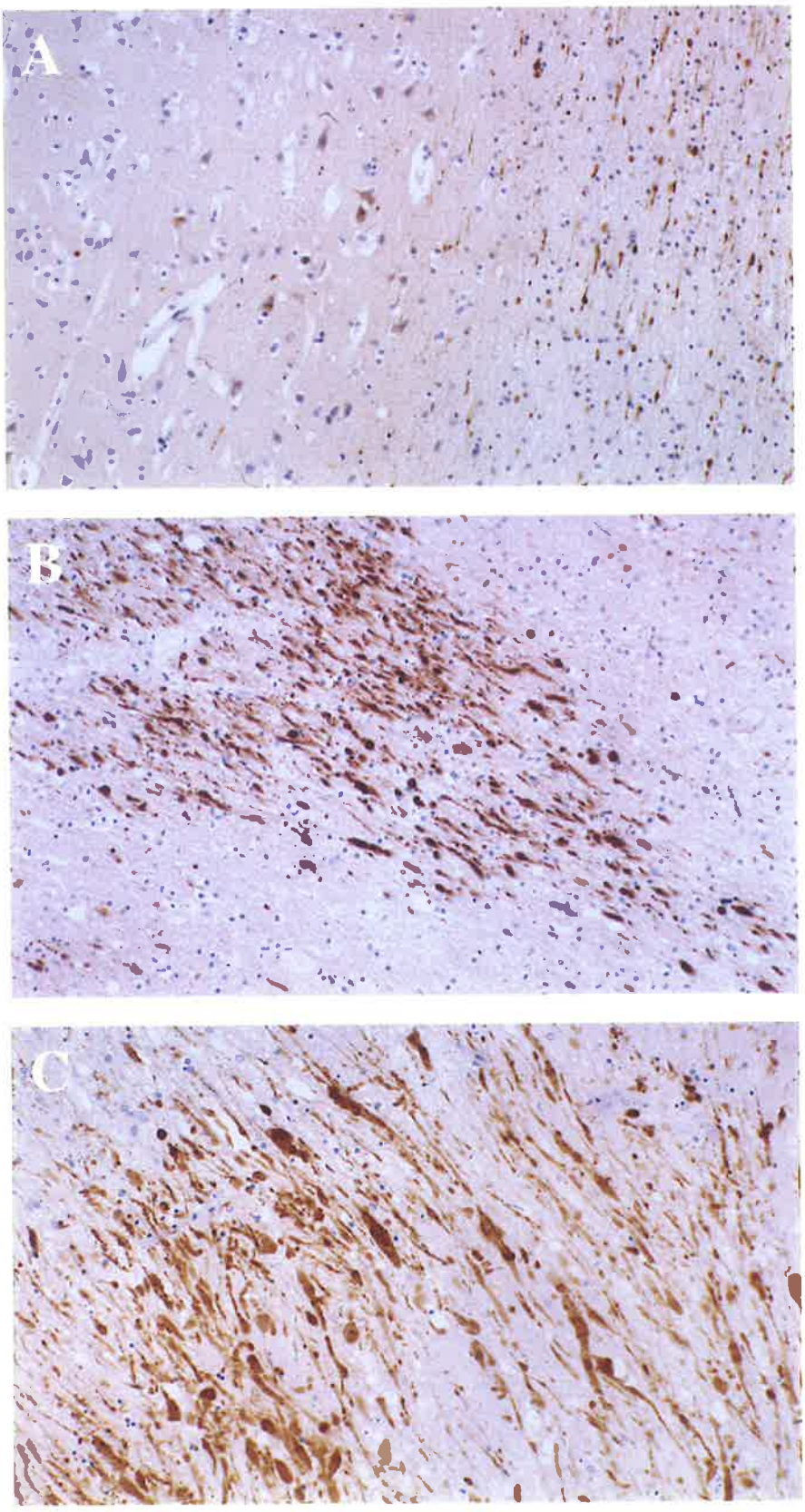


Figure 8.3.1: Histogram showing the percentage brain area with APP positive neuronal cell bodies and APP positive axons in 12 fatal human head injury cases with varying survival times from 2.5 hours to 7 days and 5 uninjured control cases. Survival times of cases; case 1, 2.5 hrs; case 2, 3.5 hrs; case 3, 4.5 hrs; case 4, 7 hrs; case 5, 16 hrs; case 6, 2 days; case 7, 2.5 days; case 8, 3 days; case 9, 3.25 days; case 10, 4 days; case 11, 6 days; case 12, 7 days. C1-C5 represent controls, numbers above bands indicate the percentage brain area.

Figure 8.3.2: APP immunostaining of AI in human cases at varying survival times. Typical appearance of APP positive axons *(orange/brown staining)* with some positive neuronal cell bodies in case number 2 who survived for 4.5 hrs (A). (B) shows prominent axonal staining in case number 6 who survived for 2 days and case number 12 who survived for 7 days (C). (x200).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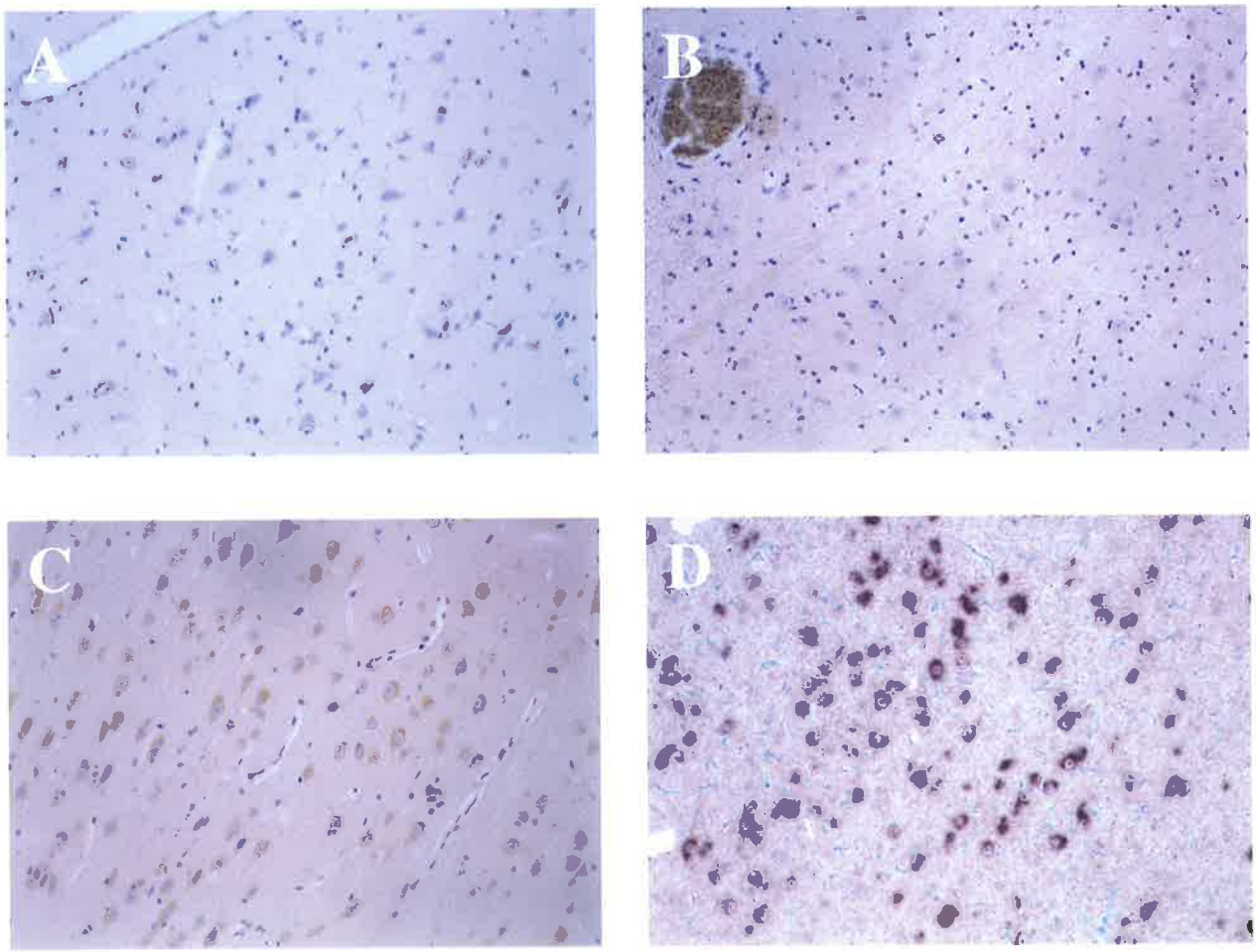
8.3.4- Neuronal APP mRNA expression

Undetectable levels of APP mRNA were found in the 3 control cases analyzed (Figure 8.3.3B). Increased neuronal APP mRNA expression was found in all of the TBI cases examined with survival times ranging from 2.5 hrs to 3.25 days. Upregulation of APP mRNA was evident in all regions sampled at 2.5 hrs after injury, in a similar distribution to APP antigen (Figures 8.3.3C and 8.3.3D). APP expression was similarly increased in all other cases sampled with survival times ranging from 16 hrs to 3.25 days.

8.3.5- βA4 immunoreactivity

Within the TBI group, βA4 immunoreactivity was seen in only 1 case, aged 88 years. This individual had widespread neurofibrillary tangles and βA4 immunoreactive plaques and. Two of the non-injured control cases (numbers 3, aged 60 years and 5, aged 88 years) also showed βA4 immunoreactive plaques.

Figure 8.3.3: Differences in APP antigen and mRNA expression in the left temporal cortex of controls and head injury cases at differing survival times. (A) shows typical undetectable levels of APP antigen in a control case with undetectable levels of APP mRNA in the same case (B). APP immunoreactive neuronal cell bodies were evident at 2.5 hrs post-injury *(orange/brown staining)* (C) with corresponding upregulation of APP mRNA in same case *(brown/black staining)* (D). (x200).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8.4- DISCUSSION

This study demonstrated the presence of widespread APP immunoreactive NCBs in the 12 human cases following TBI. Positive NCBs were seen as early as 2.5 hrs after injury and persisted for at least 7 days. These observations are in agreement with those of other groups that have qualitatively observed a general increase in APP immunoreactivity in NCBs following TBI in humans (Gentleman *et al.*, 1993b; McKenzie *et al.*, 1994; Roberts *et al.*, 1994) but this is the first topographical semi-quantitative study to analyze the NCB and axonal APP response in the entire brain in a selection of human TBI cases of varying survival times.

As described in Chapter 2, there are difficulties in interpreting the histopathological results in human TBI cases. In human TBI, the exact mechanics and site of impact, duration and magnitude of impact forces and the physiological responses of the patient are often unknown. Therefore, assessing the resulting pathology and distinguishing between the primary mechanical pathological changes and the secondary complicating factors is difficult. In particular, the APP response in human cases is difficult to assess as APP immunoreactivity within NCBs and axons is not specific to mechanical injury. APP immunoreactivity may also occur as a result of secondary complicating factors such as hypoxia, ischaemia and infarction (Abe *et al.*, 1991b; Ohgami *et al.*, 1992; Stephenson *et al.*, 1992; Kalaria *et al.*, 1993a, 1993b; Blumbergs *et al.*, 1994, 1995; Pluta *et al.*, 1994; Suenaga *et al.*, 1994; Tomimoto *et al.*, 1995; Yam *et al.*, 1997, 1998) which are frequently found following human head injury (Graham and Adams, 1971; Graham *et al.*, 1989). Therefore, it is unknown whether the APP antigen and mRNA response found in

these cases is due to the primary mechanical deformation or an additive effect of secondary complicating factors.

The variability in the types of injury and resulting pathology of the human cases reinforces the benefits of using a controlled experimental model to study the responses to TBI. In particular, it highlights the advantages of the ovine head impact model which overcomes many of the inherent problems found in studying human TBI.

Previous human TBI studies on the APP response have primarily focused on the possible link between overexpression of APP as a risk factor for βA4 deposition. These studies have shown that a single episode of traumatic brain damage may result in widespread deposition of βA4 (Roberts *et al.*, 1991, 1994; Gentleman *et al.*, 1993b; Graham *et al.*, 1995b). Similarly, experimental studies have found that head injury may accelerate the onset of AD-like pathology in transgenic mice (Hsiao *et al.*, 1996; Nalbantoglu *et al.*, 1997; Smith *et al.*, 1998) and that βA4 deposition may occur more rapidly than normal in predisposed transgenic mice (Games *et al.*, 1995; Johnson-Wood *et al.*, 1997). Therefore, it has been proposed that the expression of APP as an acute phase response to injury may initiate a cascade of events resulting in Alzheimer's disease (AD) in susceptible individuals (Roberts *et al.*, 1994; Graham *et al.*, 1995b). However, Adle-Biassette *et al.*, (1996) who studied a group of head-injured patients aged 10-63 years found no βA4 deposits in any case below the age of 63 years.

βA4 deposition following TBI was not the focus of our studies and was not systematically examined. However, in the limited survey undertaken βA4 plaques were

only seen in 1 case aged 88 years. βA4 plaque deposition was also found in 2 of the control (uninjured) cases, both over the age of 60 years. No conclusion can be drawn from these few cases and further systematic studies in this area would be of interest.

CHAPTER 9

Final discussion

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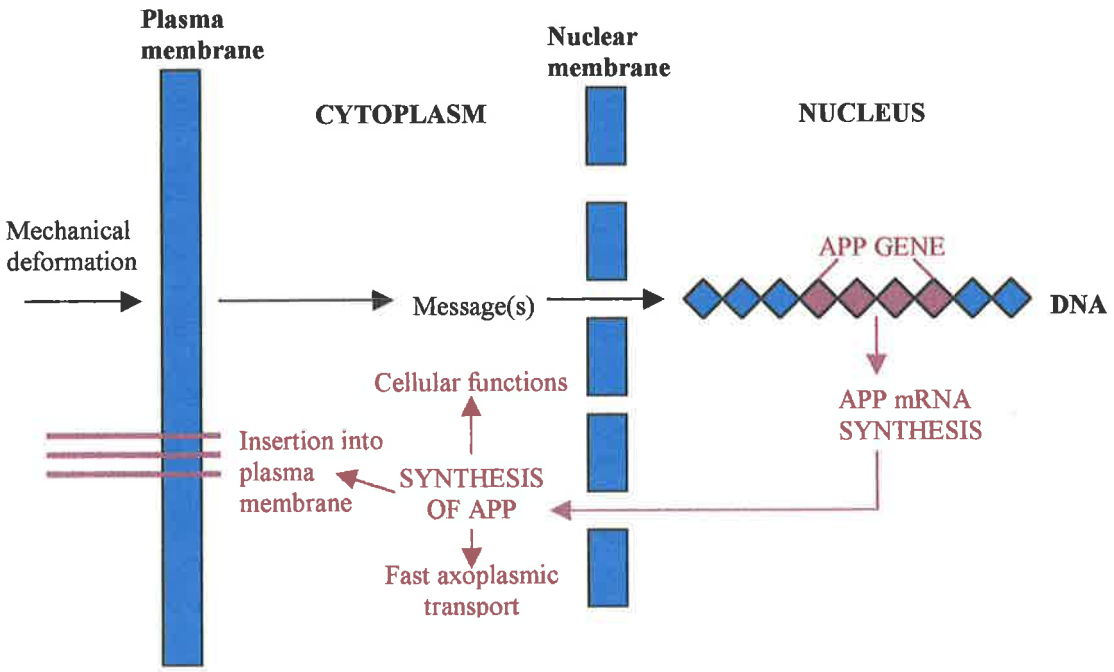
9.1- SUMMARY AND FUTURE DIRECTIONS

Previous human and experimental studies in our laboratory have used APP as a sensitive and early marker of axonal injury (AI) (Blumbergs *et al.*, 1994, 1995; Lewis *et al.*, 1996; Abou-Hamden *et al.*, 1997; Finnie *et al.*, 1999). During the course of these studies it was noted that more neuronal cell bodies (NCBs) showed APP immunoreactivity than axons and that no direct correlation existed between the number of APP positive NCBs and axons. This suggested that the subcellular mechanisms in axons leading to APP immunoreactivity may be different from those in the neuronal cell body (NCB) and as the changes in the NCBs were so widespread throughout the brain this seemed worthy of further investigation as the subject of this thesis.

The central hypothesis for these studies was that mechanical deformation, which is not severe enough to cause immediate cellular death, results in increased transcription of APP mRNA and a subsequent increase in APP antigen expression as an acute phase response to injury.



APP immunocytochemical and in-situ hybridization (ISH) studies were undertaken on a selection of human traumatic brain injury (TBI) cases (see Chapter 8). A novel semi-quantitative graticule system was developed to provide a topographical overview of the NCB and axonal APP immunoreactivity and ISH was undertaken in various regions to study APP mRNA expression in response to injury. Analysis of 12 cases of varying survival times from 2.5 hrs to 7 days showed that there was widespread APP immunoreactivity and increased APP mRNA expression within NCBs in all the injured brains compared to control (uninjured) cases. APP positive axons were also present in all injured brains but to a much lesser extent than the APP positive NCBs. However, the variability in the type and severity of the head impacts, survival times and secondary complicating factors such as raised intracranial pressure (ICP), hypoxia and ischaemia were difficult to standardize and produced a spectrum of pathological changes in the brain. As hypoxic-ischaemic damage also produces NCB and axonal APP positive immunoreactivity the interpretation of the APP changes in the human cases was problematic.

In order to overcome these limitations we developed an ovine head impact model which provided a means of closely monitoring and controlling important physiological parameters such as blood gases, blood pressure, ICP etc (see Chapter 2). Sheep were chosen because their gyrencephalic brains are much larger than the smaller almost lissencephalic brains of laboratory rodents frequently used in head trauma studies. Pathological analysis of the sheep brains following head impact showed that axonal and vascular damage similar to that seen in human TBI was produced in this model (Lewis *et al.*, 1996; Van Den Heuvel *et al.*, 1998; Finnie *et al.*, 1999). From our detailed

histological studies we believe that the current model produces a mild to moderate head injury with contusions and cellular necrosis only representing mild components of the resultant brain damage. Therefore, as the impact is not severe enough to cause immediate cell death the evolution of the neuronal APP response can be studied in this model.

Pilot studies using systematic semi-quantitative assessment of NCB APP immunoreactivity (Mab 22C11) in the ovine head impact model 2 hrs after injury revealed increased APP antigen expression within NCBs diffusely involving the whole brain (see Chapter 3). These studies were the first to demonstrate NCB APP expression as early as 2 hrs after trauma. We reasoned that increased immunoreactivity within NCBs could be due to (i) accumulation of APP in the NCBs by inhibition of normal axoplasmic transport system of APP; (ii) alterations in the availability of APP epitopes detectable by the antibody used or (iii) increased transcription of APP.

The most obvious reason for increased APP immunoreactivity was increased transcription of APP mRNA possibly to aid in neuroprotective or neuroreparative processes following TBI as there is evidence that APP may have neuroprotective and neurotrophic functions (see Chapter 1, Section 1.2.5) and APP transcription is known to be influenced by many genes and proteins which are acutely affected following trauma (see Chapter 1, Section 1.2.3). This hypothesis was addressed in Chapter 4 by assessing and comparing the neuronal APP mRNA and antigen expression at varying times ranging from 15 mins to 2 hrs using APP immunocytochemistry and in-situ hybridization (ISH) for APP mRNA. These studies using Mab 22C11 and a non-isotopic specific APP mRNA riboprobe (PV-Cap-1) demonstrated that there was a time dependent evolution of

APP mRNA and antigen response and APP mRNA upregulation was seen earlier than previously thought i.e. 30 mins post-impact. ISH and immunocytochemistry studies using a specific antibody and riboprobe to the KPI-containing APP isoforms showed that APP-695 was the predominant isoform affected (see Chapter 4). The striking correlation between APP immunoreactivity and APP mRNA in the injured brains strongly suggested that increased transcription of APP mRNA was the predominant cause of the increased APP immunoreactivity. However, these studies do not necessarily indicate that this is the sole cause of the immunoreactivity.

As there is evidence that APP has neurotrophic functions we wanted to see whether the APP response in lamb brains differed from that of the adult. We modified the adult ovine head injury model to produce a lamb head injury model to study the NCB APP response following head impact in the young not fully developed brain. We hypothesized that APP expression would also be increased in the lamb brain as an acute phase response to trauma. Our results demonstrated that there was widespread and intense NCB APP immunoreactivity and increased APP mRNA expression in the lamb brain 2 hrs after impact (see Chapter 7). These results show that head impact produces an APP response in the lamb brain similar to that of the adult sheep brain. However, quantitation of the APP mRNA using northern blotting would be of great interest to assess whether there is a greater APP response in the developing brain.

To quantitate the APP mRNA response we utilized northern blot analysis (see Chapter 5). The aim of these studies was to quantitate and compare the APP mRNA response over time in injured adult animals and controls using the ovine head impact model. Northern

blot analysis was performed on tissue obtained from the thalamus which was determined to consistently show the greatest APP response (see Chapter 5). The quantitative results demonstrated a 3 fold increase in APP mRNA in the thalamus in injured animals 2 hrs post-impact compared to controls. This is the first study to quantitatively demonstrate such an early increase in APP mRNA following TBI. Increased transcription of the KPI-containing isoforms of APP could not be shown.

The northern blot studies were of particular interest in that they demonstrated that the APP mRNA response was of the same magnitude in the thalamus under the conditions applying to our model. This suggested that the quantitation of APP mRNA in this model may be useful in analyzing the effects of putative neuroprotective therapeutic agents.

We hypothesized that as the widespread NCB upregulation of APP may be related to a possible neuroprotective function of APP, then some neuroprotective agents may act by increasing APP mRNA and antigen expression. We chose magnesium (Mg^{2+}) to test this hypothesis as there was evidence in the literature that Mg^{2+} therapy may have a neuroprotective effect (see Chapter 6, Section 6.4). Therefore, the aims of these studies described in Chapter 6 were to topographically assess and quantitate the effects of magnesium sulphate ($MgSO_4$) treatment on APP antigen and mRNA expression 2 hrs after a controlled head impact. Quantitative results showed that post-traumatic administration of $MgSO_4$ 30 mins after head impact resulted in a 2.3 fold increase in APP mRNA in the thalamus of treated impacted animals compared to untreated animals. An interesting finding was that there was also a 1.6 fold increase in APP mRNA in $MgSO_4$ treated non-impacted control animals compared to untreated controls. Topographical

assessment of the APP mRNA and antigen responses by ISH and immunocytochemistry showed that there was upregulation of mRNA and antigen in both treated and untreated impacted animals but only weakly detectable levels of APP mRNA and scattered positive NCBs in controls with treatment. These results are the first to link $MgSO_4$ therapy (a putative neuroprotectant) with upregulation of neuronal APP mRNA and antigen expression. The exact implications of this stimulatory effect by $MgSO_4$ are not known, but it may reflect a "non-specific" regulatory synthesis of APP mRNA, as demonstrated by an increase in APP mRNA in controls. The greater APP mRNA response following $MgSO_4$ administration after injury may be an additive effect of the mechanical deformation. Our evidence supports the concept that both Mg^{2+} and APP may play important roles in the complex cellular events which occur following TBI. Exactly how they interact requires further study.

Studies to determine whether treatment of animals with $MgSO_4$ prior to injury and different treatment protocols would be of interest as would be long term survival studies for which we have not been able to get ethical approval.

We also plan to study the APP mRNA response to $MgSO_4$ in different regions of the brain using RT-PCR and to use this model to assess the effects of other putative therapeutic agents.

9.2- CONCLUSIONS

1) Our topographical and quantitative studies of APP in this ovine head impact model have demonstrated a rapid increase in APP expression after head injury. Detailed molecular pathological studies of the APP mRNA response demonstrated an increase in the APP mRNA production as early as 30 mins after head impact in sheep and after 2.5 hrs in human head injury.

2) It was also shown that $MgSO_4$ (a putative neuroprotectant) is able to upregulate APP mRNA and antigen. The exact implications of this early APP response following TBI and the exact implications for a link between a putative neuroprotectant and APP are unknown. Exactly where APP fits into the complex cellular processes following injury is also unclear and requires further study, but these results provide novel information regarding the early cellular responses of APP in accordance with previous human and experimental studies suggesting that APP may be upregulated as an acute phase cell-stress response to injury (Gentleman *et al.*, 1993a; Ciallella *et al.*, 1994; Roberts *et al.*, 1994; Graham *et al.*, 1995b). Further studies to assess possible interactions of APP with *c-fos, c-jun,* heat shock proteins and other molecules involved in acute stress responses would be of interest.

3) Our observations support the hypothesis that mechanical deformation, which is not severe enough to cause immediate cellular death, results in increased transcription of APP mRNA and a subsequent increase in APP antigen expression as an acute phase response to injury.

APPENDIX

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APPENDIX A

Buffers and solutions

Unless stated otherwise, all solutions were made using deionized water purified by reverse osmosis (RO water) provided by the Media Production Unit, Infectious Diseases Laboratories, IMVS and most chemicals were purchased from Ajax, BDH and Sigma. Concentrations given as percentages indicates % weight per volume for dissolved solids or % volume per volume for liquids. Buffers and solutions are listed alphabetically

2TY broth: 16 g/l tryptone, 10 g/l yeast extract, 85 mM NaCl (Difco).

2TY agar: 1.5% technical grade agar in 2TY broth.

Acid Wash: 100 ml concentrated sulphuric acid in 340 mM potassium dichromate.

Acetylation solution: 0.25% Acetic Anhydride, 0.1M Triethanolamine pH 8.0.

Bacteria: All plasmids were grown in *Escherichia coli* strain DH5α (Sambrook *et al.*, 1989)

BCIP (X-Phos) solution: 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (X-Phosphate), toluidinium salt in 100% dimethylformamide.

Citrate buffer: 0.1M Citric acid, 0.1M Sodium citrate pH 6.0.

Competent bacteria (*E coli* strain DH5α): a 200 ml culture of log phase DH5α (OD_{550} approximately 0.3), grown in 2TY broth was cooled on ice for 15 mins before removing the broth by centrifugation and replacing it with 64 ml cold transforming buffer 1 (100 mM RbCl, 50 mM $MnCl_2$, 30 mM potassium acetate pH 7.5, 10 mM $CaCl_2$, 15% glycerol, final pH 5.8). Bacteria were incubated in transforming buffer 1

for 15 min on ice, then this solution was replaced with 32 ml cold transforming buffer 2 (10 mM MOPS (3-[N-Morpholino] propanesulphonic acid) free acid, 10 mM RbCl, 75 mM $CaCl_2$, 15% glycerol).

CSL water: Cell culture grade sterile deionized water (Commonwealth Serum Laboratories).

Denhardts solution (50x): 1 mg/ml polyvinyl pyrrolidone, 2 mg/ml bovine serum albumin, 2 mg/ml ficoll 400.

Digoxigenin (DIG) buffer 1: 100 mM Tris pH 7.5, 150 mM NaCl.

DIG buffer 2: 1% (w/v) Blocking reagent for nucleic acid hybridization (Boehringer Mannheim) dissolved in DIG buffer 1.

DIG buffer 3: 100 mM Tris pH 9.5, 100 mM NaCl; 50 mM $MgCl_2$.

DNA gel loading buffer (10x): 50% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol.

Guanidine acetate buffer (GAB): 6 M guanidine HCL, 200 mM sodium acetate pH 5.5.

Hybridization solution for in-situ hybridization: 50% deionized formamide, 1 x SSC, 1 x hybridization buffer, 0.5 mg/ml sheared denatured salmon sperm DNA, 0.5 mg/ml

tRNA, 20 mM DTT, 1u/µl RNAsin (ribonuclease inhibitor, Promega), DIG labelled riboprobe.

Hybridization solution for northern blot hybridization: 1x Denhardts solution, 5x SSC, 0.1% SDS, 50% deionized formamide, 100 µg/ml sheared and denatured salmon sperm DNA, 10% dextran sulphate, 2.5 x 10^6 cpm/ml denatured ^{32}P-labelled DNA probe.

MOPS buffer (1x): 20 mM MOPS free acid, 5 mM sodium acetate, 1 mM disodium EDTA pH 7.0.

Nitroblue tetrazolium chloride (NBT) solution: 75 mg/ml NBT in 70% (v/v) dimethylformamide.

PBS (phosphate buffered saline): 140 mM NaCl, 3 mM KCl, 1 mM KH_2PO_4, 8 mM Na_2HPO_4 pH 7.4 (Sambrook *et al.*, 1989).

Prehybridization solution for northern blot hybridization: 5x Denhardts solution, 5x SSC, 0.1% SDS, 50% deionized formamide, 200 µg/ml sheared and denatured salmon sperm DNA, 50 mM Na_2HPO_4, 50 mM NaH_2PO_4

RNA gel loading buffer: 50% glycerol, 1 x MOPS buffer, 0.25% bromophenol blue.

SSC (1x): 150 mM NaCl, 15 mM trisodium citrate pH 7.0.

TAE (1x): 40 mM Tris(hydroxymethyl)Aminomethane (Tris), 20 mM glacial acetic acid, 1 mM EDTA, pH 8.0.

TE (1x): (10:1); 10 mM Tris–HCl pH 8.0, 1 mM disodium EDTA pH 8.0, (20:50), 20 mM Tris – HCl pH 8.0, 50 mM EDTA pH 8.0.

UREA-SDS buffer: 7 M urea, 350 mM NaCl, 50 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 0.2% SDS.

APPENDIX B (i)

Test 1-Effect of injury on the proportion of positive grids with APP positive neuronal cell bodies in each of the 4 locations tested from Chapter 3.

Impacted animals

Location/ parameter tested	Mean estimate	Asymptomatic standard error
Left hemisphere	0.55	.05
Right hemisphere	0.50	.04
Cerebellum	0.71	.04
Brainstem	0.67	.04

Control animals

Location/parameter tested	Mean estimate	Asymptomatic standard error
Left hemisphere	.05	.01
Right hemisphere	.05	.01
Cerebellum	.05	.01
Brainstem	.05	.01

Z values comparing impact group to controls over all 4 locations tested

Controls / **Impacted**	LH	RH	Cerebellum	Brainstem
LH	Z= 10*	-	-	-
RH	-	Z=11.25*	-	-
Cerebellum	-	-	Z=16.5*	-
Brainstem	-	-	-	Z=15.5*

* statistically significant increase in the proportion of grids with APP positive neuronal cell bodies in all locations tested in impacted group compared to controls.

Test 2- Effect of skull fracture on the proportion of grids with APP positive axons in impacted animals.

Location/parameter tested	Mean estimate	Asymptomatic standard error
No fracture	.01	.02
Linear fracture	.07	.01
Depressed fracture	.14	.02

Z values analyzing the effect of skull fracture on APP positive axons

Skull fracture	No fracture	Linear fracture	Depressed fracture
No fracture	-	-	-
Linear fracture	Z=3*	-	-
Depressed fracture	Z= 6.5*	Z= 3.5*	-

* Statistically significant increase in the proportion of grids with APP positive axons with the presence of a linear or depressed skull fracture.

Appendix B (ii)

Test 1- Effect of injury on the proportion of positive grids with APP positive neuronal cell bodies in each of the 4 locations tested in Chapter 7.

Impacted animals

Location/parameter tested	Mean estimate	Asymptomatic standard error
Left hemisphere	0.51	.06
Right hemisphere	0.23	.06
Cerebellum	0.74	.06
Brainstem	0.36	.06

Control animals

Location/parameter tested	Mean estimate	Asymptomatic standard error
Left hemisphere	.05	.01
Right hemisphere	.05	.01
Cerebellum	.05	.01
Brainstem	.05	.01

Z values comparing impact group with controls over all 4 locations tested

Controls / **Impacted**	LH	RH	Cerebellum	Brainstem
LH	Z=7.8*	-	-	-
RH	-	Z=3*	-	-
Cerebellum	-	-	Z=11.6*	-
Brainstem	-	-	-	Z=5.3*

* Statistically significant increase in the proportion of grids with APP positive neuronal cell bodies in all locations tested in the impacted group compared to controls.

Z values analyzing the effect of location on APP positive neuronal cell bodies

Locations	Left hemisphere	Right hemisphere
Left hemisphere	-	Z=7.8*

* There was a statistical difference between the proportion of grids with APP positive neuronal cell bodies in the left (impacted hemisphere) compare to the right (contralateral) hemisphere.

Appendix B (iii)

Quantitation: inter-observer variability using a Kappa test

APP immunopositive neuronal cell bodies from 16 randomly selected cases were semi-quantitated by an independent observer to assess inter-observer variability.

κ = the proportion of agreement after chance agreement is removed from consideration.

i.e. $$\kappa = \frac{p_o - p_c}{1.00 - p_c}$$

and p_o = the proportion of grades of AI in which there was agreement
p_c = the proportion of grades of AI for which agreement is expected by chance

		Judge A								
	neurons	**LH**		**RH**		**cerebellum**		**brainstem**		**Total B**
		0	**+**	**0**	**+**	**0**	**+**	**0**	**+**	
	0	3862	49	0	0	0	0	0	0	3911
	+	0	1216	0	0	0	0	0	0	1216
	0	0	0	3909	0	0	0	0	0	3909
Judge B	**+**	0	0	80	1147	0	0	0	0	1227
	0	0	0	0	0	982	0	0	0	982
	+	0	0	0	0	32	492	0	0	524
	0	0	0	0	0	0	0	1107	43	1150
	+	0	0	0	0	0	0	0	232	232
	Total A	3862	1265	3989	1147	1014	492	1107	275	13151

		Judge A								
	neurons	**LH**		**RH**		**cerebellum**		**brainstem**		**piB**
		0	**+**	**0**	**+**	**0**	**+**	**0**	**+**	
	0	.29	0	0	0	0	0	0	0	.30
	+	0	.092	0	0	0	0	0	0	.09
	0	0	0	.30	0	0	0	0	0	.30
Judge B	**+**	0	0	0	.087	0	0	0	0	.09
	0	0	0	0	0	.075	0	0	0	.08
	+	0	0	0	0	0	.038	0	0	.04
	0	0	0	0	0	0	0	.08	0	.09
	+	0	0	0	0	0	0	0	.018	.02
	piA	.29	.10	.31	.09	.08	.04	.084	.02	Σpi = 1.00

And $p_o = 0.98$
$P_c = 0.214$

$$\kappa = \frac{p_o - p_c}{1 - p_c} = \frac{0.98 - 0.214}{1.00 - 0.214} = 0.97 = 97\%$$

Thus a 97% proportion of agreement was reached between the two individual judges semi-quantitating APP positive neuronal cell bodies after chance agreement was removed.

Appendix B (iv)

Quantitation: intra-observer variability using Kappa test

APP immunopositive neuronal cell bodies from 16 randomly selected cases were re-quantitated to assess intra-observer variability.

Count 1

neurons	LH		RH		cerebellum		brainstem		Total B
	0	**+**	**0**	**+**	**0**	**+**	**0**	**+**	
0	3431	188	0	0	0	0	0	0	3619
+	0	923	0	0	0	0	0	0	923
0	0	0	3349	0	0	0	0	0	3349
+	0	0	197	911	0	0	0	0	1108
0	0	0	0	0	852	0	0	0	852
+	0	0	0	0	89	400	0	0	489
0	0	0	0	0	0	0	806	101	907
+	0	0	0	0	0	0	0	153	153
Total A	3431	1111	3546	1011	941	400	806	254	11400

Count 2

Count 1

neurons	LH		RH		cerebellum		brainstem		piB
	0	**+**	**0**	**+**	**0**	**+**	**0**	**+**	
0	.30	0	0	0	0	0	0	0	.32
+	0	.08	0	0	0	0	0	0	.08
0	0	0	.30	0	0	0	0	0	.30
+	0	0	0	.08	0	0	0	0	.10
0	0	0	0	0	.07	0	0	0	.07
+	0	0	0	0	0	.035	0	0	.04
0	0	0	0	0	0	0	.07	0	.08
+	0	0	0	0	0	0	0	.01	.01
piA	.30	.10	.31	.08	.082	.035	.07	.02	$\Sigma pi = 1.00$

Count 2

And $p_o = 0.945$

$P_c = 0.216$

$$\kappa = \frac{p_o - p_c}{1 - p_c} = \frac{0.945 - 0.216}{1.00 - 0.216} = 0.93 = 93\%$$

Thus a 93% proportion of agreement was reached between the two individual counts semi-quantitating APP positive neuronal cell bodies after chance agreement was removed.

APPENDIX C

ImageQuant volume integration for Chapter 5

Northern blot hybridization of impacted and non-impacted sheep over time

Quantitation of corrected density volumes of each band following normalization with GAPDH. Quantitation was done using a phosphoimager and volume integration function of ImageQuant v 3.3 software (Molecular Dynamics, Sunnyvale, Ca.).

1) Brain regions in an impacted animal using PV-Cap-1-Bam HI

Thalamus	Temporal cortex	Frontal cortex	Brainstem	Cerebellum
2633941	1462117	1304621	1096211	1142741

2) Impacts compared to controls with 2 hr survival times using PV-Cap-1-Bam HI

Impacts	Controls
2742132	831112
2531466	861727
2411726	846555
2362142	834119
Sum= 10047466	Sum= 3373513
Mean= 2511866	Mean= 843378
St Dev= 169166	St. Dev= 13939

3) Impacts compared to controls with 1 and 2 hr survival times using PV-Cap-1-Bam HI

2 hr impacts	2 hr controls	1 hr impacts	1 hr controls
2944400	874211	1993412	894630
2731162	863417	1970660	900132
3004721	880014	2014991	
3103410	870143	1993100	
Sum= 11783693	Sum= 3487785	Sum= 7972163	Sum= 1794762
Mean= 2945923	Mean= 871946	Mean= 1993040	Mean= 897381
St. Dev.= 157463	St. Dev.= 6981	St. Dev.= 18100	St. Dev.= 3890

APPENDIX C

ImageQuant volume integration for Chapter 5

4) Impacts compared to controls with 1 and 2 hr survival times using APP-KPI probe

2 hr impacts	2 hr controls	1 hr impacts	1 hr controls
1235621	1145878	1002000	1062542
1102398	904253	965842	1021542
1302547	1192140	1142513	
1152145	1132132	1124521	
Sum= 4792711	Sum= 4374403	Sum= 4234876	Sum= 2084084
Mean= 1198178	Mean= 1093600	Mean= 1058719	Mean= 1042042
St. Dev.= 88671	St. Dev.= 128815	St. Dev.= 87928	St. Dev.= 28991

5) Impact cases versus control cases with survival times of 15 and 30 mins using PV-Cap-1-Bam HI

15 min impact	15 min control	30 min impact	30 min control
845632	821115	803215	845211

APPENDIX C

ImageQuant volume integration for Chapter 6

Northern blot hybridization of impacted sheep with and without $MgSO_4$ therapy and controls with and without $MgSO_4$ therapy.

6) Impacts with and without $MgSO_4$ therapy using PV-Cap-1-Bam HI

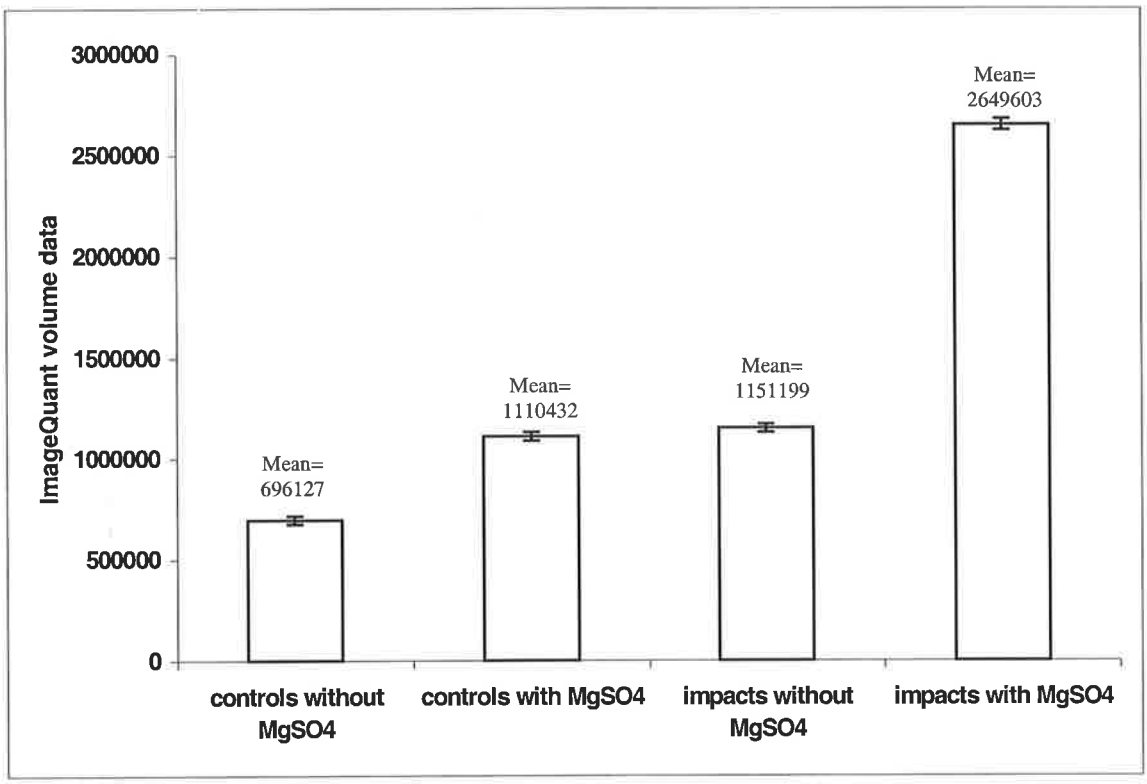
Impacts without $MgSO_4$	Impacts with $MgSO_4$
1114884	2671751
1156788	2684688
1169847	2624321
1160091	2633304
1154385	2616480
	2667074
Sum= 5755995	Sum= 15897618
Mean= 1151199	Mean= 2649603
St Dev= 20420	St. Dev= 22198

7) Controls with and without $MgSO_4$ therapy using PV-Cap-1-Bam HI

Controls without $MgSO_4$	Controls with $MgSO_4$
726272	1097774
703614	1096831
673144	1108402
694622	1148569
682983	1122995
	1088021
Sum= 3480635	Sum= 6662592
Mean= 696127	Mean= 1110432
St Dev= 21136	St. Dev= 28385

APPENDIX C

ImageQuant volume integration for Chapter 6



Histogram showing the mean volumes of the band densities of each group ± the standard deviation after GAPDH normalization.

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