Presynaptic Pathology after Acute Brain Injury

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Declaration

I declare that this thesis comprises my own original work and has not been accepted in any previous application for a degree. The work was performed by myself, except as acknowledged in this thesis. All sources of information have been suitably referenced.

Iain Murdoch

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ABSTRACTS

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Abbreviations

β-amyloid precusor protein	βAPP
Amyloid β-protein	A-β
Anterior cerebral artery	ACA
Acetylcholine	ACh
Acetylcholinesterase	AchE
Alzheimer's disease	AD
Apolipoprotein E	ApoE
Acetylcholine receptor inducing activity	ARIA
Bilateral common carotid artery occlusion	BCCAo
Basic fibroblast growth factor	bFGF
Bovine serum albumin	BSA
Boundary zone	Bz
Calcium/calmodulin protein kinase II	CAMKII
Choline acetyltransferase	ChAT
Central nervous system	CNS
Diffuse axonal injury	DAI
Enhanced Chemiluminescence	ECL
Extradural haematoma	EDH
Epidermal growth factor	EGF
Female	F
Growth-associated protein of 43kDa	GAP-43
Glyeraldehyde-3-phosphate dehydrogenase	GAPDH
Glial growth factor 2	GGF-2
Haematoxylin and eosin	H/E
Heregulin	HRG
Intracranial haematoma	ICH
Insulin-like growth factor 1	IGF-1
Luxol fast blue and cresyl violet	LFB/CV
Male	M
Microtubule-associated protein 2	MAP-2
Middle cerebral artery	MCA
Nicotinic acetylcholine receptor	nAChR

nucleus basalis of Meynert	nbM
Neu differentiation factor	NDF
Nerve growth factor	NGF
Phosphate buffered saline	PBS
Phosphate buffered saline + 0.5% defatted milk	PBS-M
Phosphate buffered saline + 0.1% Tween-20	PBS-T
Postmortem	PM
Peripheral nervous system	PNS
Reactive oxygen species	ROS
Sodium dodecyl sulphate – polyacrylamide gel electrophoresis	SDS-PAGE
Synaptosomal associated protein of 25kDa	SNAP-25
Subdural haematoma	SDH
Traumatic brain injury	TBI
Tris buffered saline + 0.1% Tween-20	TBS-T
Tris buffered saline + 0.1% Tween-20 + 0.5% defatted milk	T-TBS-M
Vesicular acetylcholine transporter	VAChT

Summary

This thesis addresses presynaptic pathology after acute brain injury. The integrity of presynaptic terminals after human head injury is not known and there are inconsistent reports regarding the sensitivity of presynaptic terminals in animal models of acute brain injury. Using a range of presynaptic markers, including high affinity nicotinic receptors and proteins specifically located within the nerve terminal, the question of presynaptic terminal integrity was studied in human postmortem material and compared to that in animal models of acute brain injury.

[^{3}H]-Nicotine binding

Deficits in cortical cholinergic neurotransmission have been implicated in the neurochemical sequelae of a head injury. Low nanomolar concentrations of [^{3}H]-nicotine were used to detect the density of high affinity nicotine binding sites in 3 cortical regions in controls and head-injured patients. No significant difference in the level of [^{3}H]-nicotine binding was detected in the cingulate gyrus, superior parietal cortex and the inferior temporal gyrus of head-injured patients compared with controls.

Presynaptic proteins after human head injury

A number of proteins, which include: synaptophysin, SNAP-25, syntaxin, synaptotagmin and synapsin I are specifically located on either the synaptic vesicle or associated with the nerve terminal membrane, and have been routinely used as markers of presynaptic terminal density. In homogenates of cingulate gyrus and the superior parietal cortex of controls and head-injured patients, a number of presynaptic proteins were detected by Western blotting. Synaptophysin, SNAP-25 and syntaxin immunoreactivity were significantly reduced by 28%, 36% and 43% respectively in the cingulate gyrus of head-injured patients compared with controls. However, synaptotagmin and synapsin I were not significantly different between groups. In the superior parietal cortex, there was no significant difference in the level of any of the presynaptic proteins in head-injured patients compared with controls, although all of the proteins showed a trend towards a loss. The association between cortical ChAT activity and synaptophysin levels in both the cingulate gyrus and the superior parietal cortex of head-injured patients, suggests that there is a loss of at least a proportion of cortical cholinergic presynaptic terminals after head injury.

nbM pathology after human head injury

The cortical mantle receives a widespread cholinergic innervation from Ch4 neurons of the nbM in the basal forebrain. Macroscopic evidence of damage to the nbM territory was evident in coronal sections from head-injured patients. Histological examination and semi-quantitative scoring of neuronal damage in the nbM demonstrated morphological changes including plastic creep, ischaemic cell change and infarction, in the nbM of 8 out of 12 head-injured patients. In contrast, none of the controls possessed any evidence of neuronal damage in the nbM. Correlation of the neuronal damage score attributed and ChAT activity in 3 cortical regions indicated that head-injured patients with low ChAT activity had extensive neuronal damage in the nbM. Immunohistochemistry with an antibody to ChAT verified that the nbM neurons with morphological abnormalities after head injury were cholinergic in origin. Association of Ch4 neuronal damage did not correlate with levels of any of the presynaptic proteins in the cingulate gyrus and the superior parietal cortex from head-injured patients. These data indicate that loss of cortical ChAT activity after a head injury, primarily reflects loss of Ch4 neurons in the nbM and not cortical presynaptic terminals.

Presynaptic pathology in animal models of acute brain injury

To obtain a temporal profile of the sensitivity of presynaptic terminals to brain injury, the levels of synaptophysin, SNAP-25 and syntaxin were determined in brain regions susceptible to ischaemic damage in rodent models of cerebral ischaemia. Western blot analyses indicated that levels of all three presynaptic proteins were preserved in the ipsilateral middle cerebral artery territory and cingulate gyrus compared with the contralateral hemisphere after 24h permanent middle cerebral artery occlusion in the rat. Immunohistochemistry combined with MCID image analyses was used to assess levels of staining for these presynaptic proteins in the caudate and a number of presynaptic inputs within the hippocampal formation after bilateral common carotid artery occlusion in the mouse. None of the presynaptic proteins were altered by either focal or global ischaemia in areas where there was evidence of neuronal damage. These data suggest that presynaptic proteins, and therefore presynaptic terminals are resistant to acute and chronic phases of brain damage in these animal models of brain injury.

Postsynaptic damage after brain injury.

The integrity of presynaptic terminal proteins was compared with that of MAP-2, as a marker of postsynaptic structures. In both the human postmortem brains of head-injured patients and global ischaemia in the mouse, early changes in the pattern of MAP-2 immunostaining were evident. Specifically, fragmentation and swelling of dendrites, and accumulation of MAP-2 immunoreactivity around neuronal perikarya. Moreover, an early loss of MAP-2 immunostaining was observed in anatomical regions displaying preservation of presynaptic proteins. This suggests that the major pathological changes following head injury and cerebral ischaemia are postsynaptic.

GGF-2 after human head injury

GGF-2 is known to promote nicotinic acetylcholine receptor subunit expression at the neuromuscular junction. The existence of GGF-2 in the human brain was demonstrated by Western blot analyses and immunohistochemistry. The level of GGF-2 was significantly increased in the cingulate gyrus of head-injured patients compared with controls. In head-injured cases, GGF-2 immunoreactivity accumulated in the neuronal cytoplasm of shrunken cell bodies and was prominent in glia. Although the level of GGF-2 was not increased in superior parietal cortex, the majority of head-injured patients possessed a level of GGF-2 immunoreactivity above that of the controls. Neuropathological evidence of presence or absence of ischaemic damage had no effect on the level of GGF-2 in head-injured patients, suggesting it is not the sole stimulus responsible for upregulation of GGF-2 after head injury. The lack of a GGF-2 response in the cingulate gyrus of Alzheimer's disease and the correlation with survival time in head-injured patients with a left subdural haematoma, imply that the elevation in GGF-2 is limited to acute (head injury) and not chronic brain injury (Alzheimer's disease).

GGF-2 in animal models of acute brain injury

The observation from human postmortem studies that GGF-2 was increased after acute brain injury, was supported by increases in GGF-2 in animal models of acute brain injury. Using Western blot analysis, GGF-2 immunoreactivity was increased in the ipsilateral caudate nucleus compared with the contralateral hemisphere after 24h permanent focal ischaemia in the rat (Intraluminal thread model). Similarly, GGF-2 immunoreactivity was significantly increased in the ipsilateral and contralateral cortex

4h after induction of subdural haematoma in the rat. These findings suggest a role for GGF-2 in the pathophysiological processes associated with acute brain injury.

CHAPTER 1: INTRODUCTION

1.1 Human Head Injury

1.1.1 Frequency and Financial Cost of Human Head injury

Head injury is an international clinical problem and remains the subject of ongoing research in the UK. Head injury is the leading killer in the productive period through age 40, and between the ages of 5 and 34 years exceeds all other causes of mortality combined (Baker et al., 1984). In the UK, there are an estimated 9 deaths from head injury per 100,000 population, accounting for 1% of all deaths and 25% of deaths from trauma (Graham and Gennarelli, 1997). Fortunately, the majority of head injured patients make an uneventful recovery, but others sustain irreversible brain damage. The incidence of disabled survivors of head injury is 100-150 per annum per 100,000 of the UK population, accounting for one family in 300 having a member with such a disability (Graham and Gennarelli, 1997).

In the USA, the estimated cost of head injuries ranges from $1 billion to $25 billion per annum (Pope and Tailor, 1991; Kraus et al., 1994), not to mention the social and economic stresses posed upon affected families. In view of the cost and sheer frequency of incidents, head injury is highlighted in 'The Health of the Nation' government white paper, as an important health issue with targets set aimed at reducing the number of deaths resulting from a traumatic event (MRC Field Review, 1998).

1.1.2 Types of Head Injury

Brain damage resulting from a head injury commonly referred to as traumatic brain injury (TBI) is complex, comprising of a number of causes, types and distribution. Head injuries are of two types: missile and non-missile (blunt), with the mechanisms of brain damage different in each type (Adams and Graham, 1988). Missile head injury involves objects that fall or are propelled through the air, frequently with entry of the object into the cranial cavity. A relatively poor prognosis is associated with missile head injuries. For example, gunshot wounds to the head frequently cause severe brain injuries with the mortality rate in some series exceeding 70% (Grahm et al., 1990; Stone, et al., 1995).

In comparison to the largely penetrative injuries associated with missile head injury, in blunt head injury it is the rapid acceleration/deceleration conditions existing at the moment of impact that initiate damage. Brain damage resulting from a blunt head injury accounts for the majority of brain injuries in modern society. For this reason, from this point onwards blunt head injury will be solely considered.

1.1.3 Causes of Human Head Injury

Based on a comprehensive neuropathological assessment of 635 fatal head injuries encountered in Glasgow over a 15 year period, the two commonest causes of head injuries were road traffic accidents (53%) and falls (35%), with the remainder largely comprised of assaults and sporting injuries (Graham and Gennarelli, 1997). Evidence of recent ingestion of alcohol was noted in 22% of head injured patients in Scotland (Strang et al., 1978).

1.2 Classification of Brain Damage

The classification of brain damage has to take into account two extremes: the patient who remains unconscious from the moment of injury until death; and the patient who is apparently normal after the initial injury but who, as a consequence of complication/s, lapses into fatal coma. From a neuropathological point of view, brain damage as a result of head injury is classified into two categories, namely primary damage which occurs at the moment of injury and takes the form of fracture of the skull, surface contusions and diffuse axonal injury; and secondary damage which is produced by complicating processes initiated at the moment of injury but do not present clinically for a duration of time after injury - these include brain damage due to raised intracranial pressure, ischaemia, intracranial haematoma, infection and swelling (Graham et al, 1995). In a series of over 1300 fatal blunt head injuries, Adams et al., 1980a, reported the frequency of principal primary and secondary types of head injury (Table 1).

The primary injury is influenced by a variety of predisposing factors such as age, undetected diseased state and a number of exogenous drug substances, particularly alcohol (Zink et al., 1993). Brain damage as a direct consequence of secondary damage has a profound effect on outcome after head injury.

Blunt Injury	Percentage
Primary Damage	
Skull fracture	75
Cerebral contusion and laceration	95
Diffuse axonal injury	
(using silver impregnation)	30
(using β-APP immunohistochemistry)	90
Secondary damage	
Intracranial haematoma	60
Cerebral oedema and brain swelling	53
Ischaemic brain damage	55
Infection	4
Secondary to raised intracranial pressure	75

Table 1: Classification and frequency of the principal types of blunt head injury.

Over recent years, improved awareness and prevention of secondary damage has reduced the mortality from severe head injury (Andrews et al., 1990; Murray et al., 1993). Recovery and functional outcome of head injured patients are the products of the primary damage, its delayed consequences, the secondary damage and the repair and rehabilitation achieved.

1.2.1 Primary Brain Damage

1.2.1.1 Skull Fracture

Presence of a skull fracture indicates the impact was of considerable force, but many individuals with a fracture of the skull do not sustain brain damage. The occurrence of a skull fracture depends on the material properties of the skull, the magnitude and direction of the impact, the size of the impacted area, and the thickness and strength of the skull in various areas. Patients with a skull fracture have a much higher incidence of intracranial haematoma (Servadei et al., 1988; Cooper, 1993) than patients without a skull fracture. Impact against a flat surface will produce fissure fractures, whereas, impact against small, irregular objects will produce a more localised, depressed fracture (Adams and Graham, 1988). There are a number of additional types of fracture with varying degrees of severity, and both highly localised and extensive distribution over the skull surface. These include hinge, coup, contrecoup, linear and basal fractures.

1.2.1.2 Cerebral Contusions

Surface contusions are common and are traditionally considered to be the hallmark of brain damage due to blunt head injury. Contusions occur at the moment of injury, brought about by the surface of the brain coming into contact with the bony proturbances in the base of the skull. They characteristically occur at the crests of gyri, but may also extend to deeper layers of the cortex, with or without involvement of subcortical white matter. Initially contusions present as groups of punctate haemorrhages, or streaks of haemorrhage perpendicular to the cortical surface (Graham and Gennarelli, 1997). Thereafter, the haemorrhage becomes more extensive, permeating into adjacent white matter. Irrespective of the site of injury contusions present a characteristic distribution in the brain, namely, the frontal lobes, the temporal poles, the lateral and inferior surfaces of the temporal lobes and the cortex above and below the Sylvian fissures (Graham and Gennarelli, 1997). Various identities have been ascribed to surface contusions: coup, contrecoup and gliding contusions to name a few. In an attempt to quantify both the number and distribution of cerebral contusions, the contusion index (Adams et al., 1980b, 1985) and the sector scoring method (Ryan et al., 1994) have been developed.

1.2.1.3 Diffuse Axonal Injury (DAI)

Although currently recognised as DAI, historically this injury has been defined by a host of descriptive terms including shearing injury (Peerless and Rewcastle, 1967), diffuse damage of immediate impact type (Adams et al., 1977) and diffuse white matter shearing injury (Zimmerman et al., 1978). It occurs particularly in patients who have been involved in road traffic accidents or as a result of falls from a considerable height (Adams et al., 1984) and may lead to a brief period of concussion or prolonged coma and ultimately an incomplete recovery (Graham and Gennarelli, 1997). The principal mechanical loading associated with induction of DAI is angular and rotational acceleration of the brain resulting from unrestricted head movement in the instant after injury (Gennarelli et al., 1982a; Thibault et al., 1990). This inertial loading to the brain induces shear, tensile and comprehensive strains leading to dynamic tissue deformation (Smith et al., 1997). Critical factors in the amount of axonal damage are the magnitude, duration and onset rate of the angular acceleration, as well as the direction of head motion: DAI is produced by long acceleration loading in the range of 20-25ms (Graham and Gennarelli, 1997).

In a review of 122 head-injured cases, Adams et al., 1989, devised a grading system for DAI. In grade 1 DAI, abnormalities are limited to histological evidence of axonal damage throughout white matter, without evidence of lesions in the corpus callosum or brain stem. Grade 2 DAI accounts for widely distributed axonal injury, along with focal lesions in the corpus callosum. Grade 3 DAI represents the most severe end of the spectrum and takes the form of widespread axonal damage, and focal lesions in the rostral brain stem in addition to the corpus callosum. The majority of blunt head injury cases sustain Grade 3 DAI (Adams et al., 1989).

Detection of diffuse injury to axons can only be determined microscopically. Historically, conventional histological techniques of haematoxylin and eosin, and silver impregnation were used. Using these techniques a survival after injury of 18-24 hours is required for the incontrovertible identification of axonal damage, i.e. axonal bulbs (Graham et al, 1995). However, adoption of more sensitive immunohistochemical techniques has allowed a diagnosis of DAI in patients who survive for only a short time after injury. A number of axonally transported proteins have been suggested as possible markers of axonal damage. In a comparative study using antibodies to a number of these proteins, immunostaining for β-amyloid precursor protein (β-APP) produced the most sensitive and reliable staining of axonal damage (Sherriff et al., 1994). The same group reported that axonal β-APP immunoreactivity was present in all patients who survived 3 hours or more. Consequently, β-APP is regarded as an excellent general marker of axonal disruption (Gentleman et al., 1993). Confirmation of the reliability of β-APP staining for detecting axonal injury has been provided by the reanalysis of twenty-five cases in which DAI had been diagnosed in eight using silver impregnation (Gentleman et al., 1995). The results generated indicated that the frequency of axonal injury had been grossly underestimated using conventional silver protocols and that axonal injury may in fact be a universal consequence of fatal head injury. In addition to staining techniques, computer tomography and magnetic resonance imaging are now being used to investigate DAI after head injury (Parizel et al., 1998).

1.2.2 Secondary Brain Damage

1.2.2.1 Intracranial Haematoma

Intracranial haematoma is a frequent complication of blunt head injury and represents the commonest cause of deterioration and death in patients who experienced a lucid interval after their injury. Although classified as a form of secondary damage, the haemorrhage is believed to begin at the time of injury - i.e. a primary event-, but the diagnosis of complications reflects an interval between the onset of the haemorrhage and appearance of clinical features of the expanding lesion. Multiple forms of intracranial haematomas exist, and these result in bleeding into the extradural, subdural or subarachnoid spaces of the brain, or into the ventricles. Extradural, subdural and intracerebral haemorrhages are the usual causes of expanding intracranial lesions after head injury.

1.2.2.2 Extradural Haematoma (EDH)

EDH occurs in approximately 2% of all types of head injury (Jamieson and Yelland, 1968; Lindenberg, 1971) and was present in 8% of cases in the Glasgow database (Graham and Gentleman, 1997). A fracture of the skull exists in the majority of patients presenting with an EDH. The artery most often damaged is the middle meningeal artery at the point where it runs across the squamous part or the temporal bone (Adams and Graham, 1988). As the haematoma enlarges it strips the dura from the skull to form a circumscribed ovoid mass which indents the adjacent brain tissue (Graham and Gentleman, 1997). Over a period of several weeks, the EDH may increase in size by up to 50%, thereafter becoming smaller, and completely resolved by 4-6 weeks after the injury (Graham and Gentleman, 1997).

1.2.2.3 Subdural Haematoma (SDH)

SDHs are present as thin smears of blood in the subdural space, which, unlike the localised nature of the EDH, spreads diffusely over the entirety of the affected hemisphere. A working classification defines SDH as acute when the haematoma is composed of blood, subacute when there is a mixture of clotted and fluid blood and chronic when the haematoma is fluid (Jennett and Teasdale, 1981). The majority of SDH arise as a result of rupture of the parasaggital bridging veins, although some have their origin from a cortical artery (Bullock and Teasdale, 1990). If the haematoma is not evacuated it may become encapsulated in a membrane and slowly increase in size, possibly due to repeated small haemorrhages into it (Markwalder, 1981). In these untreated cases, death is usually attributable to brain damage secondary to raised intracranial pressure and subsequent compression of the brain stem.

1.2.2.4 Intracerebral Haematoma (ICH)

ICHs generally occur in association with contusion and therefore exist particularly in the frontal and temporal lobes (Graham et al., 1995). Occasionally they are found in cerebellum and basal ganglia. Their precise pathogenesis is unclear, but it seems likely that they are caused by direct rupture of intrinsic cerebral blood vessels.

1.2.2.5 Cerebral Oedema and Brain Swelling

Three main types of brain swelling exist. Firstly swelling adjacent to contusions and ICHs. Common in white matter, a zone of damaged blood vessels exist where there is increased permeability at capillary level, resulting in loss of normal physiological regulation (Dietrich et al, 1994). Tornheim and McLaurin, 1978, reported an increase in water content of brain tissue around cerebral contusions, an observation later coined vasogenic oedema (Klatzo, 1979). Secondly, diffuse swelling of one cerebral hemisphere. There is a close association between diffuse swelling of this type and an ipsilateral SDH (Adams et al., 1980a). If during treatment the haematoma is evacuated, the brain expands to fill the empty volume created (Graham and Gentleman, 1997). Finally, diffuse swelling of the entire brain is normally seen in adolescents and children (Graham et al., 1989a). If one or more of these types of brain swelling persist, ultimately breakdown of the blood brain barrier will occur leading to cerebral oedema.

1.2.2.6 Infection

Although rare, infection exists in a small proportion of blunt head injury cases. In comparison, infection is relatively common in missile head injury as a consequence of infected material being carried directly into the cranial cavity. Nevertheless, meningitis is a well-recognised complication of a blunt head injury, due to spread of microorganisms through an open fracture of the skull (Adams and Graham, 1995). In a small number of head injuries a brain abscess may develop. Other complications include subdural empyema and cranial osteomyelitis (Graham and Gennarelli, 1997).

1.2.2.7 Raised Intracranial Pressure (ICP)

This is recognised as the most common cause of death and neurologic disability in patients with severe head injuries (Miller et al., 1977). Specifically, ICPs of 20-25mmHg are associated with a significant increase in morbidity and mortality (Miller, 1982; Marmarou, 1992). The usual causes are haematomas, contusions and associated brain swelling. As the mass lesion expands, further structural changes occur within the already injured brain. Generally, the surface of the brain becomes dry and flattened, there is a shift of the midline structures, the cingulate gyrus herniates under the free edge of the falx to produce selective displacement of the pericallosal arteries away from the midline (a supracallosal hernia) (Miller and Ironside, 1997), the parahippocampal gyrus is displaced through the tentorial incisura (a tentorial hernia) and there is compression of the brain stem (Graham et al, 1995) (See Figure 1). Despite increasing awareness of management and treatment of raised ICP, brain damage secondary to this is present in 75% of cases (Graham et al., 1987) and remains a common cause of deterioration and coma in patients who die within weeks of a severe head injury.

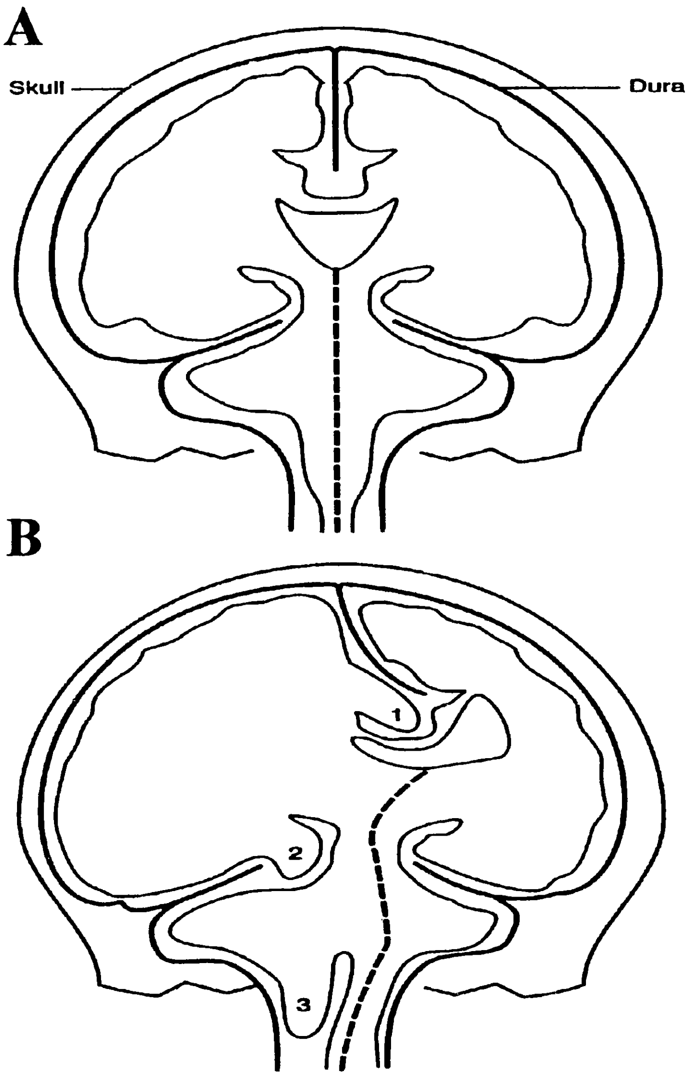


Figure 1. Diagrammatic representation of (A) normal intracranial compartments and (B) the effects of an expanding lesion within a cerebral hemisphere after head injury, showing anatomical distortion and shift of the midline structures. The locations of (1) subfalcine (cingulate gyrus), (2) transtentorial and (3) foramen magnum herniae are shown (Modified from Graham and Gennarelli, 1997).

1.2.2.8 Ischaemic Brain Damage

Of all the pathologic mechanisms that may operate in severely head-injured patients, ischaemic brain damage is a pervasive finding. Ischaemic brain damage is estimated to be present in approximately 90% of head-injured patients (Graham et al., 1978) and remains an important determinant of mortality and morbidity (Graham et al., 1989b). Cerebral ischaemia frequently occurs at the depths of sulci as a result of vascular compression between swelling gyri or between swollen hemispheres and adjacent structures (Rosenblum, 1989). Anatomically, cerebral ischaemia is most frequent in the hippocampus, basal ganglia, cerebral cortex and in the cerebellum (Graham and Gennarelli, 1997). Global insults such a hypoxia and hypotension, and focal events such as haemorrhage and raised ICP are believed to contribute to the structural evidence of ischaemic brain damage present after head injury (MRC Field review, 1998).

1.3 Experimentally-induced brain damage

1.3.1 Animal Models

Experimental models are crucial, not only for testing therapeutic agents, but for elucidating the mechanisms of brain damage and identification of novel agents (MRC Field review, 1998). The variability in the biochemical events present in human head injury has contributed to the wide variety of animal models used to study this phenomenon (Hayes and Ellison, 1989). The same authors emphasise the need for animal models to allow for the imposition of secondary insults given that human head injury involves insults of multiple aetiology. Animal models of head injury generally fall into 3 categories, namely percussion, acceleration and injection models. Percussion models are subdivided into 2 categories, fluid and rigid percussion models. The fluid model involves injection of fluid using an impacted fluid column or by rapid pump infusion (Lindgren & Rinder, 1969). The rigid percussion model (cortical impact) involves use of a solid indenter, applied to the dura at various velocities and depths of indentation. In the rat, Dixon et al., 1991, demonstrated this could produce a variable sized contusion in the parasagittal cortex beneath the impacts and coma of variable duration. Depending on conditions, acceleration models can produce relatively pure SDH (Gennarelli et al., 1982a), brief disturbances of consciousness or prolonged coma and DAI (Gennarelli et al., 1982b). The injection of blood or other fluids intracranially has been used to study various types of haematoma. Injection of blood into the subdural space mimics acute SDH (Miller et al., 1990; Bullock et al., 1991), and there are characteristically large areas of ischaemic necrosis underlying the SDH, of the type frequently seen in man. Neuropathological evidence of ischaemic brain damage is reported to be present in the majority of head-injured patients (Graham et al., 1978). Ischaemia per se has been studied using a number of permanent and transient models of focal (intraluminal thread, diathermy, microclips and endothelin) and global models (bilateral common carotid artery occlusion) of cerebral ischaemia in rodents (for review see Macrae, 1992). These rodent models provide a controlled environment to study the temporal profile and mechanisms involved in ischaemic brain damage. More recently, the pathological fluxes of a number of ions in ischaemic brain have been modelled *in vitro* using cultured neurons and hippocampal slices deprived of oxygen and glucose (Strasser and Fischer, 1995a,b).

Application of these animal models allows the routine use of histopathological, electrophysiological and biochemical techniques to study individual mechanisms of brain injury. However, clinically human head injury is rarely as pure as generated in these experimental models (Graham and Gennarelli, 1997). From a clinical series, solitary lesions in human brain occurs in 26% of cases, while two, three or more than three types of lesions occurs in 26%, 21% and 27% respectively (Gennarelli et al, 1983). As injury severity worsens, the multiplicity of different types of lesions increases and, except for very acute deaths, the incidence of single lesions in an autopsy series of head injured patients will be almost zero. The extrapolation to humans of data on head injury derived from animals must proceed cautiously (Hayes and Ellison, 1989), given the concern that some models may be studying traumatic phenomena rather than traumatic disease (Graham and Gennarelli, 1997). Associated with use of animal models is the assumption that species difference can be ignored, a concept difficult to accept given the anatomical, physiological and genomic differences existing between species (Graham and Gennarelli, 1997). These uncertainties emphasise the need for research on human tissues wherever possible.

Despite the complexity of animal models, they remain a key tool for the development of neuroprotective agents, providing an essential test-bed on which to determine efficacy, dose, safety and tolerability and hence therapeutic potential. Combined use of human head injury tissue and tissue generated using animal models has helped to understand the pathophysiology of head injury, resulting in the identification of key processes and a possible cascade of events that is likely to contribute to brain damage after blunt head injury.

1.3.2 Mechanisms of Traumatic Brain Damage

The physiological response to brain injury is extremely complex and involves the activation of an overlapping network of humoral, tissue and cellular pathways. The primary injury triggers a cascade of events that can lead to tissue and cellular death (Figure 2). These cellular changes may include alteration in neurotransmitter systems/receptor uptake, calcium-mediated damage, free radical effects and inflammatory events.

1.3.2.1 Neurochemical Changes

The excitatory amino acid neurotransmitters glutamate and aspartate produce irreversible damage in both neurons and glia *in vitro*, effects now termed excitotoxic (Rothman and Olney, 1986). Studies of mechanisms underlying selective neuronal vulnerability implicate the neurotoxic action of glutamate through activation of N-methyl-D-aspartate (NMDA) receptors and blockade of this receptor is associated with neuroprotection (Shohami et al., 1997). Using the subdural haematoma models in the rat, Bullock and colleagues, 1991, reported a correlation between the amount of glutamate released and the volume of ischaemic damage. Increases in cerebrospinal fluid (Baker et al., 1993) and brain (Zainer and Bullock, 1995) glutamate have been reported up to 4 days in head injured patients with focal brain injury. Competitive NMDA receptor blockade and modulation have been addressed in a variety of models as a strategy for treatment of head injury (for review see McIntosh et al., 1997). For several decades it has been known that there is an increase in the amount of acetylcholine in the brain and CSF of patients after head injury (Graham and Gennarelli, 1997). Moreover, in animal models of traumatic brain injury there is altered (Jiang et al., 1994) or attenuated numbers of M_2 muscarinic receptors (Ciallella et al., 1998). However, in human postmortem tissue,

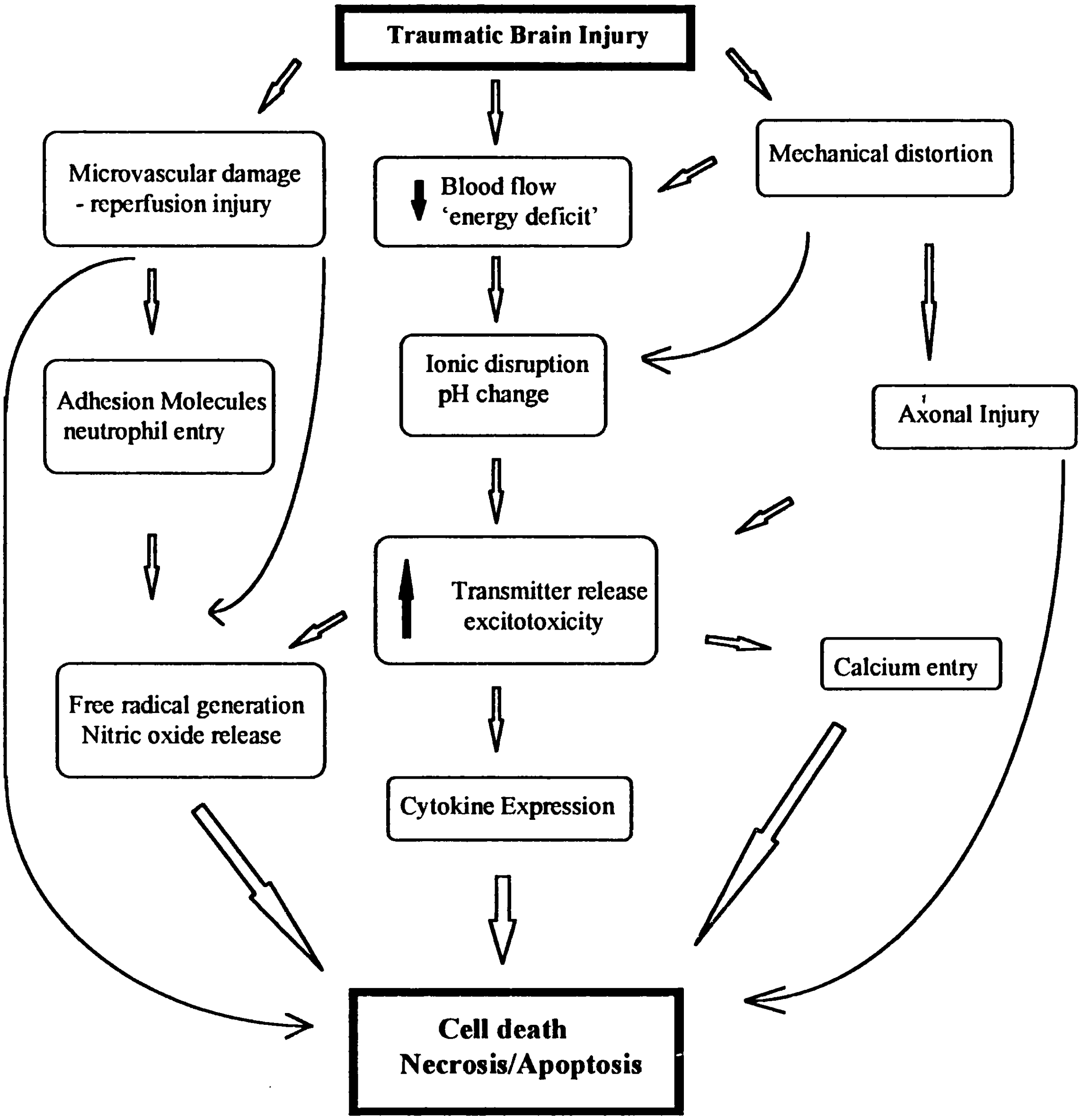


Figure 2. The cascade of events leading to cell death after blunt head injury.

Dewar and Graham, 1996, reported no change in M_2 muscarinic receptor binding after head injury.

Changes in other neurotransmitter systems have been reported in the brain after head injury. Regional alterations in the tissue concentrations of a number of monoamine and catecholamine neurotransmitters have been reported following experimental fluid percussion (McIntosh et al., 1994) and cortical impact injury (Prasad et al., 1992). In addition, Gennarelli, 1994, described changes in dopamine receptor densities after traumatic brain injury in man. Inhibitory neurotransmitter systems are also affected after trauma, given that there is a loss of GABAergic terminals in the cat lateral vestibular nucleus after head injury (Povlishok and Coburn, 1989).

1.3.2.2 Calcium-Mediated Damage

It has been suggested that alterations in Ca^{2+} homeostasis plays a major role in neuronal degeneration following trauma, and several recent studies have documented alterations in regional brain Ca^{2+} concentrations following head injury (McIntosh et al., 1997). Following a traumatic episode, Ca^{2+} can enter cells by mechanoporation, receptor-mediated or voltage-dependent mechanisms. Elevated total brain Ca^{2+} concentrations have been reported in injured areas following both fluid percussion injury (Shapira et al., 1989) and cortical contusion (Nilsson et al., 1993).

If Ca^{2+} concentrations remain increased within the cell, this will trigger a series of potentially deleterious events. Calpain, a cysteine protease is activated when Ca^{2+} concentrations exceed threshold levels (Saido et al., 1994). A number of cytoskeletal proteins are substrates of calpain and activation of calpain produces irreversible structural and functional alterations contributing to post-traumatic loss of cytoskeletal proteins such as spectrin (Kampfl et al., 1996), microtubule associated protein-2 (Taft et al., 1992), and neurofilament proteins (Postmantur et al., 1994). Dendritic damage is believed to occur more slowly or is causally linked to axonal disconnection (Folkerts et al., 1998). The progressive dissolution of these proteins causes blocks in axoplasmic transport within the cell, accumulation of axonal materials and ultimately delayed axonal disruption or secondary axotomy (Graham and Gennarelli, 1997).

1.3.2.3 Free Radical-Mediated Damage

Free radicals and oxidative stress-induced neuronal cell death have been implicated in a variety of neurological disorders. For almost two decades reactive oxygen species have been the focus of interest as possible candidates for the elicitation of various pathological responses after cerebral ischaemia or trauma. The brain uses approximately 20% of inspired oxygen (Ames, 1983). Although the majority of the oxygen is consumed for production of energy, a small proportion (approximately 5%) is used in alternative pathways, leading to the production of reactive oxygen species (ROS). The central nervous system (CNS) is particularly vulnerable to injury from ROS because of the relative lack of endogenous antioxidant defences, the abundance of stored iron (Zaleska and Floyd, 1985) and polyunsaturated lipids (Watson and Ginsberg, 1988). These highly reactive free radicals cause peroxidation of membrane phospholipids and nucleic acids, and can attack both neuronal membranes as well as the cerebrovasculature (McIntosh et al., 1997).

Despite free radical production being difficult to show in human brain, a number of animal models have shown that brain injury results in free radical production (Duckrow et al., 1986; O'Regan et al., 1994; Shohami et al., 1997; Doppenberg et al., 1998; Fabian et al., 1998). Multiple mechanisms are likely to induce production of free radicals after head injury. Likely candidates include activation of arachidonic acid release (Kontos and Povlishok, 1986), elevated intracellular calcium (Young, 1985) and endogenous kininogen and kinins (Heizer et al., 1988). The formation of these highly destructive species has been associated with a wide variety of pathologies and contributes to a worsened outcome in models of brain injury (Patel et al., 1992; Shohami et al., 1997).

1.3.2.4 Inflammation

Alterations in circulating immunocompetent cells have been reported in plasma obtained from head-injured patients (Piek et al., 1992; Quattrocchi et al., 1992). Disruption of the blood brain barrier following head injury typically allows entry of blood-borne constituents into the CNS. Specifically, the cytokines tumour necrosis factor (Goodman et al., 1990) and interleukins (IL)-1 and 6 (McClain et al. 1991; Woodroofe et al., 1991) are increased after head injury. Moreover, Yan and colleagues, 1992, reported elevated levels of IL-1 and IL-6 mRNA and receptors following mechanical brain injury, suggesting that CNS-derived cytokines may play a role in the pathophysiology of traumatic brain injury (Mocchetti and Wrathall, 1995).

Experimental identification of these injury mechanisms and elucidation of the timing of the pathologic neurochemical cascade initiated following head injury may provide enhanced opportunities for treatment with neuroprotective strategies designed to modify the synthesis, release, physiologic activity, or expression of pathogenic factors involved in these neurochemical cascades (McIntosh et al., 1997).

1.4 The Cholinergic System

1.4.1 Cholinergic Innervation of the Central Nervous System

Magnocellular neurons of the basal forebrain provide a massive and widespread cholinergic input to a number of targets within the telencephalon (Mesulam et al., 1983a). The neuroanatomy, cytochemistry, connectional topography and cortical distribution of cholinergic pathways originating in the basal forebrain have been investigated in considerable detail in the macaque monkey with the help of axonally transported tracer enzyme assays and immunohistochemistry (Mesulam et al., 1983a; Satoh and Fibiger, 1985; Kordower et al., 1989). Axonal tracing experiments in normal human brain (Selden et al., 1998) and analysis of patients with Alzheimer's disease (Mesulam and Guela, 1988) suggest projections to the human cerebral cortex is similar to that characterised in monkey.

Mesulam and colleagues (1983b) devised a nomenclature to classify bands of cholinergic neurons based on their efferent projections. The projections originate from four overlapping Ch1-Ch4 cell groups of the basal forebrain. Cholinergic neurons of the medial septal nucleus (Ch1) and the vertical limb nucleus of the diagonal band (Ch2) provide the major cholinergic input of the hippocampus; cholinergic neurons of the horizontal limb of the nucleus of the diagonal band (Ch3) provide the major cholinergic input of the olfactory bulb; cholinergic neurons of the nucleus basalis of Meynert (nbM-Ch4) provide the principal cholinergic input of the remaining cerebral cortex and amygdala (Mesulam et al., 1983b; Everitt et al., 1988) (see Figure 3). In addition, Ch5 and Ch6 neuronal groups provide the cholinergic input to multiple sites within the brain stem and especially the thalamus (Mesulam et al., 1983b; Woolf and Butcher, 1986).

All sectors of the human cerebral cortex receive dense cholinergic input. The origin of this projection is located in the Ch4 cell groups of the nbM (Selden et al., 1998). The Ch4 group provides the single major source of cholinergic innervation for the entire cortical surface, with an estimated 90% of nbM neurons cholinergic in origin (Mesulam et al., 1983b). An anatomical study by Eckenstein and colleagues (1988) illustrated the density of cholinergic innervation was uniform in all cortical areas, with the exception of

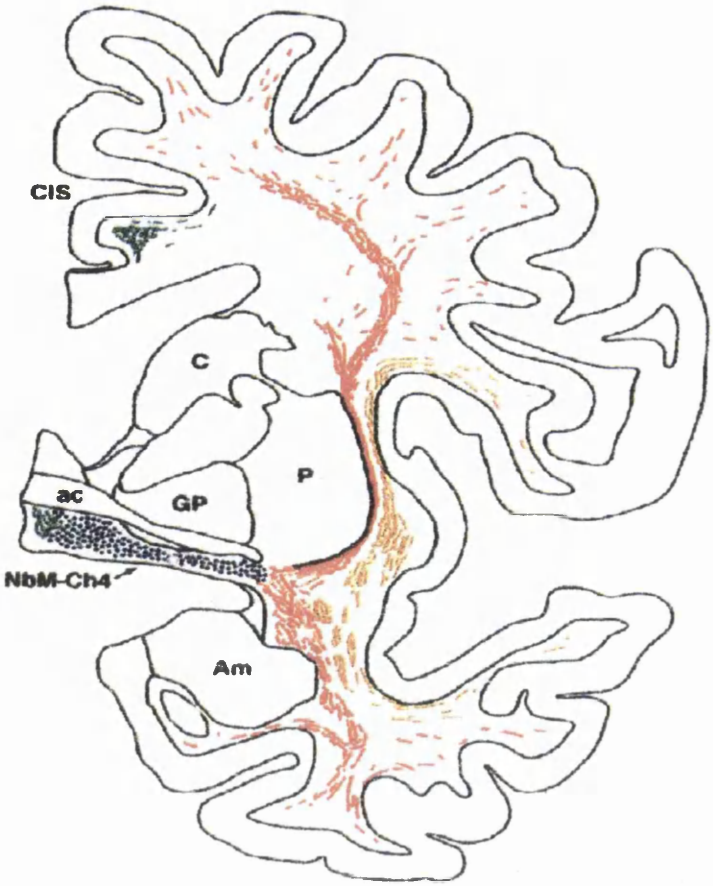


Figure 3. Schematic representation of the widespread innervation of the cortical mantle by Ch4 neurons of the nucleus basalis of Meynert. The two divisions of the lateral pathway, namely the capsular division (red) and perisylvian division (orange) are shown. The medial cholinergic pathway is represented in green and the cholinergic Ch4 neurons are shown in blue. Abbreviations: ac = anterior commissure; Am = amygdala; C = caudate; CIS= cingulate sulcus; GP = globus pallidus; P = putamen. (Modified from Selden et al., 1998).

entorhinal and olfactory cortex which show a marked increase in the number of stained terminals. Within the extensive Ch4 cell group of the nbM, two highly organised and discrete bundles of cholinergic fibres extend from the nucleus basalis to the cerebral cortex and amygdala; designated as medial and lateral cholinergic pathways. Selden and colleagues (1998) reported that the medial pathway joined the white matter of the gyrus rectus, curved around the rostrum of the corpus callosum to enter the cingulum. It supplied the paraolfactory, cingulate and retrosplenial cortices. The lateral pathway was subdivided into capsular and perisylvian divisions. Branches of the perisylvian division supply the frontoparietal operculum, insula and superior temporal gyrus, whereas the capsular division innervates the remaining parts of the frontal, parietal and temporal neocortex (Selden et al., 1998).

Although the majority of cholinergic innervation throughout the cortical mantle is provided by Ch4 neurons in the nbM, a proportion of cortical cholinergic innervation is obtained through intrinsic cortical cholinergic neurons (Eckenstein and Thoenen, 1983). In a subsequent study, Johnston and co-workers (1981) reported that these intrinsic neurons displayed no laminar pattern and that terminals of these cells represent a proportion of cholinergic terminals, possibly as much as one third of total cortical cholinergic terminals. However, more recently, Kasashima and co-workers (1999) found human cortical cholinergic neurons possessing both regional and laminal variations, particularly predominant in layers III and V.

1.4.2 Cholinergic Markers

Considerable experimental and pharmacological evidence has accumulated over the last two decades that associates a number of specific neurochemical systems in the brain with injury and loss of function. The main suspect is dysfunction of cholinergic neurotransmission, instigated through use of a host of histochemical, biochemical and immunohistochemical markers that allow for charting of the progress of cholinergic neuron status. Cholinergic neurotransmission requires biosynthesis of the acetylcholine (ACh), accumulation of ACh in synaptic vesicles, release of ACh, followed by degradation of excess ACh. A cholinergic marker protein exists for each of these cellular events.

1.4.2.1 Choline Acetyltransferase (ChAT)

ChAT is the biosynthetic enzyme for ACh, catalysing the conversion of choline and acetyl coenzymeA to ACh and coenzymeA in cholinergic neurons (Tucek, 1985). Transfer of choline, by high-affinity choline transport, is considered to be the rate-determining step (Vogelsberg et al., 1997), since the axoplasm of cholinergic neurons contains only a small supply of choline. ChAT exists in 2 isoforms, namely a cytosolic form and a membrane-bound isoform, with cytosolic ChAT accounting for the majority of the enzyme in nerve terminals. Immunohistochemistry using antibodies directed against ChAT has been used to delineate the anatomical distribution of presynaptic cholinergic elements in the CNS (Armstrong et al., 1983; Houser et al., 1983; Nagai et al., 1983).

1.4.2.2 Vesicular Acetylcholine Transporter (VAChT)

Cloned in 1994, VAChT was identified based on its similarity to the *Caellorhabditis elegans* putative ACh transporter unc-17 and the Torpedo vesamicol binding protein (Alfonso et al., 1993; Roghani et al., 1994; Varoqui et al., 1994). VAChT has since been shown to be an excellent presynaptic marker for terminal fields of the cholinergic nervous system (Schafer et al., 1995; Gilmor et al., 1996). In several species, Usdin and colleagues (1995) reported that the gene for VAChT is located within the first intron of the ChAT gene. Colocalisation of these two proteins within cholinergic neurons may suggest a common regulatory mechanism (Ciallella et al., 1998).

1.4.2.3 Choline Transport Inhibitor

Hemicholinium-3 is a very specific and potent inhibitor of the high affinity choline transport system (Guyenet et al., 1973; Simon et al., 1975). Autoradiographic distribution of [^{3}H]-hemicholinium-3 binding sites closely correlates with the localisation of presynaptic markers of the cholinergic system (Vickroy et al., 1985). Consequently, [^{3}H]-hemicholinium-3 is considered a specific marker of presynaptic cholinergic terminals (Happe and Murrin, 1992).

1.4.2.4 Acetylcholinesterase (AChE)

AChE is the destructive enzyme of the cholinergic system, responsible for hydrolysis of ACh. Although AChE is enriched in cholinergic neurons, it is also present in some nerve cells which do not utilise ACh; for this reason, AChE is not considered a marker specific for cholinergic neurons (Butcher et al., 1975). Eckenstein and Sofroniew (1983) reported AChE staining in additional neurotransmitter systems, particularly monaminergic systems in the brain.

1.5 Cholinergic Receptors

Within the central nervous system, cholinergic receptors are subdivided into either muscarinic or nicotinic, depending on agonist/antagonist profile, receptor subtypes and pre/postsynaptic distribution. In the CNS, muscarinic receptors are present as both heteroreceptors and autoreceptors regulating neurotransmitter release and regulation of higher cognitive functions (Raiteri et al., 1984). In this thesis however, the nicotinic receptor will be the subject of study in brain injury.

1.5.1 Nicotinic Acetylcholine Receptors

Neuronal nicotinic acetylcholine receptors (nAChR) are part of a gene family which include acetylcholine receptors from skeletal muscle and part of a gene superfamily which includes glycine receptors, $GABA_A$ receptors and 5HT-3 receptors (Ortells and Lunt, 1995), but not functionally related purine and glutamate receptors. Neuronal nAChRs are a family of ligand-gated cation channels that allow the passage of calcium ions. The nAChR complexes expressed in various brain regions differ from muscle nAChRs in composition, agonist and antagonist pharmacology, and biophysical profile (Role and Berg, 1996). Receptors in this superfamily are pentameric oligomers, composed of five subunits orientated around a central ion channel like barrel staves (Lindstrom et al., 1996), the staves comprising either α or β receptor subtypes (Cooper et al., 1991; Lukas and Bencherif, 1992). Application of recombinant DNA technology has revealed a rich spectrum of acetylcholine receptor subunit genes expressed by neurons (Sargent, 1993; Decker et al., 1995 and Albuquerque et al., 1997). To date, eight neuronal α (α2-α9) and three β (β2-β4) subunits have been cloned in chick and rodents (McGehee and Role, 1995) and also in human brain tissue (Anand and Lindstrom, 1990; Peng et al., 1994; Monteggia, 1995). The vast number of subunit permutations generates enormous heterogeneity of nAChR receptors (Wonnacott et al., 1996). Indeed, Alkondon and colleagues (1993 and 1994) reported that neuronal nAChR subtypes differ in their cation selectivities and permeabilities, in their activation and inactivation kinetics, and in their affinity for acetylcholine.

Of the α subunits, α8 and α9 possess somewhat different characteristics to other α subtypes. The α8 subtype has been cloned only from chicken (Schoepfer et al., 1990) and not from mammalian brain tissue. In the rat, the α9 subtype has a very restricted distribution, being localised in the pituitary and cochlear hair cells and is thought to be involved primarily in cholinergic efferent innervation of cochlear hair cells (Elgoyhen et al., 1994).

Studies of binding (Marks et al., 1986), functional neurochemistry (Wonnacott et al, 1989), electrophysiology (McGehee et al., 1995) and molecular biology (Galzi and Changeux, 1995) have shown that nAChRs and their subtypes are widely distributed in the central nervous system. nAChR are functionally subdivided into two principal classes, based on their affinity for nicotine or the snake toxin α-bungarotoxin with the anatomical distribution of α-bungarotoxin binding sites and nicotine binding sites being highly distinct in the brain. (Clarke et al., 1985).

1.5.1.1 α-Bungarotoxin Nicotinic Acetylcholine Receptor

This subset of nAChR possesses low affinity for nicotine but high affinity for α-bungarotoxin (Alkondon and Albuquerque, 1993). Unlike other nAChR, α-bungarotoxin nAChR do not consist of a plethora of subunits. In a Western blot study, Chen and Patrick, 1997, showed that α3, α5, α6, β2, β3 and β4 were not detected in the α-bungarotoxin binding nAChR purified from rat brains. When expressed in *Xenopus oocytes* only α7 and α9 form homo-oligomeric nAChRs and activation of these receptors can be blocked by α-bungarotoxin (Seguela et al., 1993). In view of the restricted distribution of α9 to hair cells, Seguela and co-workers (1993) reported that in rat brain at least, the α7 subtype is the main component of neuronal α-bungarotoxin binding nAChR. In rat brain, the distribution of α7 mRNA is similar to that of [^{125}I]-α-bungarotoxin binding sites (Clarke et al., 1985). Moreover, binding studies using [^{125}I]-α-bungarotoxin suggested that high affinity [^{125}I]-α-bungarotoxin binding sites in human brain are likely to be principally composed of α7 receptor subtypes (Breese et al., 1997).

The α-bungarotoxin nAChR is believed to be a homo-oligomer of five α7 subunits (See Figure 4B) which when expressed in various cell lines form functional homomeric ion channels that flux Ca^{2+} efficiently and desensitise rapidly (Vijayaraghavan et al., 1992; Puchacz et al., 1994).

Although the exact role of α7 nAChR in the central nervous system is unclear, there is a growing body of evidence suggesting that it is capable of diverse functions, including presynaptic modulation of transmitter release (McGehee et al., 1995; Gray et al., 1996), filtering of auditory stimuli (Rollins et al., 1993) and a possible involvement in the pathogenesis of schizophrenia (Freedman et al., 1994, 1997). In addition to a presynaptic role, Pugh and Berg (1994), proposed an extrasynaptic localisation and possible role in synaptic formation and stability.

1.5.1.2 High Affinity Nicotinic Acetylcholine Receptors

Numerous groups have used low nanomolar concentrations of $[^3H]$-nicotine to predominantly detect these high affinity nicotine binding sites. The principal receptor subunit combination that composes the high affinity $[^3H]$-nicotine binding site in both mammals and chickens consists of subunits α4 and β2 (Anand et al., 1991; Whiting et al., 1991). In immunoprecipitation studies greater than 90% of high affinity nicotinic agonist binding in the rat brain corresponds to a receptor composed of α4 and β2 subunits (Flores et al., 1992). Moreover, the distribution of α4β2 subunit combination has been reported to coincide with the distribution of high affinity $[^3H]$-nicotine binding sites in the rat brain (Wada et al., 1989). Marks and colleagues, 1992, found that α4 and β2 mRNAs predominate in the cortex and midbrain of mice, and α4 mRNA has been widely detected throughout the human cerebral cortex (Wevers et al., 1994). Although not as prevalent as α4 or β2, several studies suggest additional subunits, namely α2, and β4 may contribute to brain nicotinic cholinergic activity (Luetje and Patrick, 1991; Connolly et al., 1992). Nevertheless, nicotine has nearly 50-fold higher affinity for the brain α4β2 subtype than for non-α4β2 subtypes found in the adrenal gland (Flores et al., 1997).

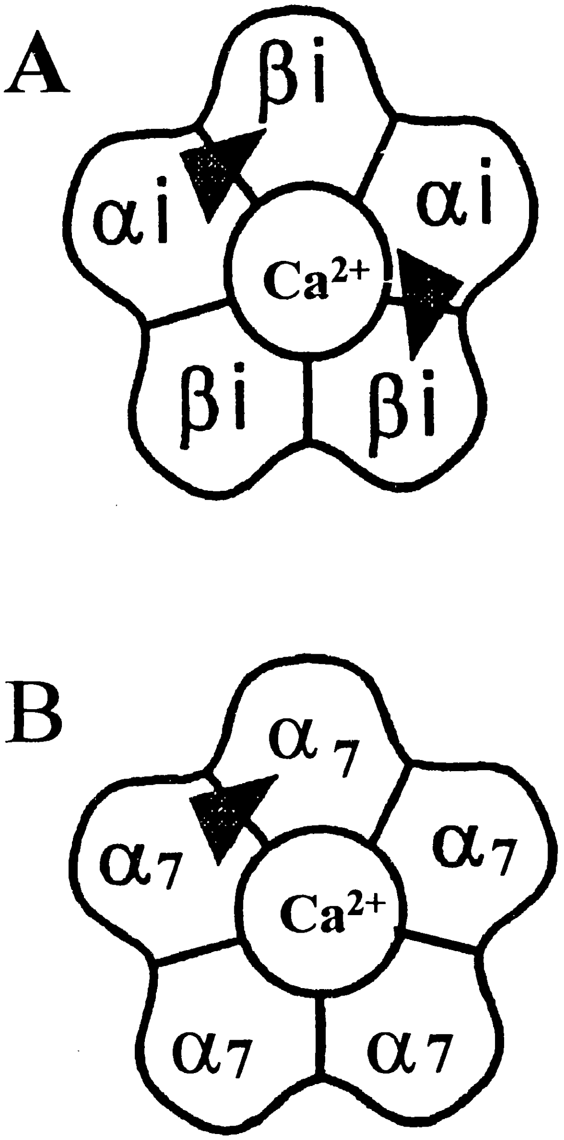


Figure 4: Pentameric models of high affinity nicotinic receptor, comprising both α and β subunits (A) and the α-bungarotoxin nicotinic receptor, comprising $\alpha7$ subunits only (B). Shaded area denotes agonist binding site.

The subunit stoichiometry of α4β2 nAChR expressed in *Xenopus laevis* oocytes has been deduced to be α4β2 = 2:3 (Anand et al., 1991; Cooper et al., 1991), with the subunits arranged around the central ion channel pore in order βαβαβ (Anand et al., 1991). (See Figure 4A). Contrary to this, Zwart and Vijierberg (1998) in a recent study reported that in *Xenopus laevis* oocytes combination of α4 and β2 subunits in subunit stoichiometry other than $(\alpha4)_2(\beta2)_3$ results in four additional forms of functional heteromeric nAChR.

Biochemical and molecular approaches have shown that the α4 subunit contains the agonist recognition site. The β2 subunit is a non-agonist binding subunit, which in combination with the α4 subunit increases agonist affinity and forms a functional nAChR (Sargent, 1993). Formation of an α4β2 subunit containing nAChR produces two agonist binding sites at the α/β subunit interfaces (Bertrand and Changeux, 1995).

1.5.1.3 Localisation of Nicotinic Acetylcholine Receptors

The exact cellular location of nicotinic cholinergic receptors in the brain, and particularly in the cortex is not yet known. Studies in humans and rodents indicate a presynaptic localisation for a proportion of nicotinic receptors (Wonnacott, 1997). However, other groups have reported the proportion of nicotinic receptors located presynaptically is small and that the majority of these high affinity nicotine binding sites are located postsynaptically (Court and Perry, 1994; Toghi et al., 1998). A hint at the cellular distribution of these receptors may be obtained by considering the function of these receptors. nAChRs are widely expressed in the nervous system, but their functions remain poorly understood. One hypothesis is that the receptors act presynaptically to modulate, rather than mediate, fast synaptic transmission in the brain (McGehee and Role, 1995). This feedback loop serves to introduce terminal regulation into the neural secretory event, representing a mechanism of fine-tuning the amplitude of chemical signals originating from the nerve ending (Langer, 1997). Considerable evidence has accumulated in favour of presynaptic nAChR that can stimulate the release of a variety of neurotransmitters in diverse brain regions (Wonnacott et al., 1990). Neurotransmitter systems affected include glutamate (Guo et al., 1998; Radcliffe and Dani, 1998), gamma-aminobutyric acid (Alkondon et al., 1997; Guo et al., 1998) dopamine (Marshall et al., 1997), serotonin and adrenaline (Li et al., 1998). Several electrophysiological studies have provided strong evidence for presynaptic nAChR by showing that nicotine applied to slice preparation increased spontaneous postsynaptic events in a manner consistent with transmitter release from nerve terminals (Lena et al, 1993; Bordey et al, 1996).

1.6 Cholinergic System in Brain Injury and Disease

1.6.1 Acute brain injury

One of the most debilitating consequences of head injury is an enduring impairment of memory and cognitive function. Oddy and co-workers, 1985, reported memory disturbances as the most frequent complaint by patients and relatives seven years posttrauma. Similarly, spatial learning impairment persists one year post-injury in rats (Pierce et al., 1998). Considerable evidence has accumulated that associates a number of specific neurochemical deficits with disrupted brain function. The importance of central cholinergic function in learning and memory was first postulated by Deutsch (1971). The body of evidence accumulated by biochemical, pharmacological and behavioural studies in the intervening years has supported the view that central cholinergic systems play a pivotal role in cognitive and mnemonic function (Bartus et al., 1982; Perry, 1986). In addition, nAChRs have been implicated in a variety of central processes, particularly learning and memory (Levin, 1992; Newhouse et al., 1994).

Dysfunction of cholinergic neurotransmission has been hypothesised as being centrally involved in memory impairment after head injury on the basis of evidence from experimental studies of TBI in rodents (Lyeth et al., 1990; Smith et al., 1991; Leonard et al., 1994). Microdialysis measurement of extracellular ACh levels have been used as a sensitive marker of cholinergic function within specific brain regions (Dixon et al., 1996). For example, Gorman et al., 1989, reported immediate release of ACh after head injury in rats. This is believed to be a transient event, in view of the reduced ability to release ACh from hippocampal cholinergic terminals from one to fourteen days after ischaemia in gerbils (Ishimaru et al., 1994).

The most compelling evidence in support of cholinergic dysfunction following head injury in human subjects comes from reports that pharmacological enhancement of cholinergic neurotransmission ameliorated memory impairment in some head-injured patients (Levin et al., 1986; Cardenas et al., 1994). Moreover, novel cholinomimetic therapeutics have been demonstrated to improve cognitive outcome following TBI in rats (Pike and Hamm, 1995; Chen et al., 1998a, 1998b). Injury-induced changes in proteins known to be involved in cholinergic neurotransmission underscores the importance of this system as a target for therapeutic intervention.

Specifically, TBI produces a time-dependent loss of ChAT activity (Gorman et al., 1996) and ChAT immunohistochemical staining (Leonard et al., 1994; Schmidt and Grady, 1995; Sinson et al., 1995). Using the global ischaemia model in gerbils, Ishimaru and colleagues, 1995, reported disappearance of ChAT immunoreactivity in the hippocampus four days after insult. Widespread loss of cortical ChAT activity has been reported in postmortem brain tissue (see Figure 5 from Murdoch et al., 1998) from patients who died following a blunt head injury. In accordance with this, high affinity [^{3}H]-choline uptake, but not available choline levels have been found to be impaired two weeks following TBI in rats (Dixon et al., 1994). The same group reported augmenting ACh synthesis, by increasing the availability of choline using CDP-choline treatment attenuated post-traumatic spatial memory performance deficits (Dixon et al., 1997a)

In addition to presumed abnormalities of presynaptic cholinergic input, alterations in cholinergic receptors following TBI in rats have been reported (Delahunty et al., 1994; Tanaka et al, 1994). In particular, altered (Jiang et al., 1994) or a time-dependent attenuation of the number of M_2 muscarinic receptors (DeAngelis et al., 1994; Ciallella et al., 1998) have been reported in experimental animal models. However, in human postmortem tissue, Dewar and Graham, 1996, reported no change in either M_1 and M_2 muscarinic binding following head injury. Integrity of nicotinic receptors following head injury may have importance in terms of cholinergic enhancement strategies given that ACh release can be modulated by nicotinic receptor activation (Marchi and Raiteri, 1996; McGehee et al., 1997).

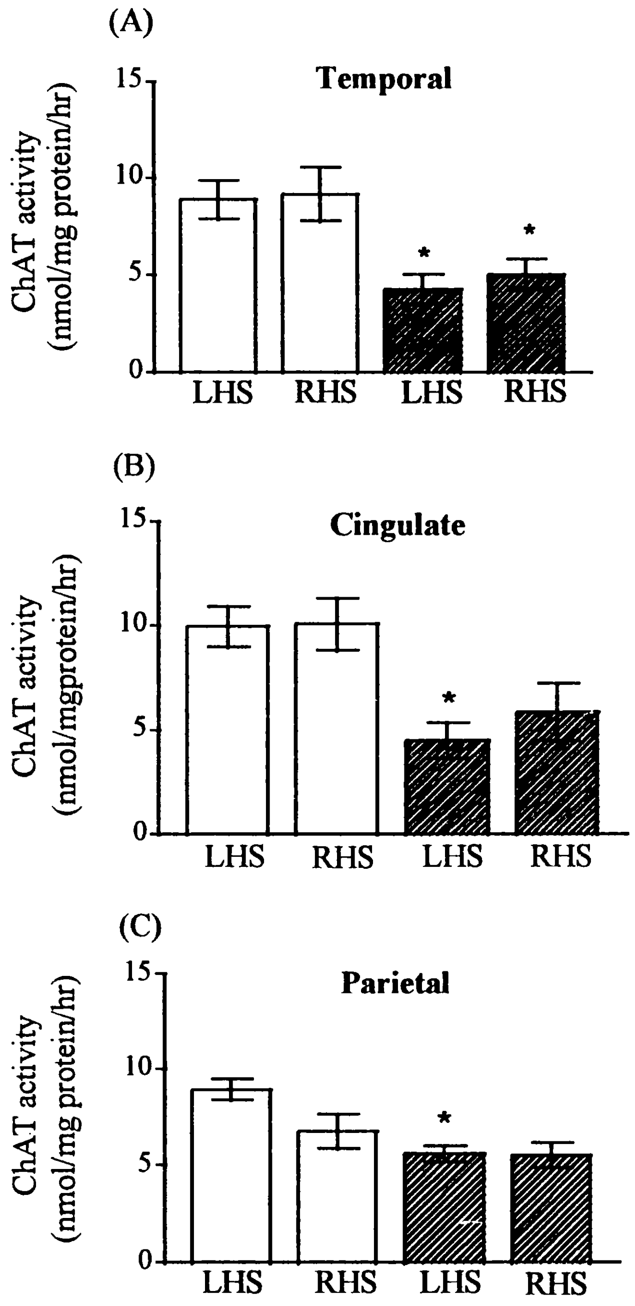


Figure 5: ChAT activity in the inferior temporal gyrus (A); cingulate gyrus (B); superior parietal cortex (C), in both the left (LHS) and right (RHS) hemispheres. Data for controls (n=8) are shown by open bars and for head-injured patients (n=16) by hatched bars. Data are mean ± SEM, with *$p<0.05$, compared to controls by unpaired two-tailed Student's *t* test. From Murdoch et al., 1998.

1.6.2 Alzheimer's disease

Abnormalities of cholinergic neurotransmission are the most consistent postmortem neurochemical finding in Alzheimer's disease (AD), in which memory deficits are central to the symptomatology (Perry, 1986). Reduced ChAT activity is the predominant neurotransmitter deficit, equivalent to a 60-90% reduction in the hippocampus and cortex of AD patients compared with age-matched controls (Bowen et al., 1976; Perry et al., 1978; Davies, 1979). Degeneration of Ch4 neurons have been reported with considerable variation in AD, ranging from an insignificant 18% reduction (Pearson et al., 1983) to an extensive 90% depletion (Whitehouse et al., 1981). Moreover, Pearson and co-workers (1983), using an antibody directed against ChAT, reported shrinkage rather than loss of Ch4 neurons. In addition, subsequent studies found no direct correlation between the ChAT deficit in cerebral cortex and extent of disappearance of cholinergic soma in the basal forebrain (Perry et al., 1982; Allen et al., 1990).

In conjunction with the ChAT deficits, nicotinic receptors have been shown to be reduced, by approximately 50% in neocortex (Whitehouse et al., 1986) and hippocampus (Perry et al., 1987) of AD brains compared to controls, with evidence emerging that specific nAChR subtypes may be involved, particularly α4β2 (Perry et al, 1995; Warpman and Nordberg, 1995; Martin-Ruiz et al., 1999). These pathological reductions in nicotine binding are believed to reflect changes in receptor numbers (βmax) and not affinity (Perry et al., 1990). Evidence of nicotinic receptor dysfunction has extended to additional neurological diseases including: Parkinson's, Senile Dementia of the Lewy Body type and Down's Syndrome (Perry et al., 1990).

In contrast, cortical muscarinic acetylcholine receptor binding have generally been found not to be decreased in AD (Nordberg, 1992). Nevertheless, there have been infrequent reports of the loss of putatively presynaptic M_2 receptors in AD (Mash et al., 1985; Aubert et al., 1992), and upregulation of postsynaptic M_1 receptors in Parkinson's disease (Perry et al., 1986). Similarly, it remains less certain whether binding to α-bungarotoxin nAChRs is reduced in the brains of AD patients, although a reduction of α-bungarotoxin binding has been reported in the thalamus of patients with dementia of the Lewy Body type (Court et al., 1999). Therefore, it appears it is the high-affinity nAChR rather than muscarinic or α-bungarotoxin receptors that demonstrate most variability during ageing and disease.

1.6.3 Overlap in Pathology of Head Injury and Alzheimer's Disease

There is increasing appreciation that the postmortem pathological correlates of a head injury share some similarities with those of AD. For example, AD-like pathology occurs in the brains of boxers (dementia pugilistica) suggesting that these two disorders share some common etiological and pathogenic mechanisms (Tokuda et al., 1991). As well as cholinergic dysfunction, additional landmark pathological features of AD are evident after head injury.

A panel of antisera that specifically stained the tangles of AD, also stained tangles in cases of dementia pugilistica (Roberts, 1988), with these tangles assumed to be the result of repetitive head injury. Extracellular deposition of amyloid β-protein (Aβ) plays a central role in the pathogenesis of AD (Hardy and Allsop, 1991; Selkoe, 1994). Furthermore, deposition of Aβ in the brain occurs in approximately one third of individuals who die shortly after a severe head injury (Roberts et al., 1994). Moreover, in brains from a small series of short-term survivors of head injury, Gentleman and co-workers (1997), found immunostaining for Aβ-42 in all parenchymal deposits, similar to the early Aβ-42 parenchymal deposits seen in AD.

Apolipoprotein E (ApoE) genotype is the most important genetic determinant of susceptibility to AD. The ApoE locus is associated with variations in the age of onset and risk of both familial (Strittmatter et al., 1993) and sporadic AD (Saunders et al., 1993). Inheritance of specific ApoE alleles accounts for more than two decades difference in the rate of disease expression (Strittmatter and Roses, 1995). Specifically, acquisition of one or two ApoE ε_4 alleles increased the rate of AD by 3-fold for each ε_4 allele inherited (Roses and Saunders, 1994). Using human postmortem material, Nicoll et al. (1995) reported the allele frequency of ApoE ε_4 in head-injured patients with A-β deposition to be higher than in most studies of sporadic AD. In human head injury, Teasdale and colleagues (1997), reported an association of ApoE polymorphism with outcome after head injury. In particular, patients possessing ApoE ε_4 were more than twice as likely as those without ApoE ε_4 to have an unfavourable outcome six months after head injury. Similarly, in boxers suffering from dementia pugilistica, inheritance of an ε_4 allele was associated with neurological impairment (Jordan et al., 1997).

1.6.3.1 Synaptic Pathology in Alzheimer's Disease

During the last ten years, increasing attention has been focused on synaptic pathology in AD, with loss of synapses now regarded as the major structural correlate of dementia in AD (Masliah, 1995). Loss or impairment of synaptic connections of the brain may produce important neurological deficits, including those of memory, cognition and behavioural functions (Hamos et al., 1989).

Several studies have shown that synaptic vesicles contain specific presynaptic proteins not enriched in other subcellular compartments (DeCamilli et al., 1983; Jahn et al., 1985). Immunodetection techniques used to identify synaptic proteins have been found to exhibit sufficient specificity for accurate quantitation of synapses (Leclerc et al., 1989; Lahtinen et al., 1993). As such, presynaptic proteins have proved valuable indexes of synaptic density and their concentrations have correlated markedly with synaptic loss (Gabriel et al., 1997).

Using an array of techniques, including direct counting at the ultrastructural level (Scheff et al., 1989; Masliah et al., 1992) and immunohistochemistry (Hamos et al., 1989; Masliah et al., 1989) or immunoblotting (Davidsson and Blennow, 1998), numerous studies exist providing evidence of synaptic degeneration in the hippocampus (Hamos et al., 1989; Davidsson and Blennow, 1998) and cortex (DeKosky and Scheff, 1990; Terry et al., 1991; Honer et al., 1992; Davidsson and Blennow, 1998). Whether these changes reflect degeneration and loss of whole synaptic elements in AD and not specific disturbances remains to be elucidated. Early indications suggest an element of differential involvement of the synaptic vesicle and presynaptic plasma membrane proteins in the pathogenesis of AD (Shimohama et al., 1997). Such findings highlight the importance of both the cholinergic system and adequacy of synaptic density in consideration of treatment of AD. It remains to be determined if the same combination of guidelines applies to the treatment of head injury.

In view of the pathological correlates of head injury and Alzheimer's disease, it seems possible that a presynaptic component may be involved in the pathogenesis of brain damage after head injury. It remains to be determined whether there is differential involvement of synaptic vesicle and presynaptic plasma membrane proteins in this process.

1.7 The Presynaptic Locus and its Associated Proteins

In the past few years, there has been major advances in understanding the molecular basis of synaptic vesicle exocytosis. The purification of vesicle-associated proteins and the isolation of their cognate genes (Jahn and Sudhof, 1994) have been facilitated by the abundance of synaptic vesicles in the brain. In 1995, Sudhof reported the identification of more than 10 presynaptic proteins (see Figure 6A). Subsequently they have been characterised and their role in synaptic transmission, through a complex array of protein-protein interactions have been elucidated (See Figure 6B).

1.7.1 Synaptophysin

Synaptophysin is the most abundant integral membrane protein of synaptic vesicles, constituting approximately 6-8% of the synaptic vesicle membrane proteins. Synaptophysin is believed to be present in most, if not all synapses of the brain (Wiedenmann and Franke, 1985), and estimated to be present in more than 95% of cortical presynaptic terminals (Navone et al., 1986). As a consequence synaptophysin is considered a representative marker protein of presynaptic terminals and has been routinely used as a reliable measure of synaptic density in postmortem brain (Hamos et al., 1989; Ince et al., 1995; Honer et al., 1997). Although synaptophysin was the first synaptic protein to be characterised in molecular terms, its function has remained unclear for a long time. The roles suggested are diverse, ranging from that of a vesicular Ca^{2+} sensor to a function in synaptic vesicle biogenesis (McMahon et al., 1995). Alternatively, synaptophysin has been proposed to be involved in the formation of a pore between the vesicle membrane and the presynaptic membrane during exocytosis (Thomas et al., 1988). Recent *in vitro* chemical cross-linking studies have suggested that synaptophysin may function as a molecular switch, controlling the association of synaptobrevin with other components of the neurotransmitter release machinery (Edelmann et al., 1995).

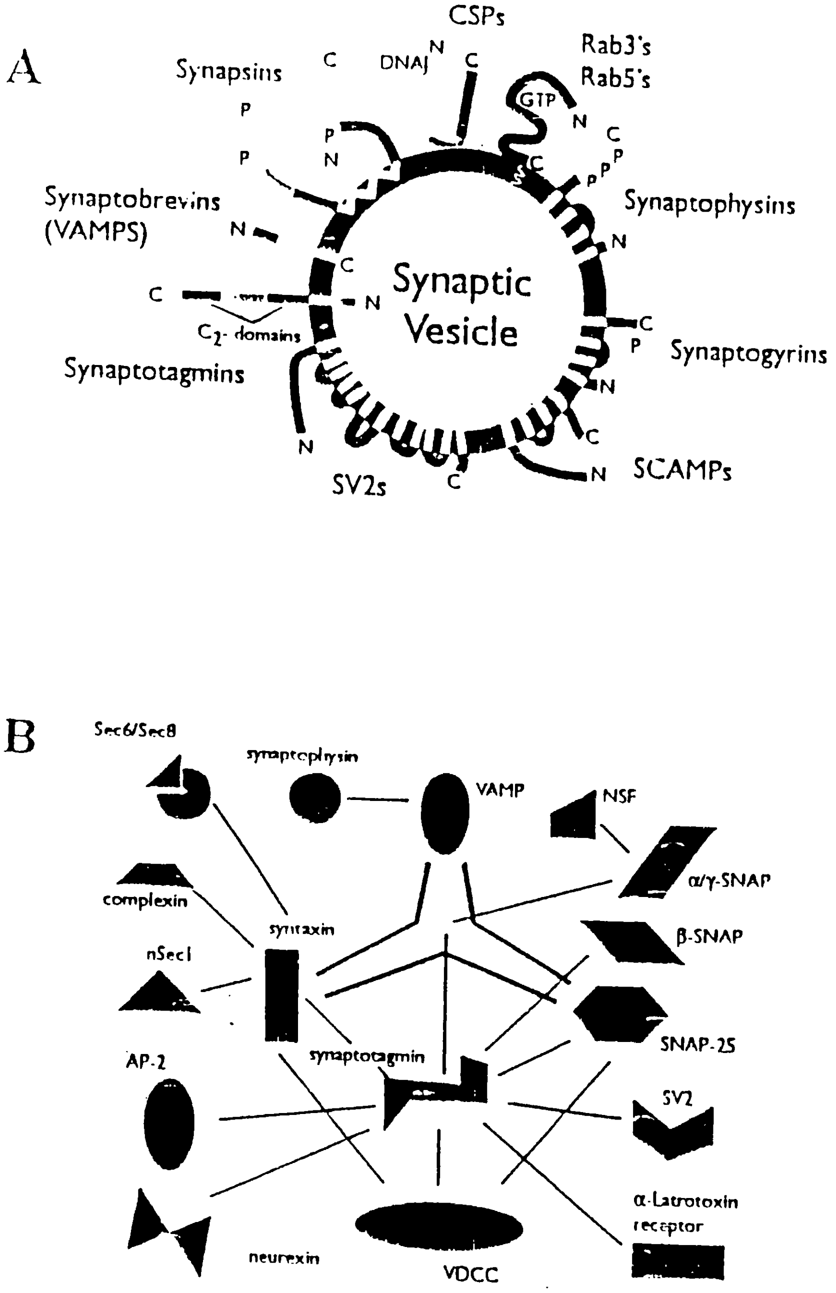


Figure 6: Schematic diagram of the major proteins located on synaptic vesicles (A), (Modified from Sudhof, 1995). Potential protein-protein interactions of proteins located on synaptic vesicles and presynaptic nerve terminal membrane (B), (Modified from Linial, 1997).

In contrast to these observations, synaptophysin knockout mice were viable and fertile, with mutant brains normal in structure and protein composition, and no detectable changes in probability of release (McMahon et al., 1995). These findings have led to the suggestion that this major synaptic vesicle protein is not essential for synaptic transmission, but merely represents an evolutionary remnant void of function (McMahon et al., 1995).

1.7.2 Syntaxin

The syntaxins comprise a growing gene family, with syntaxin 1 (syntaxin), consisting of syntaxin 1A and 1B, neuron specific, whereas others display a broader specificity (Bennett et al., 1993; Bock et al., 1996). Syntaxin is an integral membrane protein with a distribution in the CNS very similar to that of synaptosomal associated protein of 25kDa (SNAP-25) (Li et al., 1996). Syntaxin functions as a central component in regulated neurotransmitter release through interaction with proteins on the vesicle and plasmalemmal membrane. The interaction between syntaxin and other protein elements is believed to generate a core complex that forms the anchor for a cascade of protein-protein interactions leading to fusion and ultimately synaptic transmission. Moreover, via a direct association with N-type Ca^{2+} channels, syntaxin 1 is intimately involved in the structural and functional coupling of the action potential to neurotransmitter release (Sheng et al., 1994, 1998). Evidence that syntaxin participates in neurotransmitter release includes the reduction of secretion of PC12 cell generated by microinjection of a syntaxin antibody or protein fragments (Bennett et al., 1993) and selected cleavage of syntaxin by botulinum neurotoxin C (Blasi et al., 1993).

1.7.3 SNAP-25

Synaptosomal associated protein of 25kDa (SNAP-25) was first described by Wilson and colleagues as a neuron-specific protein, differentially expressed in the rat brain and localised primarily in axons and nerve terminals (Oyler et al., 1989). Two isoforms of SNAP-25 are generated by alternative splicing (Bark, 1993), namely SNAP-25a and SNAP-25b, predominant in embryonic and adult tissue respectively (Bark et al., 1995). Oyler and colleagues (1989) reported the primary structure of SNAP-25 is predominantly hydrophilic, with no apparent membrane binding domains. However, SNAP-25 is subject to covalent addition of fatty acids, particularly linkage of palmitate by thioester bonds to cysteine residues (Hess et al., 1992). Immunofluoresence and subcellular fractionation studies have shown that SNAP-25 is an integral membrane protein associated with the presynaptic plasma membrane (Oyler et al., 1989). Functionally, SNAP-25 serves as a key player firstly in axonal elongation and synapse formation (Catsicas et al., 1991; Osen-Sand et al., 1993) and secondly in membrane fusion events (Sollner et al., 1993b). In neurotransmitter release, SNAP-25 is an essential member of the release machinery, present as a tight complex with syntaxin responsible for specific trafficking of synaptic vesicles to the active zone of the presynaptic membrane (Chapman et al., 1994; McMahon and Sudhof, 1993). Moreover a number of studies have demonstrated interaction of SNAP-25 with α1a (Kim and Catterall, 1997) and α1b (Vance et al., 1999) subunits of Ca^{2+} channels located on the nerve terminals.

1.7.4 Synaptotagmin

Synaptotagmin 1 (Synaptotagmin) is an abundant synaptic vesicle protein and one of the first integral membrane proteins of the synaptic vesicle identified (Matthew et al., 1981). The brain expresses at least 8 synaptotagmins (Li et al, 1995), with different synaptotagmins differentially expressed in neurons (Sudhof, 1995). Synaptotagmin is regarded the putative Ca^{2+} sensor for neurotransmitter release, with evidence for such a role supported by a large number of genetic and microinjection studies (Littleton et al., 1994; Mikoshiba et al., 1995). In the presence of Ca^{2+}, synaptotagmin undergoes a conformational change, thereby releasing its 'fusion clamp' over neurotransmitter release (Shupliakov et al., 1996). In addition, Ca^{2+} coreceptors may cooperate with synaptotagmin in the control of the release process (DeBello et al., 1993; Sutton et al., 1995). Synaptotagmin is involved in docking of the synaptic vesicle to the presynaptic plasma membrane and exocytosis into the synaptic cleft (Brose et al., 1992). Therefore, synaptotagmin plays a dual role, as a specialised synaptic vesicle protein on the one hand and as a Ca^{2+} sensor on the other, thereby linking the signal for neurotransmitter release to the vesicle fusion machinery (Sollner and Rothman, 1996).

1.7.5 Synapsins

The synapsins consist of a family of 4 closely related proteins (synapsin Ia, Ib, IIa and IIb) (Sudhof et al., 1989), possessing extensive sequence homology in the N-terminal but divergence in the C-terminal (Benfenati et al., 1989). Accounting for approximately 6% of vesicle protein (Huttner et al., 1983), synapsin I is the most abundant of all neural phosphoproteins and is enriched on the cytoplasmic surface of synaptic vesicles. Synapsin I represents a major target for multiple protein kinases (Nicol et al., 1997). In particular, synaptic vesicle associated calcium calmodulin protein kinase II (CaMKII) seems to be the major enzyme responsible for phosphorylation of synapsin I at specific sites, resulting in changes in its structure and biological properties (Stefani et al., 1997). One major feature of synapsin I is its ability to simultaneously interact with synaptic vesicles and cytoskeletal elements, in a phosphorylation-dependent manner (Greengard et al., 1993). In *in vitro* reconstruction experiments Hirokawa and colleagues (1989) demonstrated synapsin I forms cross-bridges among synaptic vesicles and the cytoskeleton. Binding of synapsin I to synaptic vesicles and its regulation by phosphorylation is postulated to play a crucial role in the biological activity of synapsin I (Llinas et al., 1985). Phosphorylation of synapsin I by CaMKII reduces its affinity for synaptic vesicles 5-10 fold (Benfenati et al., 1992), therefore removing constraints on the availability for exocytosis. Synapsin I binds to multiple cytoskeletal elements, although, actin represents the main cytoskeletal substrate within the presynaptic terminal (Thiel, 1993) and synapsin I is believed to alter actin dynamics by binding laterally along actin filaments and cross-linking them into bundles (Ceccaldi et al., 1993; Baines et al., 1995). Interaction of synapsin I with actin is dramatically inhibited or virtually abolished by CaMKII regulated phosphorylation of synapsin I (Stefani et al., 1997). In this way, synapsin I may mediate reversible cytoskeletal anchoring of synaptic vesicles of the reserve pool to a cytoskeletal meshwork, preventing their direct contact with the presynaptic active zone.

Generally, synapsin I is assumed to have a central role in short-term regulation of neurotransmitter release and in the maturation of synaptic contacts during synaptogenesis (for review see DeCamilli et al., 1990a). Specifically synapsin I is part of a mechanism by which the nerve terminal can finely tune the efficiency of the neurotransmitter release machinery, accelerating synaptic vesicle trafficking during repeated stimulation (Rosahl et al., 1995). A role for synapsin I in synaptogenesis is demonstrated by injection of synapsin I into *Xenopus* blastomeres accelerating synapse formation (Schaeffer et al., 1994). In addition Chin and co-workers, 1995, showed that hippocampal cultures derived from synapsin I deficient mice exhibited a marked reduction in axonal elongation and rate of synapse formation. Although the identification and isolation of the core machinery mediating the release process has advanced during the past few years, it is unlikely that the entire plethora of molecular components have been identified and that the current picture will no doubt be refined. Indeed, in the last year a new generation of proteins implicated in synaptic transmission have emerged: endobrevin (Wong et al., 1998), SNAPIN (Ilardi et al., 1999), tomosyn (Fujita et al., 1998) and Vam7p (Ungermann and Winkner, 1998) to name a few.

1.7.6 Neurotransmitter Release: The SNARE Hypothesis

Neurotransmitter release requires the repeated release of neurotransmitter at high frequency at the nerve terminal. The locus of vesicular exocytosis has been ultrastructurally defined and consists of pre- and postsynaptic membrane specialisations and numerous synaptic vesicles in the nerve terminal (Tong and Jahr, 1994). Although neurotransmitter release shares a variety of properties with generalised secretion (DeCamilli and Jahn, 1990b; Bennett and Scheller, 1993), synaptic vesicle exocytosis is distinguished by unique temporal properties fundamental to its function in mediating fast, intermittent synaptic transmission.

A convergence of biochemical, genetic and cell-free studies have allowed the formulation of a model of docking and fusion: The SNARE hypothesis (Sollner et al, 1993a). Freeze-fracture studies suggest that this release apparatus is a highly structured entity (Heuser et al., 1979) ensuring the spatial specificity and short delay requirements of efficient neurotransmitter release. The SNARE hypothesis predicts that a key step in the sequence of events that leads to docking and fusion of a vesicle with the target membrane is the interaction of proteins localised on the cytosolic surface of the vesicle (v-SNAREs) with proteins localised on the cytosolic surface of the target membrane (t-SNAREs), see Figure 7. The v-SNARE is synaptobrevin and the t-SNAREs are syntaxin and SNAP-25 (Bauerfiend et al., 1996). Formation of the SNARE complex (7s complex) is followed by recruitment of the cytosolic factors α-soluble-NSF-attachment protein and Nethylmaleimide -sensitive factor to form the 20s complex. Finally the 20s complex disassembles in a reaction requiring ATP hydrolysis (Sollner et al., 1993b). As understanding of basic neurotransmitter release improves, attention will undoubtedly shift to how these molecules and events are modified to produce changes in synaptic efficacy (Sudhof, 1995).

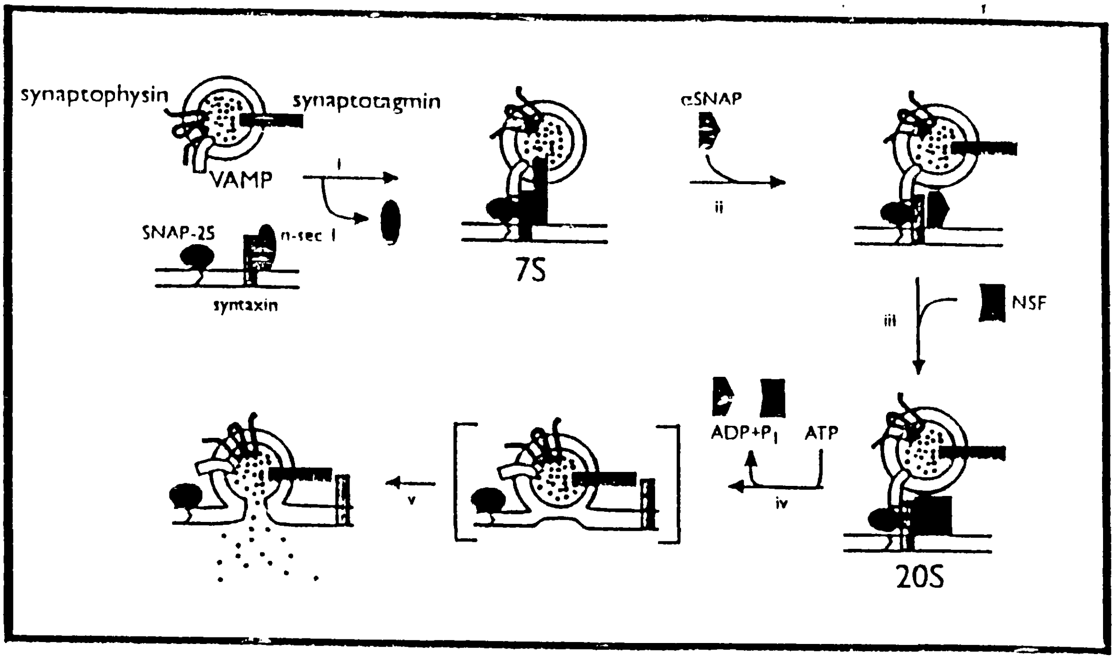


Figure 7: The SNARE hypothesis: Proposed model of molecular events involved in synaptic vesicle docking and fusion (Modified from Bennett, 1994).

1.7.7 Presynaptic Proteins in Acute Brain Injury

Release of neurotransmitter is the primary mode of communication and changes in efficacy of this process is thought to be at the basis of nervous system plasticity (Sudhof, 1995). Plasticity is believed to establish the restoration of function by changes in electrophysiological, anatomic and biochemical parameters (Stroemer et al., 1995). Synaptic plasticity has been extensively studied in the mammalian brain hippocampal formation, but the existence of CNS plasticity in the neocortex after trauma remains a controversial issue (Cotman and Nieto-Sampedro, 1982).

Focal cerebral ischaemia produces an area of infarction surrounded by neurons that may respond to nearby damage. The nerve cell is the most vulnerable to ischaemic damage in the central nervous system, although various subcellular components of neurons are thought to possess differential vulnerability (von Lubitz and Diemer, 1982), with both presynaptic and postsynaptic origins involved. A number of previous studies have used microtubule-associated protein 2 (MAP-2) as a sensitive marker of postsynaptic dendritic damage after cerebral ischaemia (Kitagawa et al., 1989; Dawson and Hallenbeck, 1996; Martinez et al., 1997). Alternatively, probes against various synaptic proteins have been used for studying neuronal plastic responses following a number of different CNS injuries (Kitagawa et al., 1992; Korematsu et al., 1993; Yamagata et al., 1995; Iwata et al., 1996). The study of the subtle molecular alterations of the synapses and of plasticity might yield important clues as to the mechanisms and pattern of damage after brain injury.

The issue of synaptic plasticity in the brain after injury has been studied in a vast number of species and experimental models, using an equally diverse range of marker proteins. Consequently, a divergent set of results exists, adding to the controversy of CNS plasticity after injury. Nevertheless, a general consensus suggests that presynaptic terminals may be relatively resistant to ischaemia in contrast to the higher susceptibility of postsynaptic sites (Kirino et al., 1990; Kitagawa et al., 1992). In contrast to the loss of MAP-2 immunoreactivity after cerebral ischaemia, Miyazawa and colleagues (1993), reported preservation of synaptophysin immunoreactivity. Similarly, Kitagawa et al., 1992, reported unchanged synapsin-1 immunoreactivity in the degenerated CA1 two months after insult. Moreover, electron microscopy studies revealed intact presynaptic terminals, abundant in synaptic vesicles, twelve months after the ischaemic event (Bonnekoh et al., 1990).

However, other groups have reported synaptic changes both in the form of a loss and increases in the level of presynaptic proteins in several models of brain injury. Synaptophysin has been reported to be increased in the cortex (Stroemer et al., 1995), the caudate (Korematsu et al., 1993) and the stratum lacunosum molecular layer and stratum oriens of the CA1 sector of the hippocampus (Miyazawa et al., 1993) after ischaemia, with the increase proportional to the severity of ischaemic injury. In situ hybridisation experiments have shown elevated growth associated protein of 43kDa (GAP-43) mRNA expression after ischaemic lesions in humans (Ng et al., 1992). In addition, kainate (Boschert et al., 1996) and ischaemia-induced lesions (Marti et al., 1998) both resulted in upregulation of SNAP-25 in the mossy fibre layer of the hippocampus. Moreover, Lynch and co-workers, 1994, reported augmented expression of three presynaptic proteins, namely, synapsin, synaptophysin and synaptotagmin, after long-term potentiation, a well studied form synaptic plasticity. In contrast, site-specific loss of presynaptic proteins have been reported following CNS injury. For example, SNAP-25 immunoreactivity is transiently reduced in the inner molecular layer of the dentate gyrus (Marti et al., 1998) and synapsin-1 is reduced in the ipsilateral caudate (Kitagawa et al., 1992) after ischaemic lesions. The spectrum of plastic changes may be a reflection of the inherent variability associated with use of different species and experimental models of inducing brain injury.

It remains essential to clarify mechanisms of damage of presynaptic terminals, since nerve terminals occupy the majority of the neuropil and their loss leads to massive destruction of nervous tissue. An understanding of the progression of molecular events that provide the basis for remodelling in the cortex after stroke/head injury will allow development of exogenous therapies to improve neuroanatomical plasticity after injury (Stroemer et al., 1995). One candidate that is thought to contribute to survival or remodelling is the endogenous production and supply of neurotrophic factors.

1.8 Neurotrophic Factors

Several peptide growth factors have been identified and implicated in the CNS response to trauma. The neurotrophin family consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3, -4/5, -6 (NT-3; NT-4/5; NT-6) (Korsching, 1993). The function of neurotrophic factors is mediated by binding to specific tyrosine kinase receptors (Chao, 1992). Each of the neurotrophins exert specific actions on distinct, but overlapping populations of developing and/or mature neurons (Ip et al., 1993). Functionally, the traditional view holds that neurotrophic factors are signaling proteins essential for neuronal survival and differentiation in the peripheral and CNS. The efficacy of neurotrophic factors to support cell survival has been most extensively studied in the cholinergic system (Hagg and Varon, 1993; Dixon et al., 1997b). In addition, neurotrophic factors have been suggested to have a more limited and specific role as retrograde modulators. Indeed, retrograde transport was first demonstrated with injection of [^{125}I]-labeled NGF into the terminal fields of sympathetic and sensory neurons in the peripheral nervous system (PNS) (Hendry et al., 1974) or cholinergic neurons in the CNS (Schwab et al., 1979). Similarly, injection of iodinated BDNF, NT-3 and NT-4 resulted in their retrograde transport to adult PNS and CNS neuron cell bodies (DiStefano et al., 1992; Sobreviela et al., 1996). Consequently, neurotrophic factors function as retrograde messengers, in doing so enhancing synaptic efficacy in targeted neuronal systems.

1.8.1 Neurotrophic Factors in Synaptic Plasticity

A novel role for the neurotrophins is their participation in the dynamic modulation of synaptic efficacy (Levine and Black, 1997). Neuronal impulse activity is known to enhance neurotrophin production and secretion (Thoenen et al, 1991), which in turn potentiates neuronal activity and synaptic efficacy. A number of recent studies have demonstrated this capacity of neurotrophic factors to enhance efficacy of synaptic transmission. In hippocampal slices, BDNF and NT-3 enhanced the strength of Schaffer collateral CA1 synapses (Kang and Schuman, 1995). Using dissociated cell culture of rat cortical neurons, Kim et al., (1994), reported NT-3 induced a 5-6 fold increase in the frequency of spontaneous action potentials. Moreover, BDNF and NT-3 potentiated evoked synaptic currents at the *Xenopus* neuromuscular junction (Lohof et al., 1993). In addition to these acute effects, long-term effects of neurotrophic factors on synaptic function, particularly plasticity have been addressed in the visual system (Gu et al., 1994).

All of the observations are in favour of a presynaptic action of neurotrophic factors, possibly through potentiation of availability of loaded vesicles or in the probability of vesicular exocytosis (Stoop and Poo, 1996). Using cultured rat cortical neurons, Takei and colleagues (1997) reported BDNF and NT-3 elevated the levels of a number of both vesicle-associated and membrane-associated proteins of the neurotransmitter release machinery. In addition, neurotrophin treated *Xenopus* neuromuscular cultures showed an enhanced expression of synapsin I and synaptophysin (Wang et al., 1995). While different cytoplasmic transduction mechanisms are triggered by various neurotrophin factors, their actions are likely to converge on common effector proteins involved in presynaptic release, possibly protein kinases downstream of putative proteins involved in trafficking, docking and exocytosis of synaptic vesicles (Stoop and Poo, 1996).

1.8.2 Neurotrophic Factors and Brain Injury

Several recent reports have suggested that neurotrophic factors are synthesised or released following brain injury, perhaps to facilitate neuronal repair mechanisms and re-establish function in injured brain (Varon et al., 1991). The evidence for involvement of neurotrophins and other peptide growth factors in neuronal plasticity evolves from observations made in complex integrated neuronal systems *in vivo* and from detailed analyses in a variety of *in vitro* systems.

NGF is the best known and most widely studied of all the neurotrophic factors in relation to acute brain injury, with evidence emerging that astrocytes are an important site of NGF production following brain injury in rats (Goss et al., 1998). NGF is reported to be acutely increased in a number of models of acute brain injury (for review see McIntosh et al., 1997). In humans, NGF is elevated in CSF following head injury (Patterson et al., 1993). Like NGF, basic fibroblast growth factor (bFGF) is postulated to participate in the protection and repair processes of neurons following brain injury. Regional increases in bFGF have been shown following lesioning of the entorhinal cortex and fimbria-fornix transection (Gomez-Pinella et al., 1992), and after cortical trauma (Logan and Berry, 1993). *In vivo*, bFGF attenuated infarct size following permanent focal cerebral ischaemia in the rat (Koketsu et al., 1994). Insulin-like growth factor-1 (IGF-1) has been evaluated as a potential therapeutic neuroprotective agent in several *in vivo* models. Sandberg-Nordqvist and colleagues (1996), reported upregulation of not only IGF-1, but IGF binding protein-2 and -4 mRNAs following cerebral contusion. IGF-1 has been found to improve both neurological and motor cognitive outcome following experimental brain injury (Saatman et al., 1997). Alterations in BDNF and NT-3 mRNAs have been demonstrated in rat hippocampus after experimental brain trauma (Hicks et al., 1997). Moreover, exogenous BDNF administration prevented neuronal death following global ischaemia in the rat (Kiprianova et al., 1999)

1.8.3 Neuregulins

One growth factor signaling system receiving recent attention is that involving the neuregulins. The name neuregulin reflects signaling through *neu* and related receptors and suggests involvement of these molecules in regulating neural development and function (Marchionni et al., 1997). The neuregulin family of growth factors, also known as glial growth factor (GGF) (Brockes et al., 1980), as acetylcholine receptor inducing activity (ARIA) (Falls et al., 1990), as neu differentiation factor (NDF) (Peles et al., 1992) and as heregulin (HRG) (Holmes et al., 1992) are all products of a single gene located on the short arm of human chromosome 8 (Orr-Urteger et al., 1993). The neuregulins belong to the epidermal growth factor (EGF) superfamily and exhibit some 15 membrane-attached and secreted isoforms, generated by alternative splicing and usage of 3 distinct promoters. The majority of the neuregulins are synthesised and released as biologically active, membrane-bound precursors termed pro-neuregulins which are subsequently processed to produce the soluble secreted forms of neuregulins (Burgess et al., 1995).

Neuregulins mediate their biological activities through members of the EGF receptor (or ErbB) superfamily, namely, ErbB2, ErbB3 and ErbB4. Experiments in cell culture demonstrated that neuregulin receptors can be constituted by ErbB2/ErbB3 or ErbB2/ErbB4 heterodimers and ErbB4/ErbB4 homodimers, but not by ErbB2/ErbB2 or ErbB3/ErbB3 homodimers (for review see Lemke, 1996). Following ligand binding and receptor dimerisation, transduction of cellular signals occurs through autophosphorylation of tyrosine residues. Pinkas-Kramarski and colleagues (1996) reported neuregulin-induced responses appeared to depend upon the erbB heterodimer formed and the subsequent interaction of the receptors with distinct signaling pathways.

The study of neuregulins has advanced through the generation and analysis of mutant mice. Disruption of the neuregulin gene (Meyer and Birchmeier, 1995), ErbB2 (Lee et al., 1995) and ErbB4 (Gassman et al., 1995) have shed light on the molecular interactions of this signaling system. Mice lacking functional neuregulin or erbB receptors died *in utero* due to aberrant cardiac and nervous system development. In contrast, mice heterozygous in the disrupted gene appeared healthy and fertile (Carraway, 1996). These observations imply the inactivated genes are essential and that they are not functionally or developmentally redundant.

Neuregulins are expressed by both peripheral and central neurons (Marchionni et al., 1993; Chen et al., 1994) and are believed to be involved in numerous interactions between neurons and glia. In general, neuregulins are regarded as multi-functional regulators influencing cellular proliferation, differentiation, survival or fate (for review see, Carraway and Burden, 1995). The functional properties of the neuregulin family have been in the main characterised in a number of *in vitro* systems. Consequently, the neuregulins are known to induce a variety of responses in cultured cells. For example, neuregulins have been shown to promote astrocyte survival and differentiation in cerebral cortical dissociated cell culture (Pinkas-Kramarski., 1994) and to enhance the development of oligodendrocytes from bipotential O_2A glial progenitor cells (Vartanian et al., 1994). While most of the reported effects are on non-neuronal cells, evidence is accumulating for a direct effect of neuregulins on neurons. In particular, at least one of the neuregulin receptors (ErbB4) is expressed by brain neurons in many different regions of the brain (Lai and Lemke, 1991). Moreover, Bermingham-McDonogh (1996) reported that the neuregulins act directly on retinal ganglion neurons to promote neurite outgrowth and neuronal survival.

1.8.3.1 Neuregulins as Indirect Neurotrophins

As a consequence of the specificity of peptide growth factors for tyrosine kinase receptors, no single trophic factor factors is expected to rescue all neurons afflicted in neurological disease and injury (Marchionni et al., 1996). Several lines of evidence support the concept that neuregulins act as indirect neurotrophins via induction of neurotrophin synthesis and secretion. Verdi and co-workers, 1996, reported NT-3 expression was upregulated 40-fold in glial cells treated with recombinant human GGF-2. Furthermore, conditioned media from schwann cells contained soluble neurotrophic activities, possibly NGF (Marchionni et al., 1997).

1.8.3.2 Neuregulins and Plasticity

The persistent expression of both neuregulin and its cognate receptors in the postnatal and adult heart suggests a continuing role for neuregulins in the myocardial adaption to physiologic stress or injury (Zhao et al., 1998). The continued and abundant expression of the neuregulins in the adult CNS suggests that the neuregulins are involved in the response of the brain to environmental stimuli. Indeed, Eilam and co-workers (1998) have shown that brain neuregulin and ErbB4 are induced by 3 types of stimuli, namely: use of a glutamate receptor agonist, locomotion and tetanic stimulation. These observations strengthen the argument that the neuregulin family of ligands participates in activity-dependent brain processes and in neuronal plasticity. In conjunction with the pleiotrophic actions of the neuregulins in the nervous system, they have the potential for the treatment of a variety of neurological disorders, including stroke or traumatic brain/spinal cord injury. Whether GGF-2 exists in the human postmortem brain, and at what level after head injury remains to be determined.

1.9 Aims of Thesis

The sensitivity of neurons and glial cells to brain injury has been well characterised. However, the question of whether presynaptic terminals are damaged after acute brain injury has received less attention. The integrity of presynaptic terminals after human head injury is not known and there are inconsistent reports regarding the sensitivity of presynaptic terminals in animal models of acute brain injury. Presynaptic terminals serve to regulate the highly orchestrated process of neurotransmitter release. Since neurochemical deficits have been reported in neurodegenerative disorders and after head injury, the status of presynaptic terminals is of importance regarding possible therapeutic approaches.

The main aims of the work described in this thesis were:-

(i) To determine the levels of high affinity nAChRs in the cortex after head injury.

(ii) To determine levels of individual presynaptic marker proteins in the cortex after head injury.

(iii) To investigate the existence of neuronal damage in the nbM after head injury.

(iv) To define the integrity of presynaptic terminals after ischaemic damage in animal models of cerebral ischaemia and contrast this with the sensitivity of postsynaptic structures.

(v) To explore whether GGF-2 exists in the human brain and to determine the level of GGF-2 after head injury and in animal models of acute brain injury.

CHAPTER 2: MATERIALS & METHODS

2.1 Human Postmortem Studies

2.1.1 Head-Injured Brain Tissue Collection

Fresh brain tissue was obtained at autopsy from 16 patients (12 males; 4 females) who died following a head injury and from 8 control subjects (5 males; 3 females) who died from causes other than a CNS pathology. Collection of the material was approved by the ethics committee of the Institute of Neurological Sciences, Glasgow. At autopsy, as part of a standard protocol, 11 anatomically defined regions in each cerebral hemisphere were dissected from a 1cm-thick coronal slice at the level of the lateral geniculate body. The tissue was transported to a dedicated human grade II containment suite where all subsequent analyses of unfixed material were conducted according to defined health and safety protocols. The dissected samples for analyses were frozen in isopentane at -40°C and stored at -80°C. Brain regions for analyses were grey matter from the cingulate gyrus, inferior temporal gyrus and superior parietal cortex from the left hemisphere only and subcortical white matter from the corpus callosum. The remainder of the brain was fixed in 10% formalin for neuropathological examination of brain damage resulting from head injury (Adams et al., 1980a; Graham et al., 1995) and for Alzheimer type pathology (Khachaturian, 1985). Case details of control and head-injured patients are summarised in Table 2. The survival time after injury in the head-injured group ranged from 1 to 300h. Postmortem delay ranges were: controls = 22 to 63 and head injured = 17 to 120h. The mean ± SEM ages were: control = 57 ± 4 and head-injured = 45 ± 4 years. Control patients died from a number of non-CNS related disorders including: ischaemic heart disease; aspiration pneumonia and pulmonary embolism. The neuropathological features of the head-injured patients are detailed in Table 3. Of the 16 head-injured patients, numbers presenting with particular findings were: skull fracture 13; oedema 11; DAI 8; contusions 15; raised ICP 14; SDH 12; ischaemic brain damage 12. Oedema and SDH were bilateral, right and left with 7 of the head-injured patients with SDH having undergone surgical evacuation of the haematoma prior to death. Varying grades of DAI and contusions were reported in cases, and ischaemic damage was either localised to brain regions supplied by specific cerebral vessels or diffuse throughout the brain.

Table 2: Case Details of Head-Injured and Control Patients

Case	Age	Sex	Survival (h)	Cause of Death	PM Delay (h)
H1	52	M	96	Raised ICP due to head injury	120
H2	61	M	11.5	Raised ICP due to head injury	36
H3	59	F	<24	Raised ICP due to head injury	72
H4	44	M	55	Raised ICP due to head injury	68
H5	69	F	92	Raised ICP due to head injury	46
H6	21	M	216	Raised ICP due to head injury	67
H7	20	F	96	Raised ICP due to head injury	36
H8	27	M	264	Raised ICP due to head injury	36
H9	28	F	10	Raised ICP due to head injury	34
H10	37	M	4	Severe diffuse ischaemic brain damage	96
H11	41	M	3	Severe diffuse ischaemic brain damage	120
H12	47	M	80	Raised ICP due to head injury	69
H13	51	M	300	Raised ICP due to head injury	67
H14	62	M	27	Raised ICP due to head injury	17
H15	41	M	17	Raised ICP due to head injury	52
H16	70	M	27	Raised ICP due to head injury	91
C1	59	M	-	Myocardial infarction	50
C2	29	F	-	Pulmonary thromboembolism	48
C3	68	F	-	Ischaemic heart disease	22
C4	52	M	-	Ischaemic heart disease	63
C5	71	M	-	Aspiration pneumonia	57
C6	62	M	-	Pulmonary embolism	54
C7	64	F	-	Ischaemic heart disease	31
C8	57	M	-	-	22

Survival time for the head-injured cases is the number of hours (h) between the time of injury and death.

Postmortem (PM) delay is the number of hours between death and freezing of the tissue. F, female; M. male; ICP, intracranial pressure.

Table 3: Neuropathological Findings in Head-Injured Cases

Case	Skull Fracture	Intracranial Haematoma	Contusions	DAI	Oedema	Raised ICP	Ischaemic Brain Damage
H1	Y	L SDH (E)	0	N	Bilateral	Y	N
H2	Y	L SDH (E)	12	N	N	Y	R ACA/R MCA BZ
H3	N	Bilateral (R>L) SDH	5	N	R	Y	RACA/R MCA
H4	Y	Bilateral EDH (E)	6	Grade 1	L	Y	L ACA/L MCA
H5	Y	Thin Bilateral SDHs	23	Grade 1	L	Y	N
H6	N	L SDH (E)	8	Grade 3	Bilateral	Y	Severe Diffuse
H7	Y	N	24	Grade 3	Bilateral	Y	Severe Diffuse
H8	Y	R ICH (E)	16	Grade 1	N	Y	N
H9	Y	N	12	N	L	Y	Severe Diffuse
H10	Y	N	14	N	N	N	Severe Diffuse
H11	Y	N	6	Grade 3	N	N	Severe Diffuse
H12	N	L SDH (E)	8	N	Bilateral	Y	Severe Diffuse
H13	Y	L SDH (E)	18	N	L	Y	Severe Diffuse
H14	Y	L SDH	20	Grade 1	L	Y	L ACA
H15	Y	R SDH	24	N	N	Y	N
H16	Y	Thin EDH SDH	13	Grade 1	Bilateral	Y	ACA/ MACA BZ

Abbreviations: L, Left; R, right; SDH, subdural haematoma; EDH, extradural haematoma; (E), surgically evacuated; DAI, diffuse axonal injury; ICH, intra-cerebral haematoma; ICP, intracranial pressure; ACA, anterior cerebral artery; MCA, middle cerebral artery; BZ, boundary zone; Y and N signifies the presence and absence of specific neuropathological features.

2.2 Presynaptic Pathology after Head Injury in the Human Brain

2.2.1 [^{3}H]-Nicotine Receptor Binding

Membrane preparations for receptor binding assays were derived from the: inferior temporal gyrus; cingulate gyrus and superior parietal cortex. Grey matter was dissected from the blocks, homogenized 1:10 in 50mM Tris HCl pH7.4, containing 8mM $CaCl_2$ and centrifuged (26,000g, 15min, 4°C). The supernatant was discarded and the pellet washed twice. The final membrane preparation was stored at -70°C until use. [^{3}H]-nicotine binding was determined by resuspending membranes (30mg/ml original weight/ml, 50mM Tris buffer, pH7.4) and incubating for 60min at 25°C with 4nM L-(-)-[*N*-methyl ^{3}H]-nicotine (specific activity 64.4 Ci/mmol, Dupont, NEN) in the absence or presence of 0.1mM carbachol to detect non-specific binding. The reaction was stopped by placing the samples on ice for 10min after which the membranes were filtered through GF/C filters pre-soaked in 0.05% polyethyleneimine using a Brandel M24R cell harvester. Filters were washed three times with 3ml of cold buffer (as above) and radioactivity in the filters determined by liquid scintillation counting. Protein content of the samples was measured by the method of Lowry et al., (1951), see section 2.2.2.

2.2.2 Western blotting

Grey matter was dissected from the cingulate gyrus and superior parietal cortex of controls and head-injured patients. Samples were rapidly homogenized 1:10 (weight:volume) in Hepes buffer (5mM Hepes, 0.32M sucrose, benzamidine 5mM, β-mercaptoethanol 2mM, EGTA 3mM, $MgSO_4$ 0.5mM, sodium vanadate 1mM, phenylmethyl-sulphonyl fluoride 0.1mM, leupeptin 2μg/ml, pepstatin A 5μg/ml, aprotonin 2μg/ml, pH8), using a glass-glass homogeniser. Samples were then centrifuged for 5min at 13000 rpm using a Beckman Microfuge E with the supernatant retained and stored at -70°C. Protein assays were performed according to Lowry et al., (1951). Briefly, samples were diluted 1:5 and 1:10 in homogenisation buffer, 25μl of which was added to 1ml of 2% sodium carbonate in 0.1M NaOH containing copper sulphate and potassium sodium tartrate (both 0.02% aqueous solutions). 100μl of Folin and Ciocalteu's reagent (Sigma, UK) was then added, followed by vortexing and the reaction mixture was allowed to stand for 20-30min at room temperature. Bovine serum albumin

(BSA) standards were prepared at concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0mg/ml in homogenisation buffer. Absorbance of standards and samples were measured at 680nm, using a spectrophotometer (Pharmacia LKB, Ultrospec III) for detection. A typical example of the standard curve obtained using the Lowry assay is shown as Figure 8. After calculation of protein content in brain homogenates, samples were prepared in Laemmli buffer (0.1M Tris-HCl pH8.0, 5M urea, 5% sodium dodecyl sulphate (SDS), 5% dithiolthreitol and 0.1% bromophenol blue) (Laemmli, 1970) such that 1μl was equivalent to 1μg of protein.

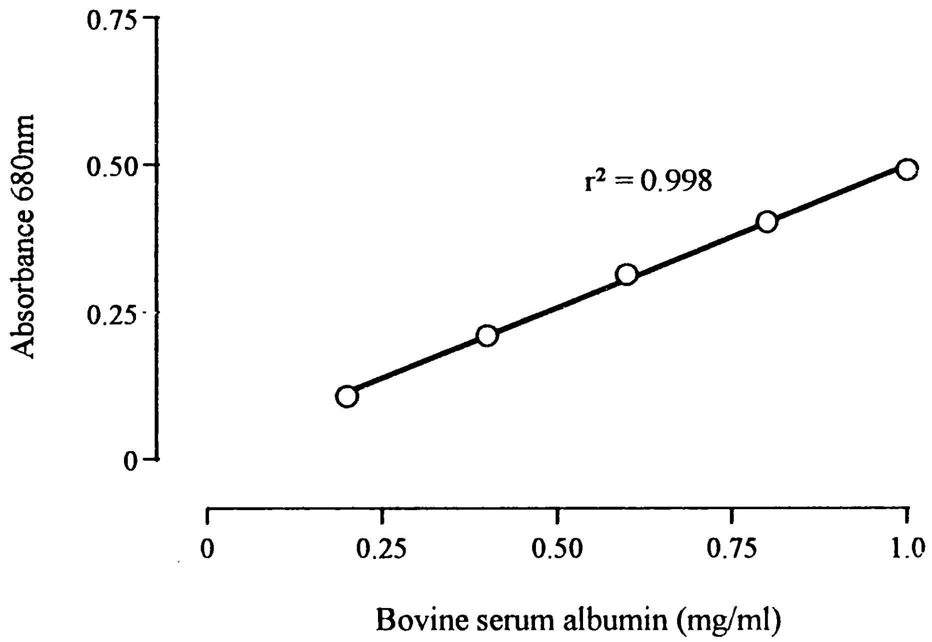


Figure 8: Representative standard curve for Lowry Protein assay.

2.2.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE gels consisted of a lower resolving and upper stacking gel contained within two glass plates. The resolving gel comprised of 10-12% acrylamide, 0.33% bisacrylamide, 0.75M Tris-base, pH8.8 and was polymerised using 0.1% ammonium persulphate (Sigma) and 0.04% TEMED (Sigma). A small volume of 0.1% SDS was added to the upper surface of the resolving gel during polymerisation and removed on addition of the stacking gel. The stacking gel consisted of 4.8% acrylamide, 0.11% bisacrylamide, 0.25M Tris-base, pH6.8 and polymerisation achieved as for the resolving gel. Either a 10- or a 15-well comb was inserted into the stacking gel and a 45min polymerisation period was allowed before addition of samples. The cast gels were mounted in the electrophoresis rig and the inner and outer reservoirs filled with electrophoresis buffer: 0.025M Tris-base; 0.0192M glycine and 0.1% SDS. Combs were removed, samples boiled for 5min and spun briefly using a Beckman Microfuge E desktop centrifuge. 10µg aliquots were loaded using a Hamilton syringe (Hamilton Bonaduz, Switzerland) and proteins separated using a Mini-Protean II cell (Bio-Rad, Hemel Hempstead, UK). Broad range biotinylated and prestained standards (Bio-Rad) were diluted 1:5 in Laemmlli buffer (Laemmli, 1970) and added alongside. Samples were electrophoresed at 60volts through the stacking gel and 80volts thereafter, with current approximately 15-25mAmps throughout. Optimisation for primary antibody concentrations directed against all proteins was performed prior to quantification of protein bands.

2.2.4 Semi-Dry Blotting and Alkaline Phosphatase Detection

Gels were sandwiched between twelve pieces of filter paper and one piece of nitrocellulose, cut to the dimensions of the gel and presoaked in Transfer buffer (0.025M Tris-base, pH8.3, 0.0192M glycine and 20% methanol) and water respectively. Air-bubbles were removed by gentle rolling of the filter paper-gel-nitrocellulose-filter paper stack. Proteins were transferred to the nitrocellulose membrane (Schleicher and Schuell, London, UK) by semi-dry transfer for 15min at 15volts (Trans-Blot SD, Bio-Rad). To demonstrate equal loading of the gels, after transfer the stacking gel was removed and the resolving gel was stained for 2 days in 0.1% coomassie blue (Sigma) in water: methanol: glacial acetic acid (5:5:2 by volume). Background (non-protein) staining was removed by destaining the gel in a solution containing 12.5% isopropanol and 10% glacial acetic acid. Coomassie blue staining of protein bands were scanned and is shown in Figure 9. The nitrocellulose membranes were blocked in 5% defatted dry milk in 0.065M Tris-base, 0.1% Tween-20 (Sigma) (T-TBS) buffer for 1h at room temperature with agitation. Blots were then incubated with a primary antibody diluted in T-TBS

0.065M Tris-base, 0.1% Tween-20 (Sigma) (T-TBS) buffer for 1h at room temperature with agitation. Blots were then incubated with a primary antibody diluted in T-TBS overnight at 4°C. Following incubation, blots were washed in T-TBS, incubated with a biotinylated anti-mouse or anti-rabbit secondary antibody (Vector), diluted 1:500 in T-TBS. After washing, blots were incubated for 1h at room temperature with streptavidin alkaline phosphatase conjugate (Amersham, Slough, UK) diluted in T-TBS containing 5% defatted dried milk. Immunoreactivity was visualised following incubation with an alkaline phosphatase substrate kit (Bio-Rad), blots were rinsed in distilled water and allowed to air-dry.

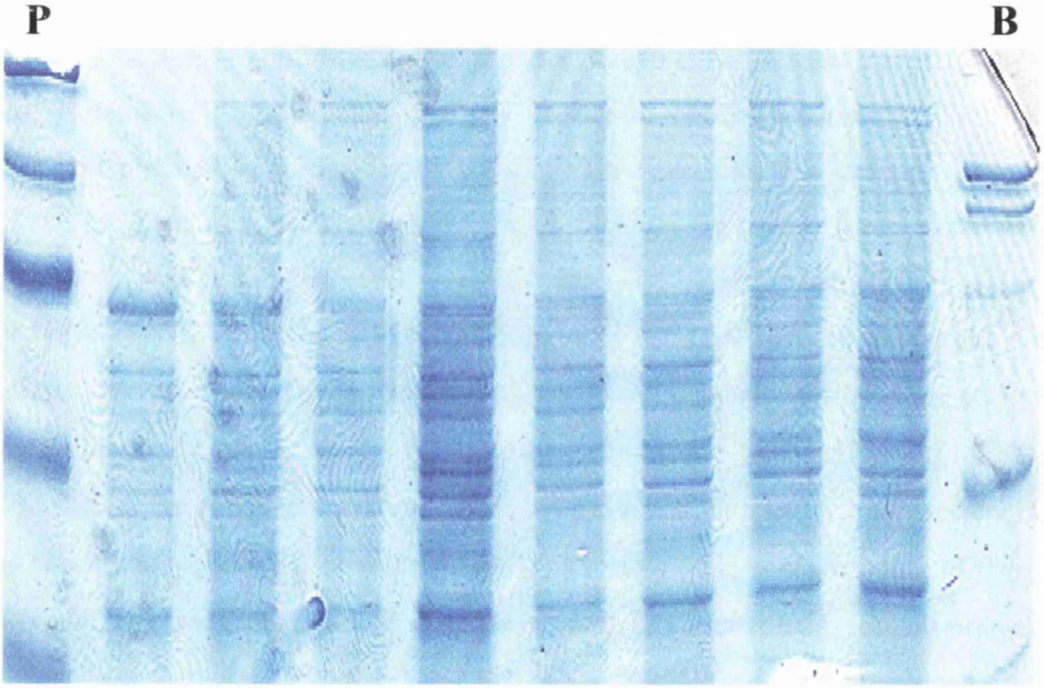


Figure 9: Coomassie blue stained SDS-PAGE gel showing protein loading and separation by Western blotting. B and P correspond to biotinylated and prestained molecular weight protein standards respectively.

2.2.5 Proteins Detected

Primary antibodies directed against a number of presynaptic proteins were used to assess presynaptic pathology after head injury. Concentrations of primary antibodies used and product details are summarised in Table 4. A primary antibody to the housekeeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal loading control. 10µg of protein was added for detection of presynaptic proteins, except GAPDH and 5µg of protein was loaded to detect GAPDH. All of the antibodies used were monoclonal, with the exception of anti-ChAT, which was polyclonal.

2.2.6 Enhanced Chemiluminesence (ECL) Detection

In some Western blotting experiments to detect nitrotyrosine residues, ECL detection was used instead of alkaline phosphatase. SDS-PAGE and semi-dry transfer were performed as described in sections (2.2.3 and 2.2.4). Blots were washed in water, followed by rinsing in 50mM phosphate buffered saline (PBS) (made from PBS tablets, Oxoid, Hampshire, UK), containing 0.1% Tween-20 (PBS-T) (Sigma). Non-specific binding was blocked by incubation of blots in PBS containing 5% defatted dried milk (PBS-M) for 30min at room temperature with constant agitation. Blots were washed with water and incubated with monoclonal anti-nitrotyrosine (1:50) (Upstate Biotechnology, USA) diluted in PBS and incubated for 1.5h at room temperature with agitation. Thereafter, blots were washed with water and incubated with horse anti-mouse secondary antibody conjugated to horseradish peroxidase (Promega, UK) (1:1000) in PBS-M for 1.5h at room temperature. Blots were washed with water, followed by PBS-T and water once again. The membrane was then incubated in equal volumes of the ECL (Amersham, UK) reagents (1 and 2) for 1min exactly and excess ECL reagent drained on blotting paper. Membranes were then placed in SaranWrap and positioned in film cassettes (protein side up) and exposed to hyperfilm (Fujifilm, Japan) for initially 15 seconds, and exposure time modified thereafter to optimise signal generation. Optimum exposure time was 1min. Films were then developed, washed in water and allowed to air-dry before densitometrical analysis.

Table 4: Concentrations of primary antibodies used in Western blotting and immunohistochemistry to assess presynaptic pathology following human head injury.

Western Blotting			**Immunohistochemistry**		
Antibody	Concentration	Clone / Source	Antibody	Concentration	Clone / Source
Synaptophysin	1 : 5000	SVP38 / Sigma	Synaptophysin	1: 1500	SVP38/ Sigma
SNAP-25	1 : 5000	SP12 / Serotec	SNAP-25	1 : 4000	SP12/ Serotec
Syntaxin	1 : 1500	HPC-1/ Sigma	MAP-2	1 : 750	HM2/ Sigma
Synaptotagmin	1 : 400	ASV30/ Bioquote	ChAT	1: 2000	AB144p Chemicon
Synapsin-I	1 : 5000	A10C Serotec			
GAPDH	1 : 400,000	6C5 Chemicon			
Nitrotyrosine	1:50	Upstate			

2.2.7 Dot-Blotting

Two pieces of blotting paper and 1 piece of 0.45μm nitrocellulose (Schleicher & Schuell, Germany) were prewetted in TBS for 1min and loaded onto the Minifold I Spot-blot plate platform (Schleicher & Schuell). A pipette was rolled over the surface of the membrane and the stack was clamped in place. One μl of protein in 40μl of buffer was loaded and aspirated with a vacuum pump for 3min. The membrane was removed from the platform and after washing in T-TBS for 3x10min, non-specific binding was blocked by incubation of the membrane in T-TBS containing 5% defatted milk (T-TBS-M) for 1h with agitation. The blots were then washed in T-TBS and incubated with monoclonal anti-nitrotyrosine (Upstate, USA) diluted 1:5000 in T-TBS-M for 1.5h at room temperature. After washing, blots were incubated with horse radish peroxidase conjugated secondary antibody (Promega, UK), diluted 1:1000 for 1.5h at room temperature. After numerous washes in T-TBS and distilled water, immunoreactivity was visualised as described above for ECL detection. Optimum exposure time was 2min.

2.28 Quantification and Validation of Optical Density Analysis

Optical density of the protein signal was quantified by densitometric analysis (MCID, Imaging Research, Ontario, Canada) of the blots. A range (0.1-1.0 ODunits) of Kodak Wratten gelatin filters (Eastman Kodak Company, New York, USA) were used for calibration and calculation of optical density of the protein bands. Readings for calibration were recorded from the area exactly adjacent to the band of interest. To ensure accurate calibration, individual calibration curves were constructed for each band on a blot. To verify the linear relationship between the amount of protein loaded and the optical density of the signal generated, 1, 2, 5, 10, 20 and 30μg samples of protein from a sample of cingulate gyrus from a control patient were separated using SDS-PAGE and semi-dry transfer, incubated with anti-synaptophysin (1:5000) and developed using the alkaline phosphatase conjugate substrate. The blot and the relationship between protein loaded and optical density are shown in Figure 10.

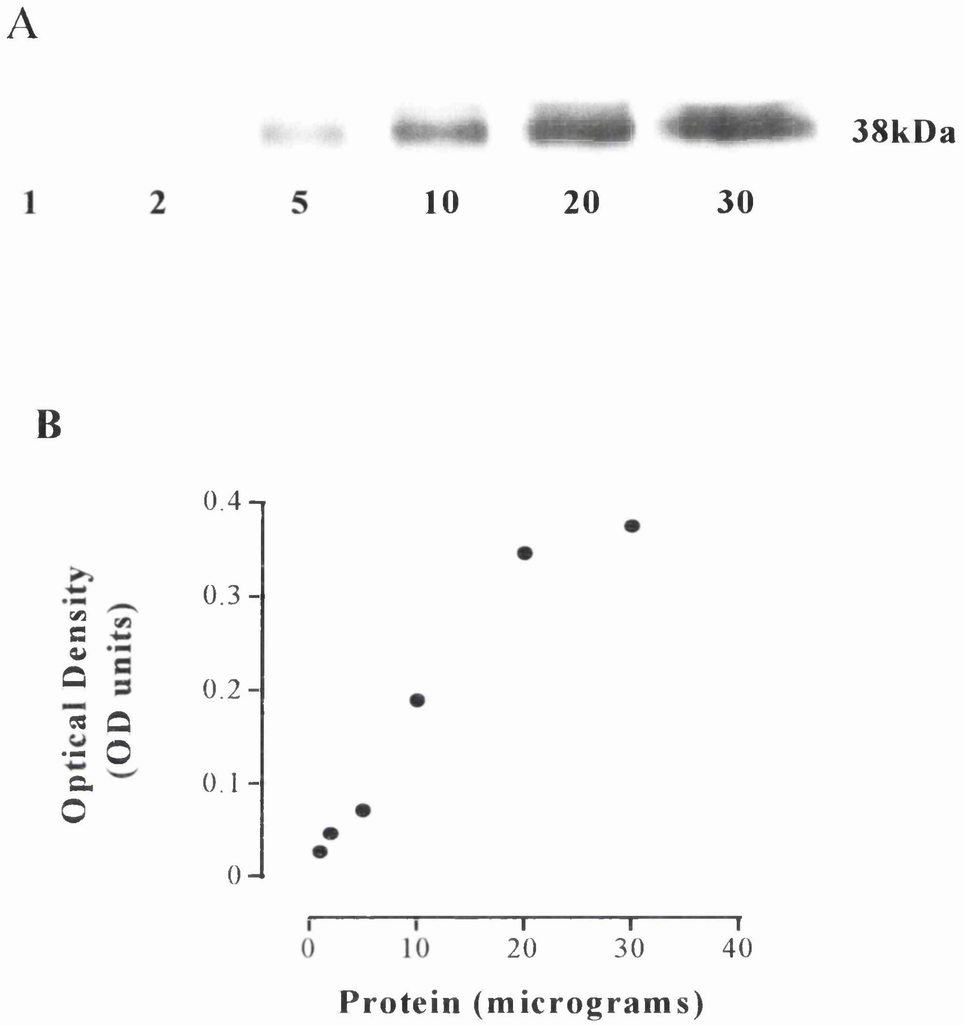


Figure 10: Validation of use of optical density image analysis.

Western blot (A), showing the signal generated using anti-synaptophysin (1:5000) and the alkaline phosphatase conjugate detection system with ascending amount of loaded protein from grey matter of the left cingulate gyrus of a control subject. (B) Relationship between quantity of protein loaded and the optical density of the bands measured using image analysis.

2.3 Immunohistochemistry

Fresh frozen, 4% paraformaldehyde-fixed left cingulate gyrus from the same group of controls and head-injured patients was cryostat cut (Microtome 3050, Bright, Huntingdon, UK) at a thickness of 30μm and mounted on to poly-L-lysine coated slides. Sections were dried and stored at -20°C until use. Sections were removed from the freezer, thawed and rinsed with two washes of PBS. Endogenous peroxidases were blocked by incubating sections with 3% hydrogen peroxide (Sigma) in methanol for 30min. After rinsing firstly in running water for 10min, followed by PBS 2x5min, sections were drawn round using a hydrophobic pen (Dako, Cambridge, UK). Non-specific binding was blocked by incubation with 350μl of blocker solution containing 0.5% bovine serum albumen (BSA) and 10% normal horse (monoclonal) or goat (polyclonal) serum in PBS for 1h at room temperature. Sections were then incubated with 350μl of primary antibody overnight at 4°C, diluted in PBS containing 0.1% BSA and 1.5% serum (see Table 4 for dilutions). The following day, sections were washed in PBS and incubated with biotinylated secondary antibody (horse anti-mouse or goat anti-rabbit) diluted 1:100 in PBS. Sections were then washed with PBS 2x5min, followed by incubation with a Vectastain ABC kit (Vector) (made up approximately 1h before use) for 1h at room temperature. Once again sections were washed with 2x5min PBS and immunoreactivity visualised by incubation of sections with the chromagen 3,3-diaminobenzidine. After detection, the reaction was stopped by rinsing in water, dehydrated through alcohols and counterstained with haematoxylin (optional) before mounting using DPX mountant (Raymond Lamb, Eastbourne, UK).

2.3.1 Quantification of Immunohistochemical Signal: Relative Optical Density

Sections were viewed using a Leitz DM RB light microscope (Leica, Germany). Immunostaining of the presynaptic proteins was restricted to the neuropil and was absent from the cell bodies (see Figure 11). Immunostaining was diffuse, consistent with the distribution of synaptic vesicles throughout the neuropil.

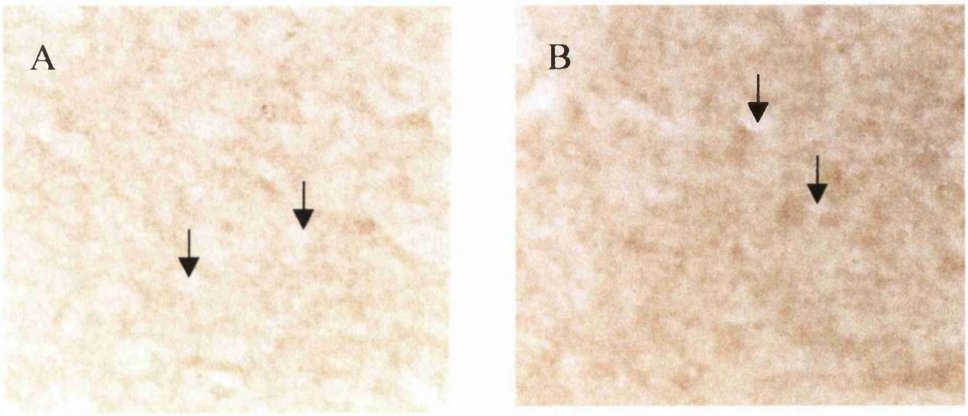


Figure 11: The pattern of immunohistochemical staining for (A) synaptophysin and (B) SNAP-25 staining in cingulate gyrus from a human control subject. Antibodies to both proteins produced diffuse and punctate pattern of immunostaining in the neuropil, but absent from cell bodies (arrows).

The intensity of the immunohistochemical signal of the presynaptic proteins, synaptophysin and SNAP-25, was measured by MCID image analysis, using a camera (Nikon, Japan) attached to a light microscope (Leica). Since the immunostaining for the presynaptic proteins was predominantly localised in the neuropil, relative optical density was measured at 10 points within 5 fields of cortical grey and subcortical white matter (background), and calculated as the mean of 50 grey matter readings subtract the mean of 50 white matter readings.

2.4 nucleus basalis of Meynert Pathology after Head Injury

2.4.1 Ch4 Neurons in the nbM

Blocks of interest were removed from specimen jars containing brain material stored in 10% formalin. Using the anterior commissure and optic tract as anatomical landmarks for the identification of the nbM (see Figure 12), as described previously (Allen et al., 1988), a 1cm-thick coronal slice containing the intermediate portion of the nbM was dissected from blocks from the same group of controls and head-injured patients, used previously to assess presynaptic pathology after head injury. Brain slices were washed, dehydrated and paraffin embedded and 8μm thick sections were serially cut using a microtome (Jung Biocut 2035, Leica, Germany). Sections were mounted onto 3-aminopropyl triethoxysilane-coated slides and dried in an oven overnight at 37°C.

Figure 12: Macroscopic identification of the nbM in human postmortem brain. Scale bar = 1cm. The arrow indicates the anterior commissure, used as an anatomical landmark for macroscopic identification of the nbM. The localisation of the Ch4 neurons of the NbM is indicated by *.

2.4.2 Histology and Semi-Quantitative Analysis

Every tenth section was stained for haematoxylin/eosin (H&E) and cresyl violet/luxyl fast blue (LFB/CV) (see Appendix 1). As described by Allen et al., (1988), Ch4 neurons of the nbM were identified through a combination of their size, morphology and hyperchromatic staining pattern (See Figure 13). Using H&E and LFB/CV stained sections, damage to Ch4 neurons of the intermediate portion of the nbM was semi-quantitatively assessed using the following scoring system:-

Scoring System: 0	No damage
1	0-10% damage
2	10-25% damage
3	25-50% damage
4	50-100% damage,

with scores representing the number of damaged Ch4 neurons as a percentage of the total Ch4 neuronal population in the intermediate portion of the nbM. Criteria considered for assessment included: cell morphology (circular or irregular); hyperchromatic Nissl substance staining; eosinophilic and/or luxophilic neurons and presence of incrustations.

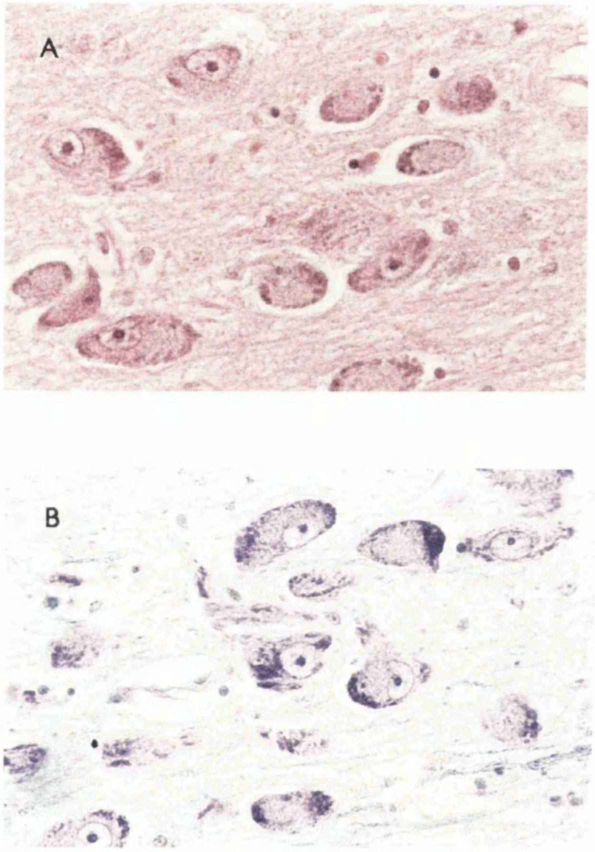


Figure 13: Histological staining of Ch4 neurons of the nucleus basalis of Meynert of a control subject. Using haematoxylin/eosin (A) and luxol fast blue/cresyl violet (B) staining in adjacent sections, Ch4 neurons were identified as large circular neurons with Nissl substances densely stained with haematoxylin or cresyl violet. Nuclear membranes and nucleoli are clearly defined with myelin counterstain (luxol fast blue) absent from healthy neurons. Magnification X400.

2.4.3 Immunohistochemistry for Choline Acetyltransferase

Adjacent sections to those used for H&E and LFB/CV staining were placed in an oven at 65°C for half hour to melt wax. Dewaxing of the sections was completed by placing sections in xylene for 2x10 min, followed by dehydration in absolute alcohol for 10 and 5min. Retrieval of antigen binding sites was achieved by microwaving sections at high power for 2x5min in 10mM citric acid and allowed to cool for 30min thereafter. The remainder of the immunohistochemistry protocol was similar to that described in section 2.3. Normal donkey serum was used in the blocking solution and sections were incubated overnight at 4°C with a polyclonal primary antibody to choline acetyltransferase (clone AB144p, Chemicon; donated by Dr D. Maxwell, Institute of Biomedical and Life Sciences, University of Glasgow), diluted 1:1000 in PBS containing 1.5% normal donkey serum and 0.1% BSA. The following day the standard protocol was used (see section 2.3), except that an ABC elite kit was used and 3,3-diaminobenzidine was used as chromagen with nickel enhancement.

2.5 Animal Models of Acute Brain Injury

A number of rodent models of cerebral ischaemia were used to assess presynaptic terminal integrity after acute brain injury.

2.5.1 Focal Cerebral Ischaemia in the Rat: Permanent Middle Cerebral Artery Occlusion: Intraluminal Thread Model

Surgery was performed by Louise Marks as detailed in Appendix 2. After a recovery period of 24h, the animals were reanaesthetised, decapitated and the brain rapidly removed. The area of cortex supplied by the middle cerebral artery was dissected from both the ipsilateral and contralateral hemispheres and snap-frozen in liquid nitrogen. The area supplied by the unaffected anterior carotid artery, including the cingulate gyrus was dissected from both hemispheres and processed as described above.

2.5.2 Focal Cerebral Ischaemia in the Rat: Permanent Middle Cerebral Artery Occlusion: Diathermy model.

Surgery was performed by Dr Rammy Gill, Roche, Switzerland using the method of Tamura et al., 1981. Animals were allowed to survive for 24h before being transcardially perfused with 4% parafomaldehyde.

2.5.3 Western blotting and Image Analysis

Membrane homogenate preparation and Western blotting were performed as described in section 2.2.2-2.2.4. Optical densities of the immunoreactive signals generated for a number of presynaptic proteins, namely, synaptophysin, SNAP-25, syntaxin and were determined by MCID image analysis as described in section 2.2.8.

2.5.4 Global Cerebral Ischaemia in the Mouse: Transient Bilateral Common Carotid Artery Occlusion

Surgery was performed by Stephen Kelly as described in Appendix 3.

2.5.5 Tissue Processing and Staining

Fixed brains were cut into 3mm blocks and processed for paraffin embedding. Specifically, blocks were dehydrated in cassettes for 30min in two volumes of 70%, 90% and 100% alcohols and for 1h in xylene. Thereafter blocks were wax-embedded via overnight incubation in paraffin wax at 65°C. 6μm thick sections were cut at the level of the caudate and dorsal hippocampus using a microtome (Leica RM2135, Germany) and mounted on to poly-L-lysine coated slides and air-dried. Paraffin embedded brain tissue was dewaxed in histoclear and dehydrated in alcohol solutions prior to H&E (see Appendix 1) and immunohistochemical staining. Immunohistochemistry and relative optical density measurements on mouse tissue was performed as described for human brain tissue in sections 2.3 – 2.3.1. Primary antibody concentrations used were: anti-synaptophysin, 1:10000; anti-SNAP-25, 1:5000; anti-syntaxin, 1:2000 and anti-MAP-2, 1:750.

2.5.6 Assessment of Neuronal Damage

The extent of neuronal damage was determined in the caudate and a number of layers within the hippocampus (CA1, CA2, CA3, hilus and dentate gyrus) of both hemispheres. The amount of neuronal damage was expressed as a percentage of the overall neuronal population within the specific brain regions, with low scores equivalent to minimal damage and high scores equivalent to considerable damage.

Scoring system:		
	0	no damage
	1	0-10% damage
	2	10-25% damage
	3	25-50% damage
	4	50-100% damage

2.6 GGF-2 in Acute Brain Injury

The response of GGF-2 to acute brain injury was assessed in human postmortem brain tissue from controls and head-injured patients and in a number of animal models of acute brain injury.

2.6.1 GGF-2 and Human Head Injury

Using the same group of controls and head-injured patients detailed in section 2.11, and Tables 2 and 3, GGF-2 levels were assessed using both Western blot analyses for the cingulate gyrus and the superior parietal cortex (see sections 2.2.2-2.2.4), and immunohistochemistry (for the cingulate gyrus (see section 2.3). In Western blotting a panel of primary antibodies was used against GGF-2, each directed against different recognition sequences of the GGF-2 protein (see Figure 14). All of these antibodies were provided by Dr Mark Marchionni, Cambridge NeuroScience and used at dilution of 1:500 in T-TBS for Western blotting. Antibody CN16 was used at a dilution of 1:500 for immunohistochemistry, as described in section 2.3.

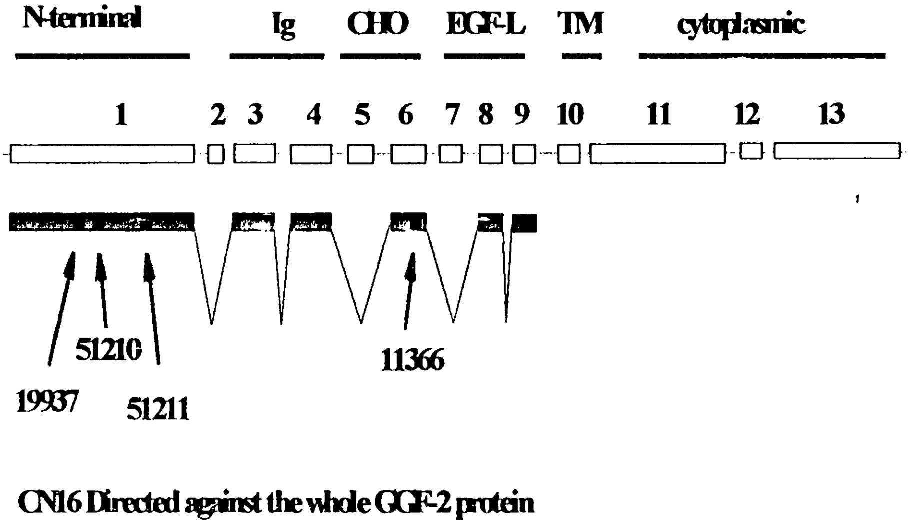


Figure 14: Primary antibodies directed against GGF-2.

Five primary antibodies, namely CN16, 11366, 19937, 51210 and 51211, recognise specific epitopes on the GGF-2 protein. The coding introns are indicated in numbers (1-13) and various domains of the proteins are shown: Ig, immunoglobulin; CHO, carbohydrate; TM, transmembrane domains and EGF-L, epidermal growth factor-like domain.

2.6.2 Subdural Haematoma in the Rat

Surgery was performed by Dr Michael Fitzpatrick (see Appendix 4) and after a 4h survival period, rats were reanaesthetised, decapitated and the brain rapidly removed. The area of cortex underlying the haematoma and the corresponding area on the contralateral hemisphere were dissected on ice and snap-frozen in liquid nitrogen. Levels of GGF-2 immunoreactivity were determined by separation of 10μg of protein on 10% acrylamide gels by Western blotting and SDS-PAGE, as described in sections 2.2.2–2.2.4. The primary antibody, 51210, was used to detect GGF-2 immunoreactivity.

2.6.3 Focal Cerebral Ischaemia in the Rat: Permanent Middle Cerebal Artery Occlusion - Diathermy Model

Surgery was performed by Dr Marc Soriano using the method of Tamura et al., 1981. The brain was rapidly removed and tissue from the cortical and striatal MCA territory was dissected on ice from both the ipsilateral and contralateral hemispheres and snap-frozen in liquid nitrogen. Membrane homogenates were prepared as described in sections 2.2.2. Western blot analysis of GGF-2 levels was performed using the primary antibody CN16 diluted 1:500 as detailed in section 2.2.2-2.2.4.

2.6.4 Alzheimer's Disease Brain Tissue Collection

Fresh brain tissue was obtained at autopsy from 8 patients (3 males; 5 females) diagnosed with Alzheimer's disease and 9 control subjects (5 males; 4 females) with no evidence of Alzheimer's disease symptomatology. Tissue collection and processing was as described previously (Dewar et al., 1990). Brain tissue was dissected from the left cingulate gyrus only. Case details of the Alzheimer's disease and control subjects are summarised in Table 5. Postmortem delay ranges were: control = 3 to 6h and Alzheimer's disease = 2.5 to 14.2h. The mean ± SEM ages were: Alzheimer's disease = 81 ± 2 years and controls 82 ± 3 years. Membrane homogenates preparation and Western blotting were performed as described in section 2.2.2.-2.2.4. GGF-2 was detected using CN16 (1:500) and optical density measured using the protocol in section 2.2.8.

Case	Age	Sex	PM Delay (h)
AD 1	78	F	2.5
AD 2	82	M	10
AD 3	81	F	6
AD 4	81	M	5
AD 5	85	F	3
AD 6	69	M	6
AD 7	82	F	14.2
AD 8	94	F	3.5
C 1	86	M	6
C 2	74	M	5
C 3	96	F	3
C 4	75	M	4
C 5	83	F	6
C 6	86	M	5.5
C 7	76	F	5
C 8	73	M	6
C 9	79	F	6

Table 5: Case Details of Alzheimer's Disease and Control Patients Postmortem delay is the number of hours between death and freezing of the tissue. F; female: M, male.

CHAPTER 3: RESULTS

3.1 Presynaptic pathology: Human postmortem brain studies

Mechanisms involved in human head injury have been studied in both rodent trauma models and use of human postmortem brain material. Involvement of various cellular compartments and pathophysiological mechanisms have been shown to contribute to the behavioural and neurochemical deficits existing after a head injury. However, whether there is damage or dysfunction of the presynaptic terminals after a head injury is not known. Therefore, this study was designed to address this issue using human postmortem brain tissue from the cortex of controls and head-injured patients. Deficits in cortical choline acetyltransferase activity are present in the human brain after head injury (Murdoch et al., 1998). However, whether this is due to a loss of cholinergic innervation or presynaptic terminals in not known. The levels of a number of presynaptic markers were assessed by receptor binding, Western blot analyses and immunohistochemistry in a group of controls and head-injured patients.

3.1.1 Nicotinic receptor binding

Mnemonic and cognitive deficits are debilitating consequences of human head injury (Oddy et al., 1985). The cholinergic system, including nAChRs have been implicated in the processes of learning and memory (Newhouse et al., 1994). Low nanomolar concentrations of [^{3}H]-nicotine were used to detect high affinity nAChRs in homogenates from 3 cortical regions; cingulate gyrus, superior parietal cortex and inferior temporal gyrus. A proportion of these nAChRs are believed to be present on presynaptic terminals (Wonnacott, 1997), thereby providing a measure of cortical presynaptic terminal integrity. 4nM [^{3}H]-nicotine binding used in this study did not detect any change in the number of nAChRs in the cingulate gyrus, the superior parietal cortex or the inferior temporal gyrus of controls and head-injured patients (Figure 15).

3.1.2 Western blotting

Western blotting analyses was performed for a number of presynaptic proteins in homogenates from the cingulate gyrus and the superior parietal cortex of controls and head-injured patients. In the cingulate gyrus, levels of synaptophysin, SNAP-25 and syntaxin immunoreactivity were reduced by 28%, 36% and 43% respectively in head-

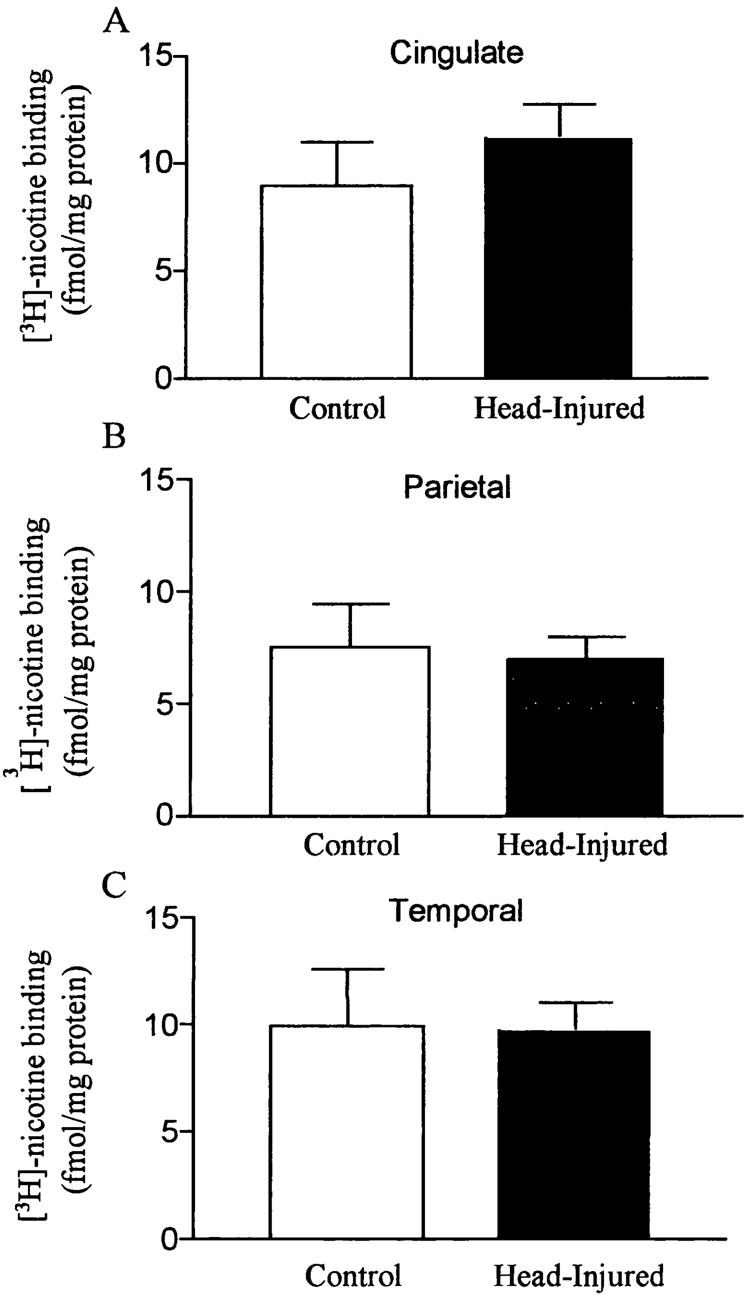


Figure 15

[^{3}H]-nicotine binding in the cingulate gyrus (A), superior parietal cortex (B) and inferior temporal gyrus (C) of controls (open bars) and head-injured patients (filled bars). Data represent the mean + SEM. Unpaired two-tailed Student's *t* tests revealed there was no difference in the level of [^{3}H]-nicotine binding in any of the cortical regions examined in head-injured patients (n=16) compared with controls (n=8).

injured patients compared to controls (Figures 16, 17, 18). Synaptotagmin and synapsin I were detected at the predicted molecular weights, but optical densities of these proteins were not statistically different between controls and head-injured patients. Synaptotagmin immunoreactivity was reduced by 18% (Figure 19) and synapsin Ia and I b were increased by 12 and 20% respectively in head-injured patients compared with controls (Figure 20), but these differences did not reach statistical significance. The housekeeping protein GAPDH is routinely used as an internal loading control for blotting techniques (Zhong and Simons, 1999). The levels of GAPDH immunoreactivity were not different between head-injured patients and controls (Figure 21), indicating equivalent amounts of protein had been loaded in the gel lanes.

In the superior parietal cortex from the same group of controls and head-injured patients as used for the cingulate gyrus analyses, none of the presynaptic proteins were significantly reduced in the head-injured group compared to controls. The mean levels of synaptophysin (Figure 22), SNAP-25 (Figure 23), syntaxin (Figure 24) and synaptotagmin (Figure 25) were reduced by 18, 26, 14 and 17% respectively in the head-injured group compared to controls, although none of these changes reached statistical significance. Similarly, the levels of both synapsin Ia and Ib (Figure 26) were no different in the superior parietal cortex of head-injured patients compared to controls. The level of GAPDH immunoreactivity was unchanged in the head-injured patients compared to control subjects (Figure 27). An overview of the presynaptic protein measurements in the cingulate gyrus and the superior parietal cortex are summarised in Table 6.

3.1.3 Immunohistochemistry for presynaptic proteins

Immunohistochemistry for synaptophysin in fresh-frozen, 4%-paraformaldehyde fixed cingulate gyrus from blocks adjacent to those used for Western blot analyses was performed for the same group of controls and head-injured patients. Synaptophysin immunostaining was predominantly present in the neuropil as a punctate pattern attributable to synaptophysin's localisation on synaptic vesicles (Navone et al., 1986). Minimal synaptophysin immunostaining was detected within cell bodies in either the controls or head-injured patients (Figure 28). The distribution of synaptophysin immunostaining in the neuropil permitted determination of relative optical density

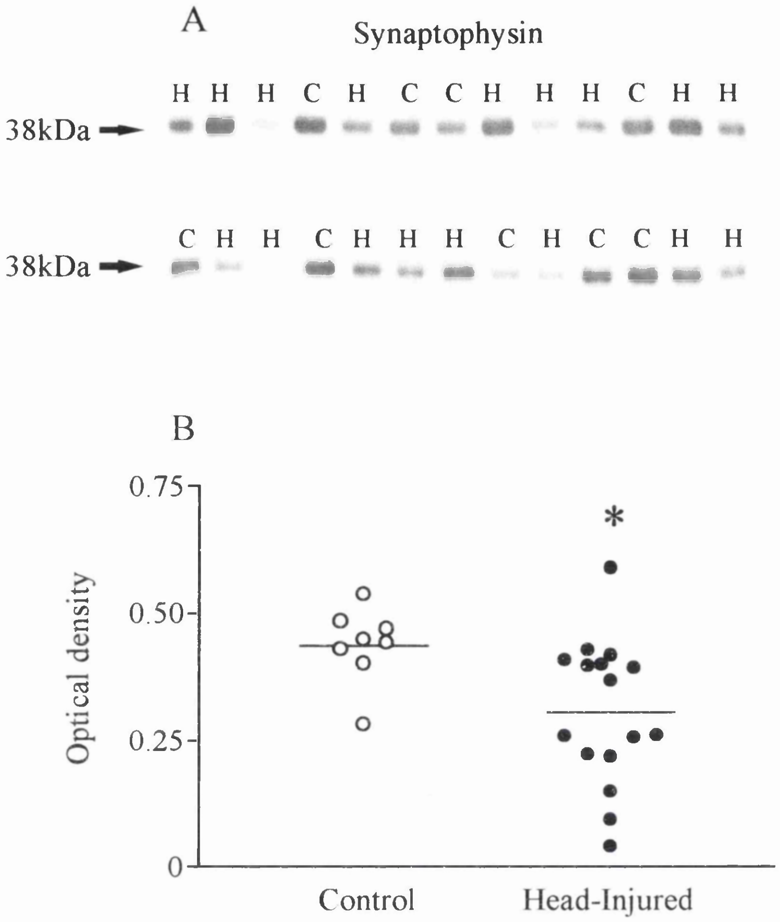


Figure 16

Synaptophysin immunoreactivity is reduced in the cingulate gyrus of head-injured patients compared with controls. A: Western blots of synaptophysin immunoreactivity in 8 controls (C) and 16 head-injured patients (H), with detection of synaptophysin at the predicted molecular weight of 38kDa. One control case and one head-injured case were loaded onto both gels accounting for 26 bands detected per experiment. B: Quantitative data of the immunoblots in A. The mean value (bar) of synaptophysin immunoreactivity in the head-injured patients (closed circles) was reduced by 28%, $^*p<0.05$, compared to controls (open circles). Unpaired two-tailed Student's *t* test was used for statistical analysis.

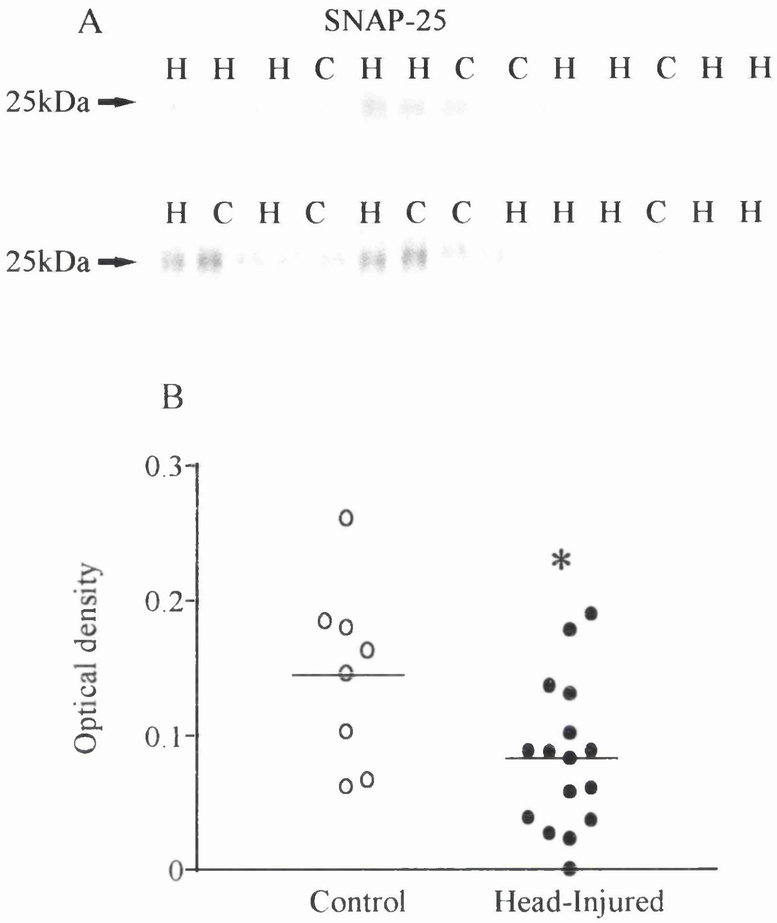


Figure 17

SNAP-25 immunoreactivity is reduced in the cingulate gyrus of head-injured patients compared with controls. A: Western blots of SNAP-25 immunoreactivity in 8 controls (C) and 16 head-injured patients (H), with detection of SNAP-25 immunoreactivity at the correct molecular weight of 25kDa. B: Quantitative data of the immunoblots in A. Unpaired two-tailed Student's *t* test showed the level of SNAP-25 immunoreactivity was significantly reduced by 36%, *$p<0.05$, in head-injured patients (closed circles) compared with controls (open circles). Bars represent the group means.

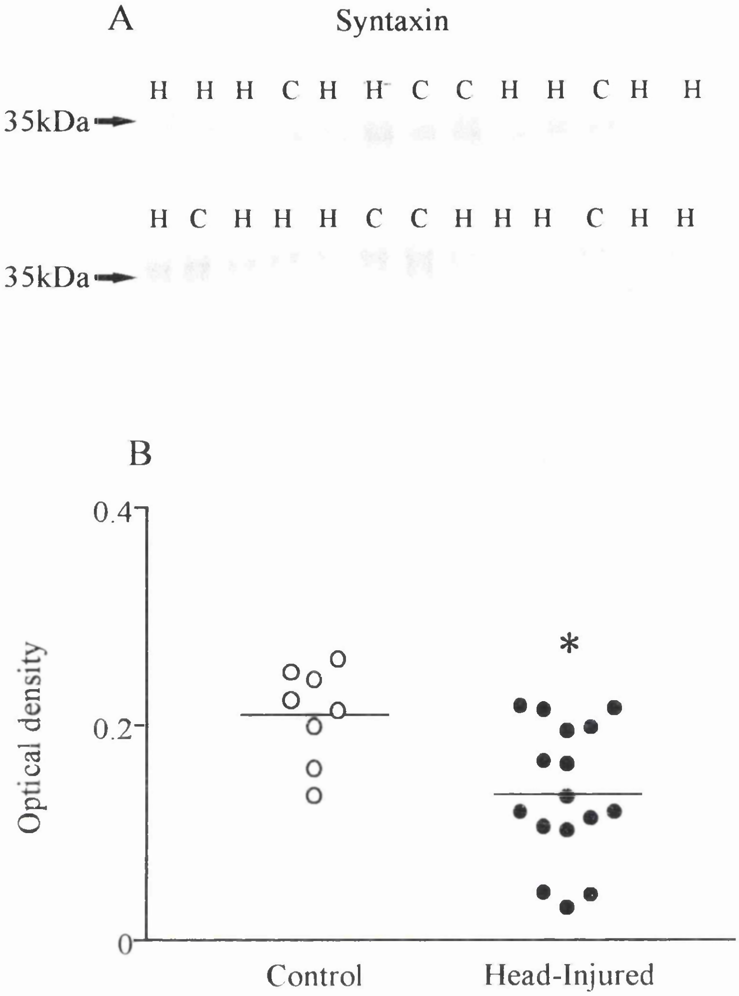


Figure 18

Syntaxin immunoreactivity is reduced in the cingulate gyrus of head-injured patients compared with controls. A: Western blots of syntaxin immunoreactivity in 8 controls (C) and 16 head-injured patients (H), with detection of syntaxin immunoreactivity at the correct molecular weight of 35kDa. B: Quantitative data of the immunoblots in A. Unpaired two-tailed Student's *t* test showed the level of syntaxin immunoreactivity was significantly reduced by 43%, *p<0.01, in head-injured patients (closed circles) compared with controls (open circles). Bars represent the group means.

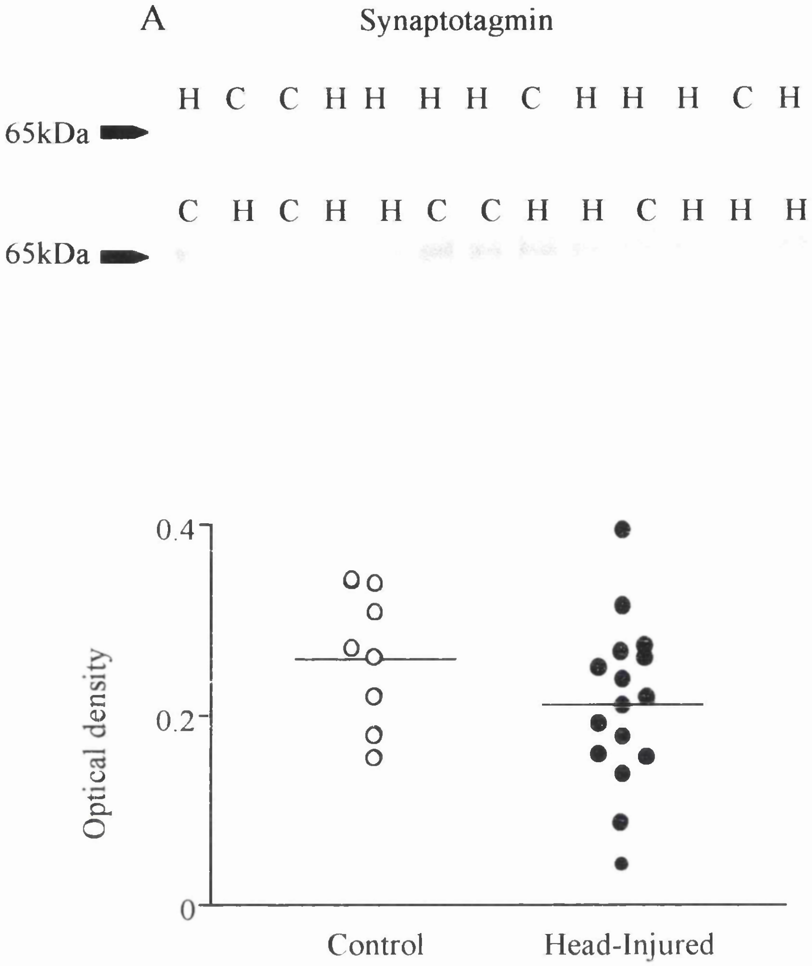


Figure 19

Synaptotagmin immunoreactivity in the cingulate gyrus of head-injured patients compared with controls. A: Western blots of synaptotagmin immunoreactivity in 8 controls (C) and 16 head-injured patients (H), with detection of synaptotagmin at the correct molecular weight of 65kDa. B: Optical density values of the immunoblots in panel A. Bars represent the group means. Levels of synaptotagmin immunoreactivity were not different between controls (open circles) and head-injured patients (closed circles).

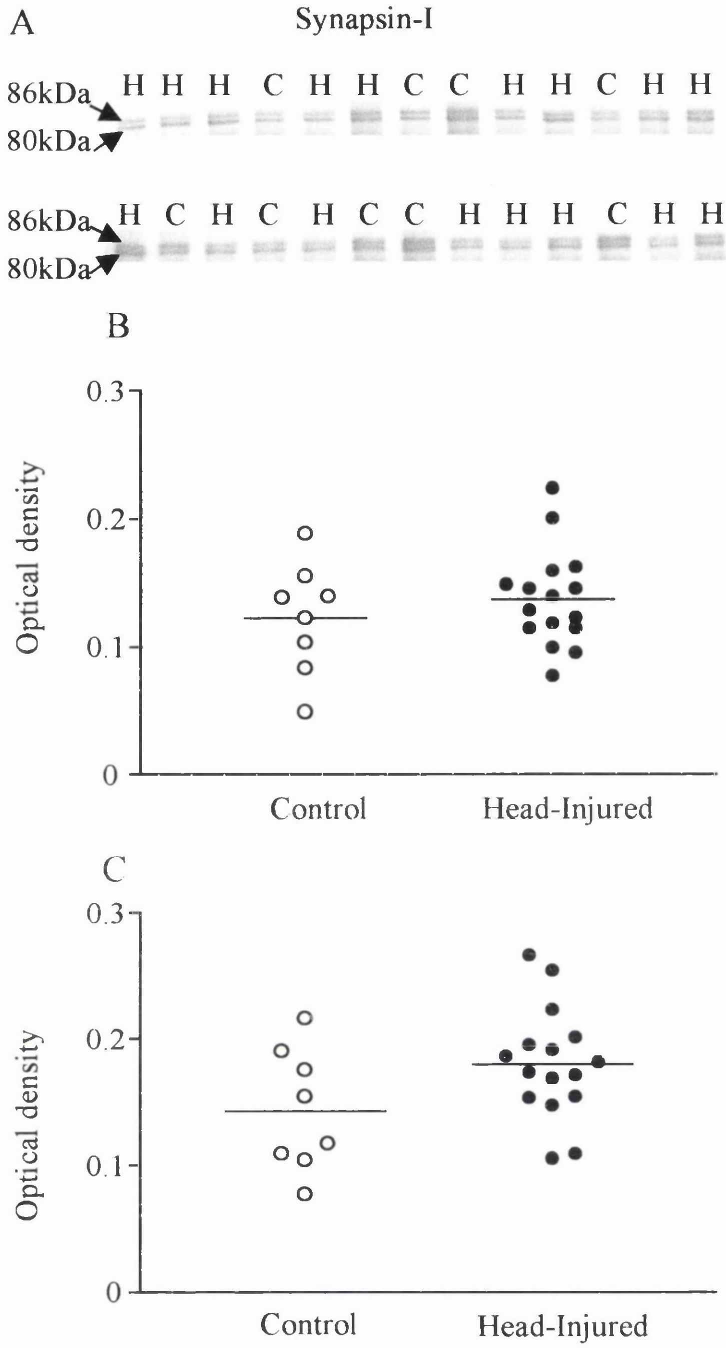


Figure 20

Synapsin I immunoreactivity in the cingulate gyrus of head-injured patients compared with controls. A: Western blots of synapsin I immunoreactivity in 8 controls (C) and 16 head-injured patients (H), with detection of the synapsin I doublet at the correct molecular weights of 86kDa and 80kDa for synapsin Ia and Ib respectively. B, C: Quantitative data for the Western blots in A. Bars represent the group means. Unpaired two-tailed Student's *t* tests revealed there was no difference in the levels of both synapsin Ia and synapsin Ib in head-injured patients (closed circles) compared with controls (open circles).

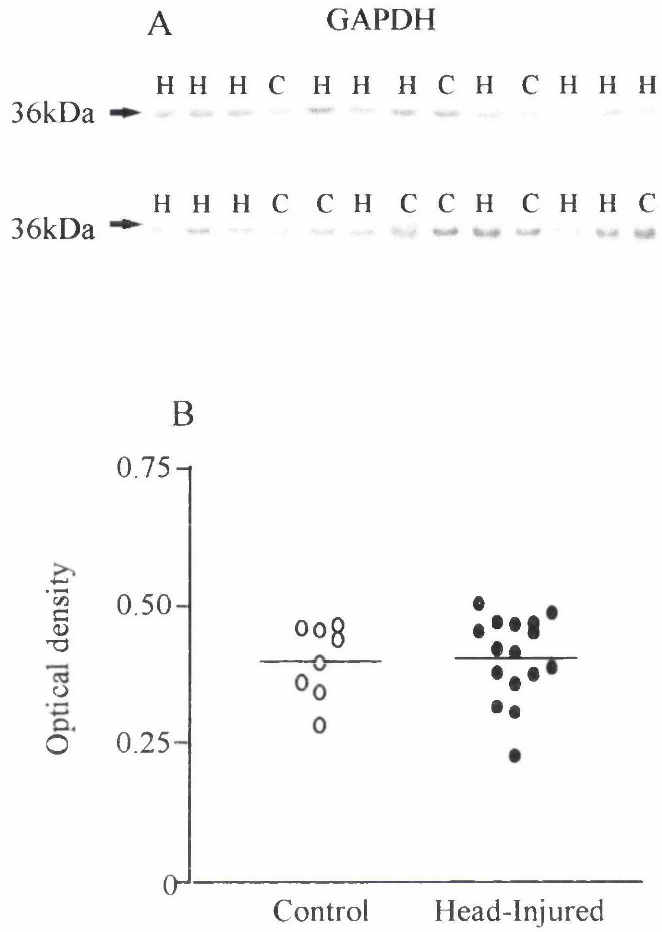
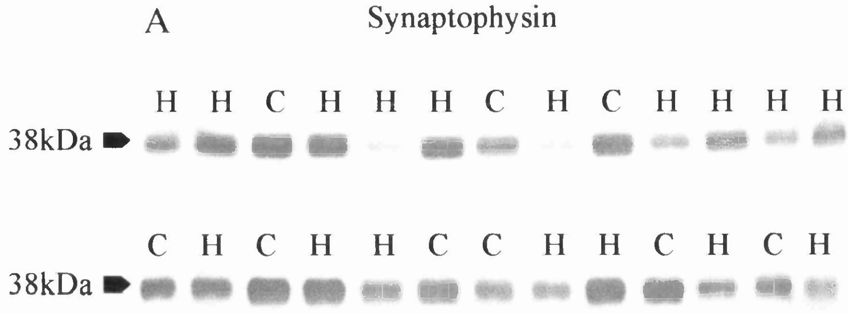


Figure 21

GAPDH immunoreactivity in the cingulate gyrus of head-injured patients compared with controls. A: Western blots of GAPDH immunoreactivity in 8 controls (C) and 16 head-injured patients (H), with detection of GAPDH immunoreactivity at the correct molecular weight of 36kDa. B: Optical density values for the western blots in A. Bars represent the group means. Levels of GAPDH immunoreactivity were unchanged in controls (open circles) compared with head-injured patients (closed circles), using the Unpaired two-tailed Student's *t* test.



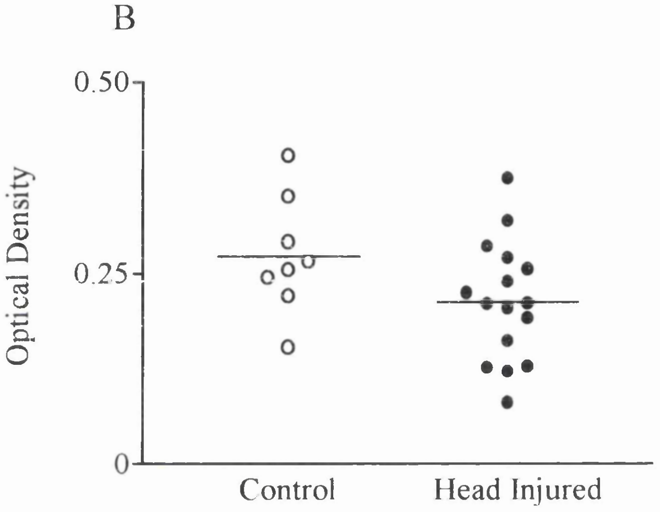


Figure 22

Synaptophysin immunoreactivity in the superior parietal cortex of head-injured patients compared with controls. A: Western blots of synaptophysin immunoreactivity in 8 controls (C) and 16 head-injured patients (H), with detection of synaptophysin at the predicted molecular weight of 38kDa. B: Quantitative data of the immunoblots in A. The mean value (bar) of synaptophysin immunoreactivity in the head-injured patients (closed circles) was no different to controls (open circles), using an unpaired two-tailed Student's *t* test for statistical analysis.

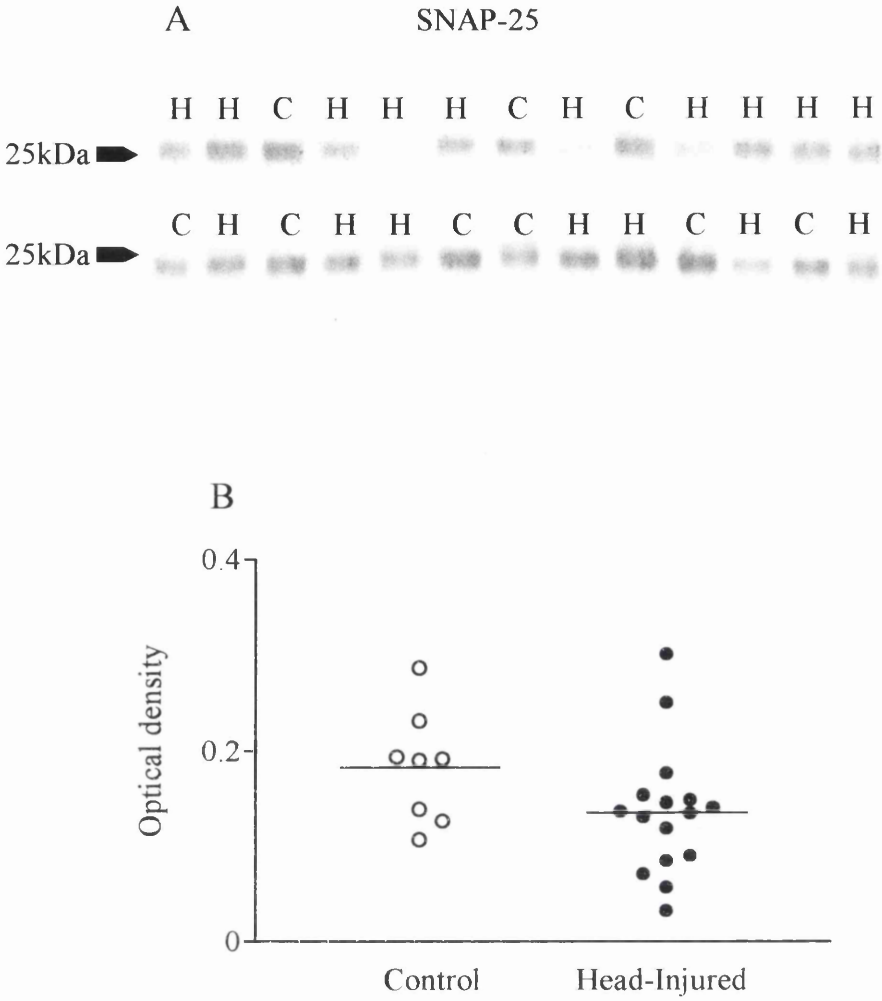


Figure 23

SNAP-25 immunoreactivity in the superior parietal cortex of head-injured patients compared with controls. A: Western blots of SNAP-25 immunoreactivity in 8 controls (C) and 16 head-injured patients (H), with detection of SNAP-25 immunoreactivity at the correct molecular weight of 25kDa. B: Quantitative data of the immunoblots in A. Unpaired two-tailed Student's *t* test revealed that the level of SNAP-25 immunoreactivity was no different in head-injured patients (closed circles) compared with controls (open circles). Bars represent the group means.

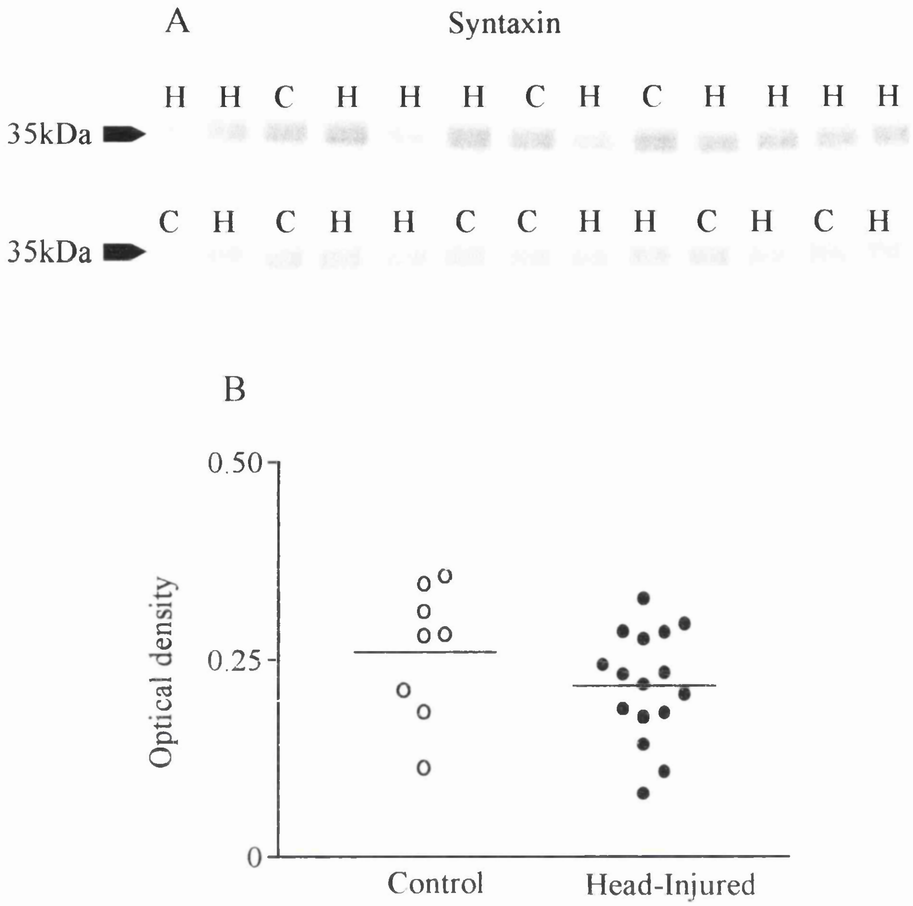


Figure 24

Syntaxin immunoreactivity in the superior parietal cortex of head-injured patients compared with controls. A: Western blots of syntaxin immunoreactivity in 8 controls (C) and 16 head-injured patients (H), with detection of syntaxin immunoreactivity at the correct molecular weight of 35kDa. B: Quantitative data of the immunoblots in A. Unpaired two-tailed Student's *t* test revealed that the level of syntaxin immunoreactivity was unchanged in head-injured patients (closed circles) compared with controls (open circles). Bars represent the group means.

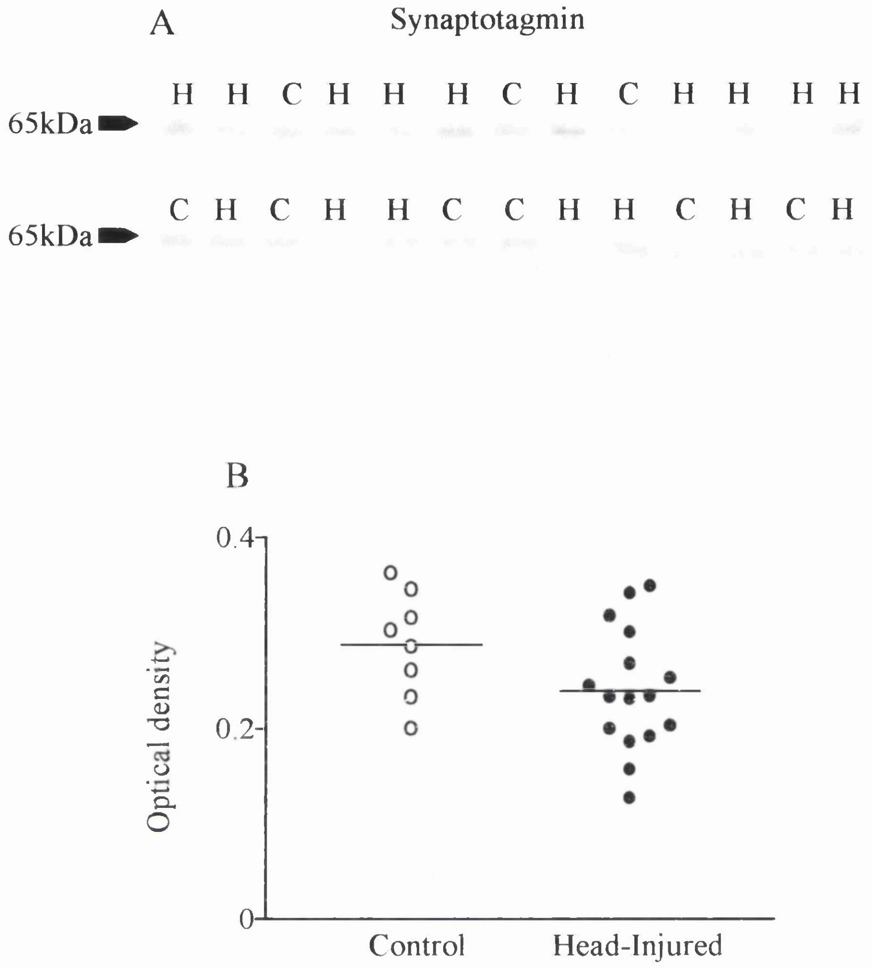


Figure 25

Synaptotagmin immunoreactivity in the superior parietal cortex of head-injured patients compared with controls. A: Western blots of synaptotagmin immunoreactivity in 8 controls (C) and 16 head-injured patients (H), with detection of synaptotagmin at the correct molecular weight of 65kDa. B: Optical density values of the immunoblots in panel A. Bars represent the group means. Levels of synaptotagmin immunoreactivity were not different between controls (open circles) and head-injured patients (closed circles), using unpaired two-tailed Student's *t* test for statistical analysis.

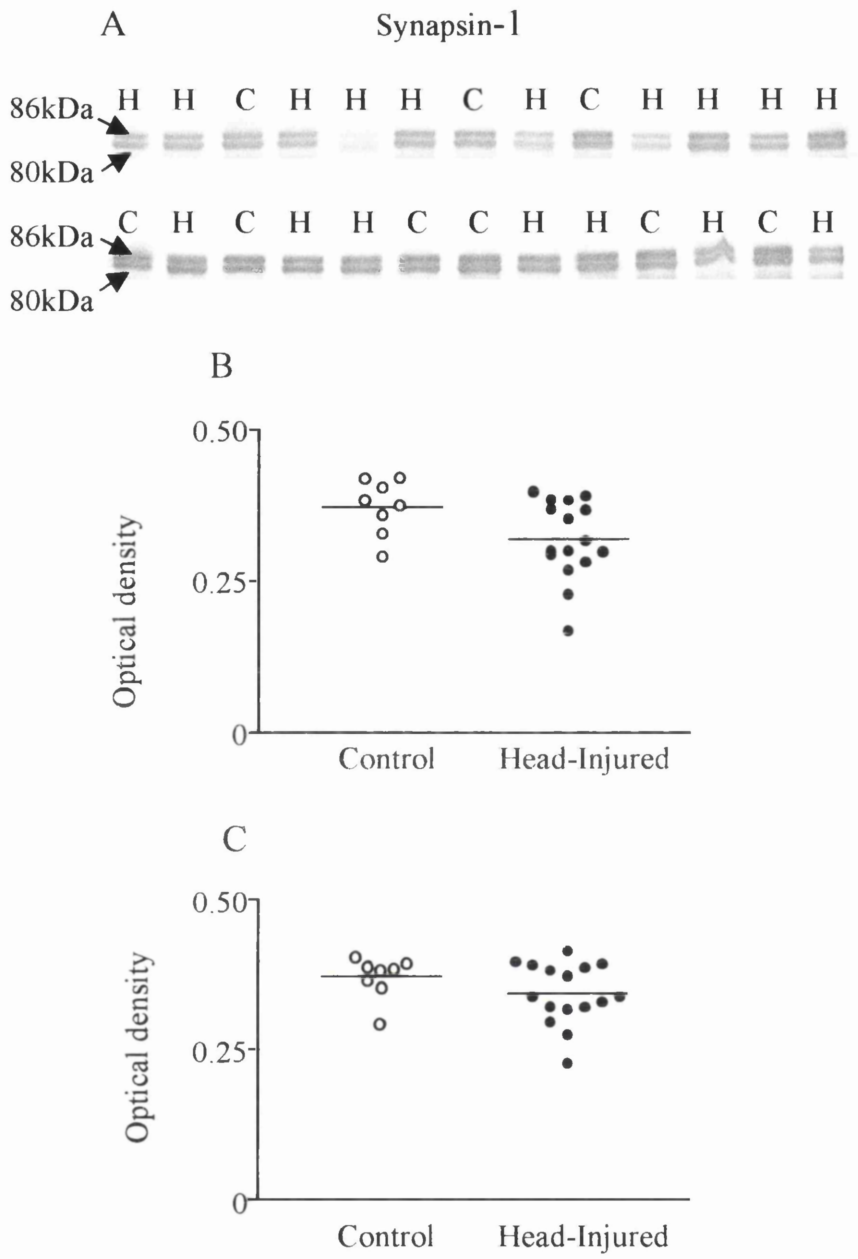


Figure 26

Synapsin I immunoreactivity in the superior parietal cortex of head-injured patients compared with controls. A: Western blots of synapsin I immunoreactivity in 8 controls (C) and 16 head-injured patients (H), with detection of the synapsin I doublet at the correct molecular weights of 86kDa and 80kDa for synapsin Ia and Ib respectively. B, C: Quantitative data for the Western blots in A. Bars represent the group means. Unpaired two-tailed Student's *t* test revealed there was no difference in the levels of both synapsin Ia and synapsin Ib in head-injured patients (closed circles) compared with controls (open circles).

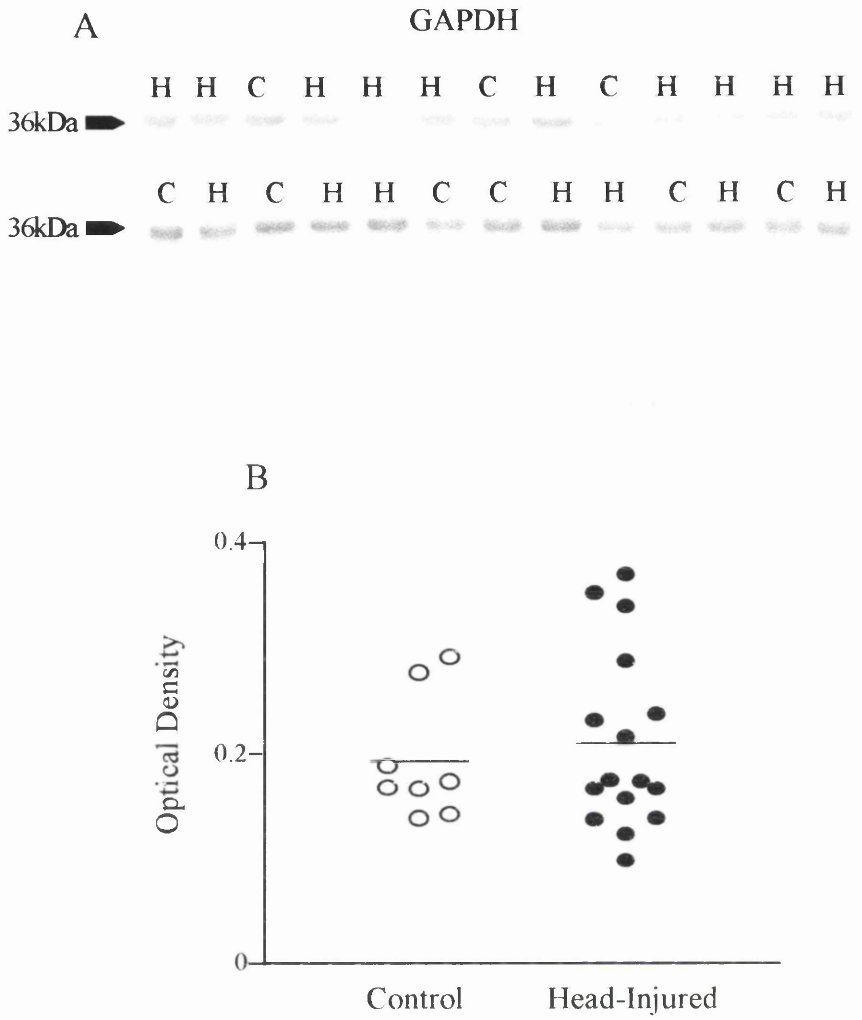
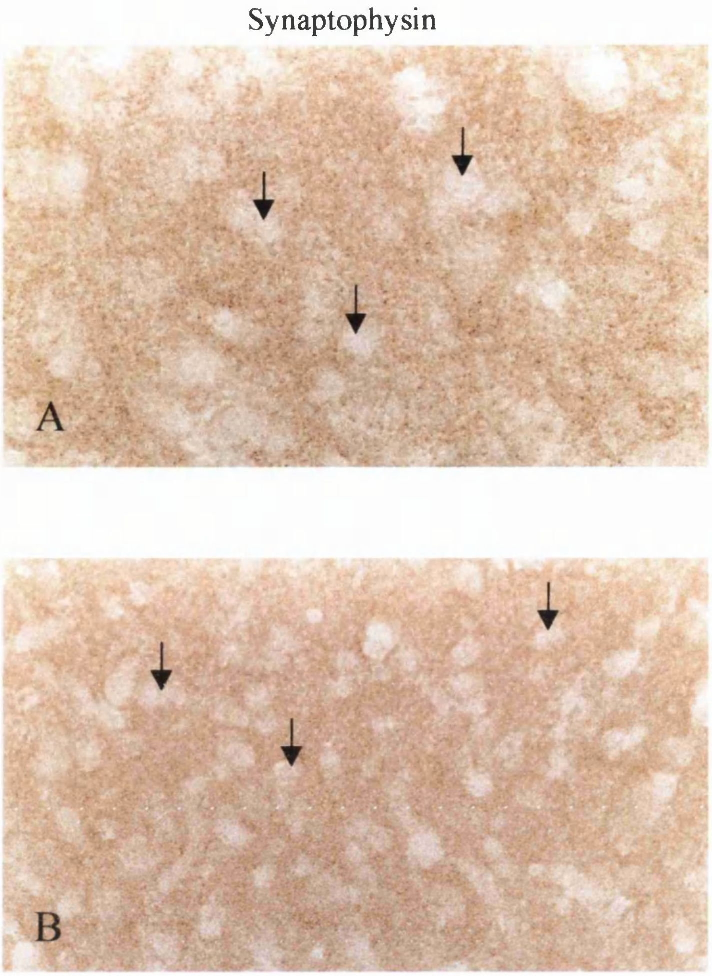


Figure 27

GAPDH immunoreactivity in the superior parietal cortex of head-injured patients compared with controls. A: Western blots of GAPDH immunoreactivity in 8 controls (C) and 16 head-injured patients (H), with detection of GAPDH immunoreactivity at the correct molecular weight of 36kDa. B: Optical density values for the western blots in A. Bars represent the group means. Unpaired two-tailed Students *t* tests revealed that levels of GAPDH immunoreactivity were unchanged in controls (open circles) compared with head-injured patients (closed circles).



Synaptophysin

Figure 28

Representative synaptophysin immunostaining in the cingulate gyrus from a control (A) and a head-injured patient (B). Immunostaining was diffuse and punctate in pattern in the neuropil, but minimal in cell bodies (arrows). Relative optical density values were measured from areas of synaptophysin immunostaining in the neuropil. Ten measurements were made per case and the means calculated. Magnification X400.

Table 6: Summary of Cortical Presynaptic Protein Pathology after Human Head Injury

	Cingulate	Parietal
Synaptophysin	-28% *	-18%
SNAP-25	-36% *	-26%
Syntaxin	-43%**	-14%
Synaptotagmin	-18%	-17%
Synapsin Ia	+12%	-8.1%
Synapsin Ib	+20%	-10.8%
GAPDH	+1.3%	+ 4.7%

Data represents the percentage difference in the mean optical density for each group of head-injured patients (n=16) compared with controls (n=8). Values represent either an increase (+) or a reduction (-) in optical density. $^{*}p<0.05$ and $^{**}p<0.01$ by unpaired two-tailed Student's *t* tests

measurements of the immunoreactive signal. In accordance with the Western blot analysis (Figure 16), synaptophysin immunostaining was reduced in the cingulate gyrus of head-injured patients compared with controls, equivalent to a 30% reduction (Figure 29). In contrast, SNAP-25 immunoreactivity was no different in the head-injured patients compared to controls.

3.1.4 Correlation of ChAT activity with synaptophysin immunoreactivity

Widespread cortical deficits of ChAT activity have been demonstrated previously for the same group of controls and head-injured patients used in the current study (Murdoch et al., 1998). Correlation of ChAT activity in the cingulate gyrus and the superior parietal cortex with synaptophysin levels (detected by Western blot analyses) for the head-injured group indicated that patients with low levels of ChAT activity tended to have low levels of synaptophysin immunoreactivity whereas those with higher ChAT

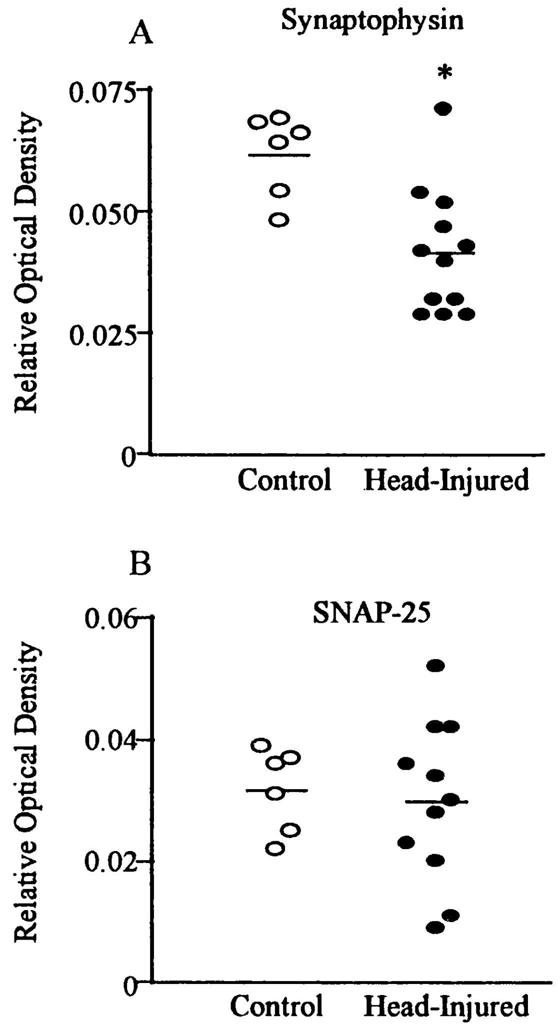


Figure 29

Relative optical densities of synaptophysin (A) and SNAP-25 (B) immunoreactivities in the cingulate gyrus of controls (open circles) and head-injured patients (closed circles). Data represent mean ± SEM and individual data points correspond to individual patients values. Unpaired two-tailed Student's *t* test revealed that the level of synaptophysin immunoreactivity was reduced by 30%, *$p<0.05$, in head-injured patients compared with controls. The level of SNAP-25 immunoreactivity was no different between controls and head-injured patients.

activity tended to have higher levels of synaptophysin immunoreactivity (Figure 30). Correlation coefficients for linear regression analyses (Pearson's product moment) were $r^2=0.260$ and $r^2=0.461$ for the cingulate gyrus and the superior parietal cortex respectively, with *$p < 0.05$ for the superior parietal cortex.

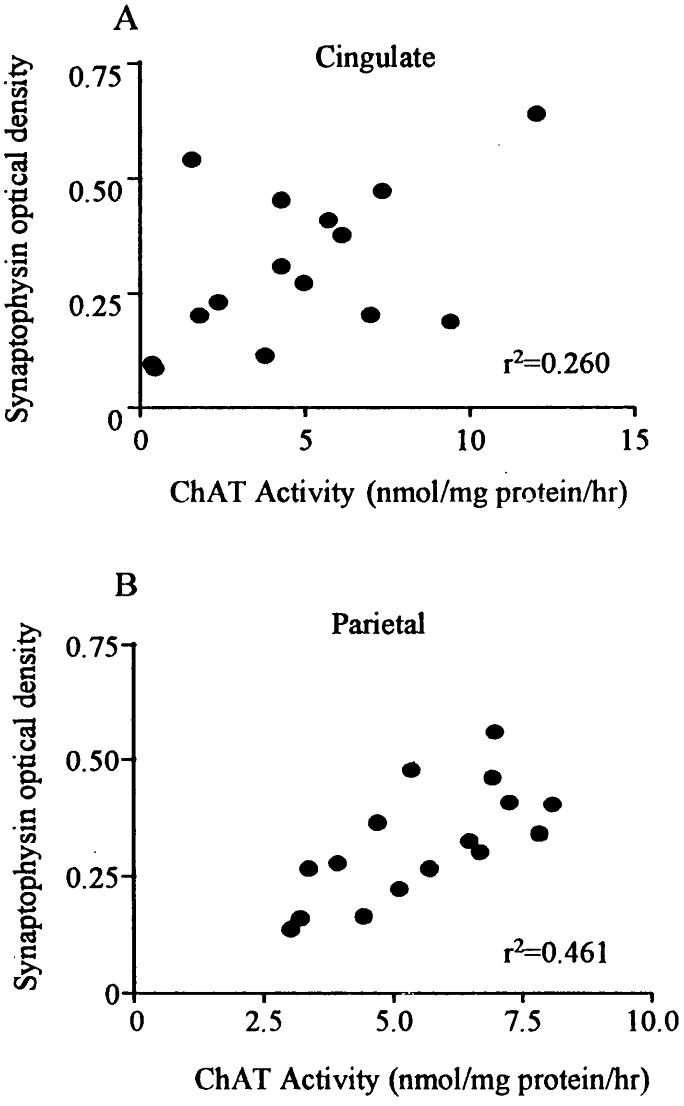


Figure 30

Relationship between synaptophysin immunoreactivity detected by Western blot analysis and ChAT activity in the cingulate gyrus (A) and the superior parietal cortex (B) of head-injured patients. The linear coefficient value is stated for both cortical regions. Linear regression analysis indicated that *$p<0.05$ for the parietal, but $p>0.05$ for the cingulate gyrus.

3.1.5 Postsynaptic changes after head injury

To contrast the presynaptic changes observed after head injury, MAP-2, a protein routinely used as a marker of postsynaptic elements after brain injury (Kitagawa et al., 1989; Folkerts et al., 1998), was detected by immunohistochemistry in the cingulate gyrus from the same group of controls and head-injured patients as those used to assess presynaptic pathology. In controls, MAP-2 immunostaining was faint and limited to narrow dendritic processes. The dendrites were not fragmented and no pinching or swelling of MAP-2 staining was seen along the length of the dendrites (Figure 31). In head-injured patients, MAP-2 immunostaining accumulated in dendrites and shrunken cell bodies. In a patient who survived 3 days after a head injury, MAP-2 was localised to swollen, fragmented dendrites, indicating a disruption of dendritic processes after head injury. In a patient who survived 4 days after head injury, MAP-2 staining was intense not only in the dendritic processes but also in shrunken neuronal perikarya.

3.1.6 Nitrotyrosine formation after head injury

Free radical-mediated neuronal cell death is implicated in the pathogenesis of brain damage (Coyle and Puttfarcken, 1993; Berlett and Stadtman, 1997). The potent oxidant peroxynitrite is implicated in diverse pathological conditions (Miller et al., 1995; Shigenaga et al., 1997). To investigate whether free radical-mediated damage was involved in the presynaptic dysfunction in the cingulate gyrus after head injury, the level of the peroxynitrite-radical byproduct, nitrotyrosine (Kooy et al., 1995; Smith et al., 1997) was detected by Western and dot blot analyses in homogenates from grey matter from the cingulate gyrus of control and head-injured patients. Using Western blot analyses, specific binding of the anti-nitrotyrosine antibody to several epitopes was detected in both controls and head-injured patients (Figure 32), although the signal was unsuitable for quantification. Dot-blot analyses produced an easily quantifiable signal, with nitrotyrosine immunoreactivity no different between control and head-injured patients. Nevertheless, there was a trend towards an increase in the head-injured group, with only 5 of the head-injured patients having a nitrotyrosine relative optical density lower than the mean of the controls (Figure 33).

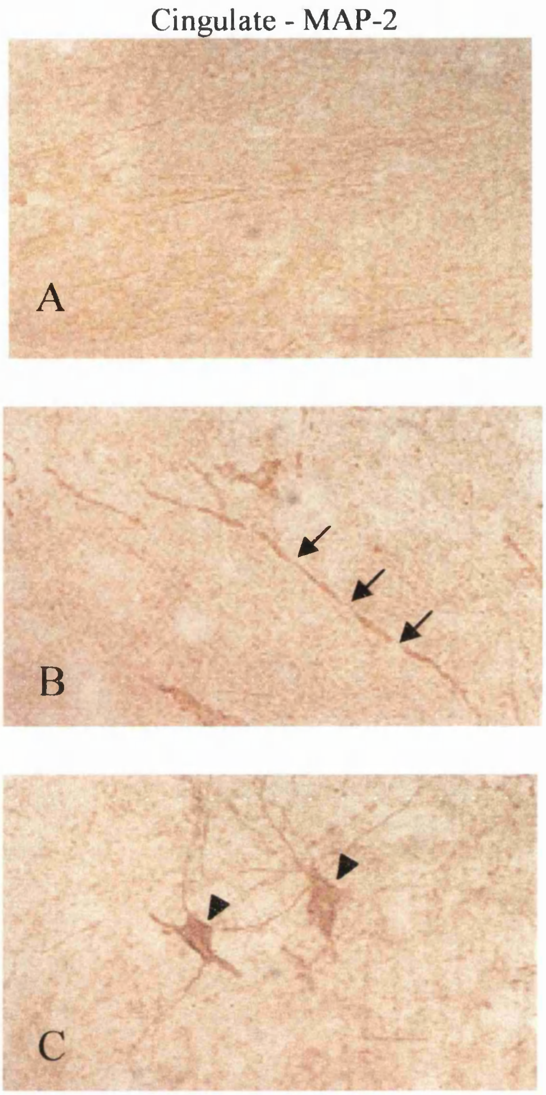


Figure 31

Representative MAP-2 immunostaining in the cingulate gyrus of controls (A) and head-injured patients (B, C). MAP-2 immunostaining was localised to thin, continuous dendrites in controls (A). In a head-injured patient who survived 3 days (B) MAP-2 immunostaining was located in swollen, fragmented dendrites (arrows). In a patient who survived 4 days after a head injury (C), MAP-2 immunostaining was present in dendrites, but also accumulated in cell bodies (arrowheads). Magnification X400.

Nitrotyrosine

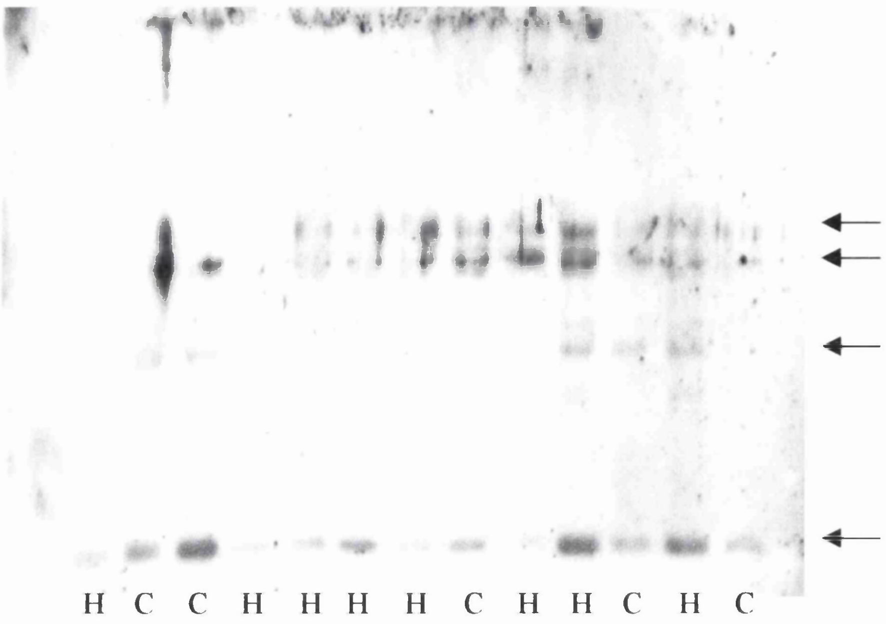


Figure 32
Western blot of nitrotyrosine immunoreactivity in the cingulate gyrus of controls (C) and head-injured patients (H). A number of bands (arrows) were detected in both controls and head-injured patients, indicating binding of the primary antibody to epitopes on proteins of various molecular weights. These results indicate that nitrotyrosine residues are present in proteins in both controls and head-injured patients.

3.2 nbM pathology after human head injury

The nbM provides a cholinergic input to the entire cortical mantle through the Ch4 subpopulation of neurons (Mesulam et al., 1983b). To elucidate whether the deficits in cortical ChAT activity after head injury reflects a loss of cortical cholinergic presynaptic terminals or alternatively loss of cholinergic input from the basal forebrain, the integrity of the Ch4 neurons in the nbM was determined in the same group of controls and head-injured patients. The coronal sections of 10%-formal saline fixed brain containing the intermediate portion of the nbM were macroscopically identified using the anterior commissure and optic tract as anatomical landmarks (Allen et al., 1988). In a head-injured case, macroscopic evidence of damage within the nbM territory

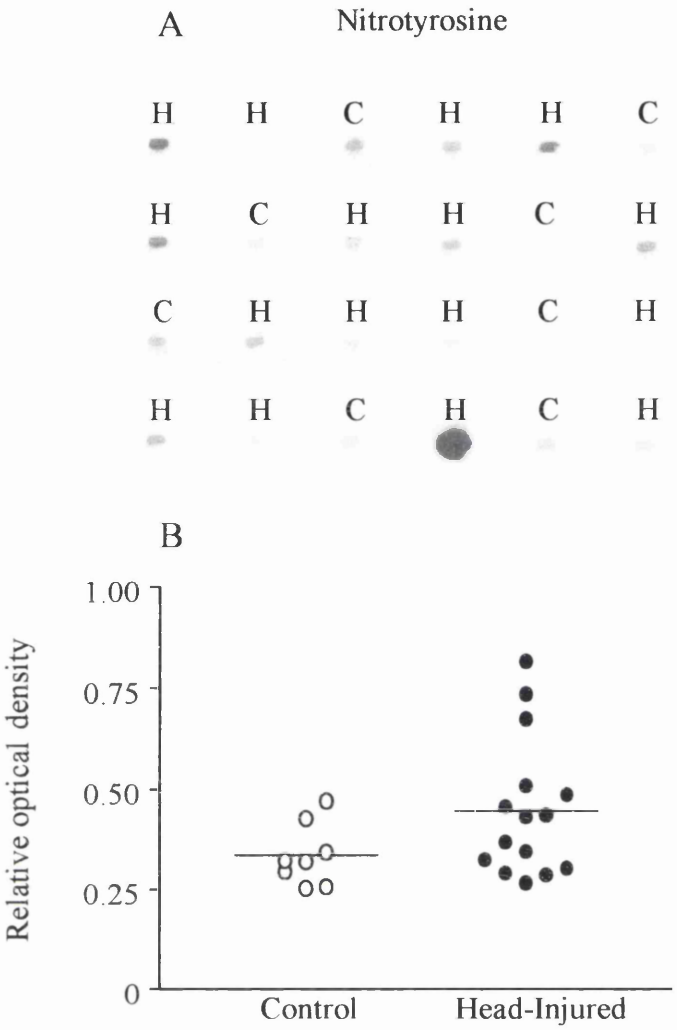


Figure 33

Quantification of nitrotyrosine immunoreactivity in the cingulate gyrus of controls and head-injured patients. A: Dot-blot of nitrotyrosine immunoreactivity for the 8 controls and 16 head-injured patients. B: Quantitative data for nitrotyrosine immunoreactivity in the cingulate gyrus of controls (open circles) and head-injured patients (closed circles). Individual data points correspond to values for individual patients and the bars represent the group means. Unpaired two-tailed Student's *t* test analysis revealed no statistically significant difference between groups.

was evident through presence of a haemorrhage and a compression of the brain structures due to raised intracranial pressure (Figure 34).

Figure 34

Photomicrograph of macroscopic identification of neuropathological findings in the region of the nbM of a head-injured patient. The coronal slice shown provides evidence of a well-defined dark core, corresponding to a brain haemorrhage (arrow), resulting in ischaemic brain damage, raised intracranial pressure and a midline shift (#) causing compression of brain structures, including the nbM (arrowhead).

3.2.1 Histological assessment

Ch4 neurons of the nbM were identified using H&E and LFB/CV stained sections (Figure 35). In controls, Ch4 neurons were large (30-40μm) and circular, although often oval. The nucleus was eccentrically placed with a distinct nucleolus and abundant cytoplasm, with marginated Nissl bodies and lipofuscin granules. In tissue from head-injured cases a range of morphological changes were seen in Ch4 neurons. Firstly, ischaemic cell change, the neurons having an intensely eosinophilic or luxophilic cytoplasm and in some cases incrustations (Figure 35). The nucleus was hyperchromatic and the nucleolus could no longer be recognised. In a few head-injured cases there was infarction of part or the entire nbM nucleus (Figure 35): in several cases the margins of the lesion could be demarcated by the boundary of cresyl violet stained and luxophilic

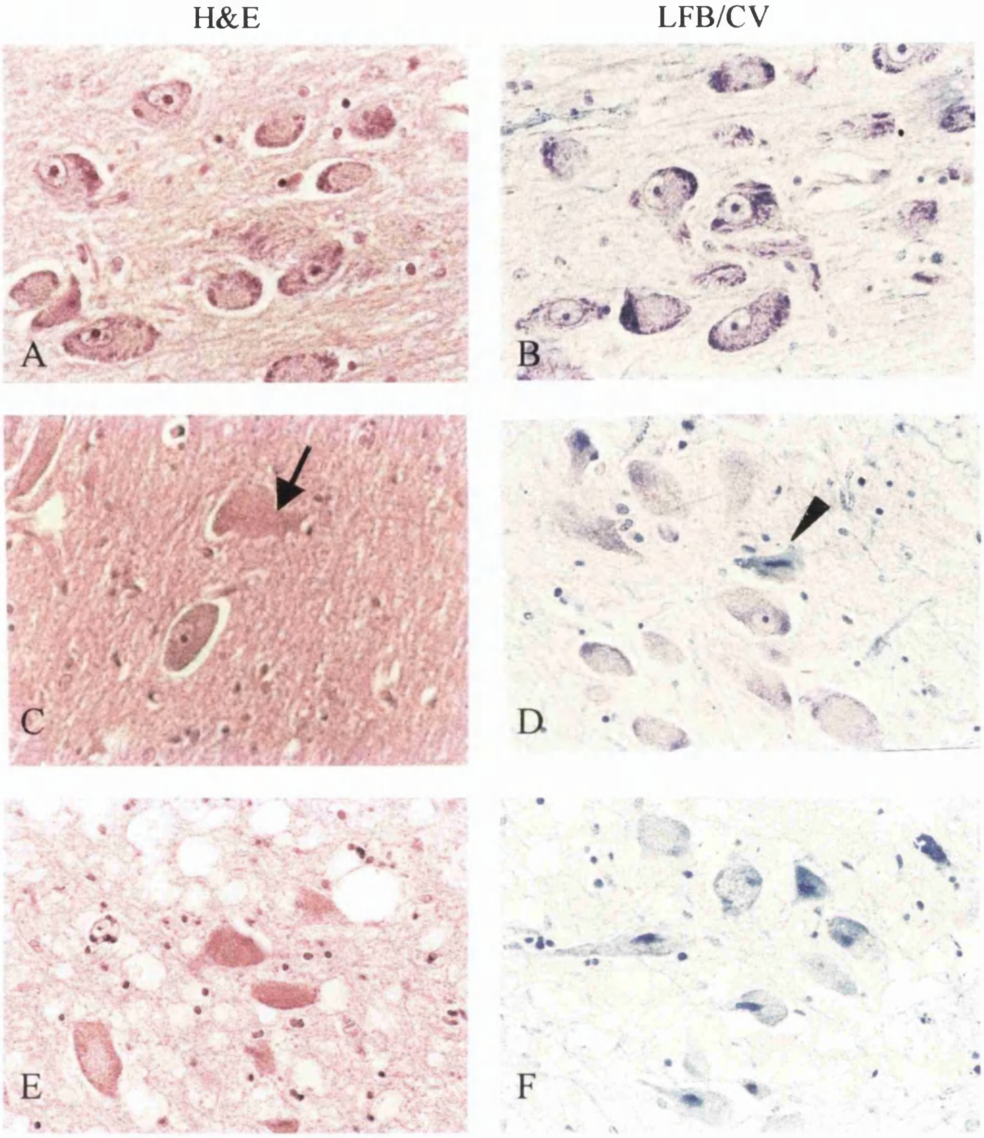


Figure 35

Photomicrograph of histological assessment of Ch4 neuronal damage in the nbM of controls (A, B) and head-injured patients (C, D, E, F) using H&E (A, C, E) and LFB/CV (B, D, F). In the control case, Ch4 neurons were large in diameter, densely staining for nissl stains, with the nucleolus clearly defined (A, B). A range of morphological changes existed in head-injured patients, including partial (C, D) or total infarction of the nbM nucleus (E, F). Ischaemic neurons were eosinophilic (arrows) and no longer positive for cresyl violet, but were luxophilic (arrowheads). Magnification X400.

neurons (Figure 36). Evidence of mechanical distortion resulting from compression of brain tissue and development of 'plastic creep' (Langfitt et al., 1964) of neurons was also observed, with neurons being transformed from a round to an elongated structure.

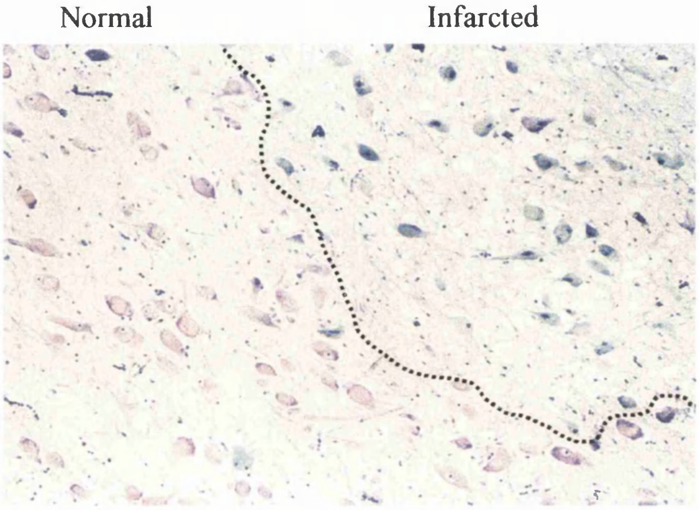


Figure 36
Photomicrograph of LFB/CV staining at the border of a normal portion of the nbM and a zone of localised infarction. Normal Ch4 neurons were generally positive for cresyl violet with the nucleolus clearly defined. Within the infarcted zone, Ch4 neurons were irregular and luxophilic, with the nucleolus and nuclear membrane less distinct. The dotted line represents the border between normal and infarcted nbM territory. Magnification X200.

Damage to the Ch4 neurons of the nbM was semi-quantitatively assessed by 2 investigators, both blind to the identity of the cases. None of the control cases examined had any evidence of Ch4 neuronal damage, all being attributed a score of 0 (Table 7). The scores given for head-injured patients ranged from 0 through to 4, with 8 of the 12 head-injured patients having evidence of neuronal damage in the nbM. There was no association between survival time after death and postmortem delay and neuronal damage in the nbM of head-injured patients (Figure 37).

Table 7: Neuronal damage in the human nbM after head injury

Neuronal damage score	Controls (n=4)	Head-Injured (n=12)
0	4	4
1	0	3
2	0	1
3	0	2
4	0	2

Number of controls and head-injured patients exhibiting neuronal damage scores of 0-4. None of the controls had evidence of Ch4 neuronal damage in the nbM. Two-thirds of head-injured patients examined had evidence of neuronal damage in the nbM.

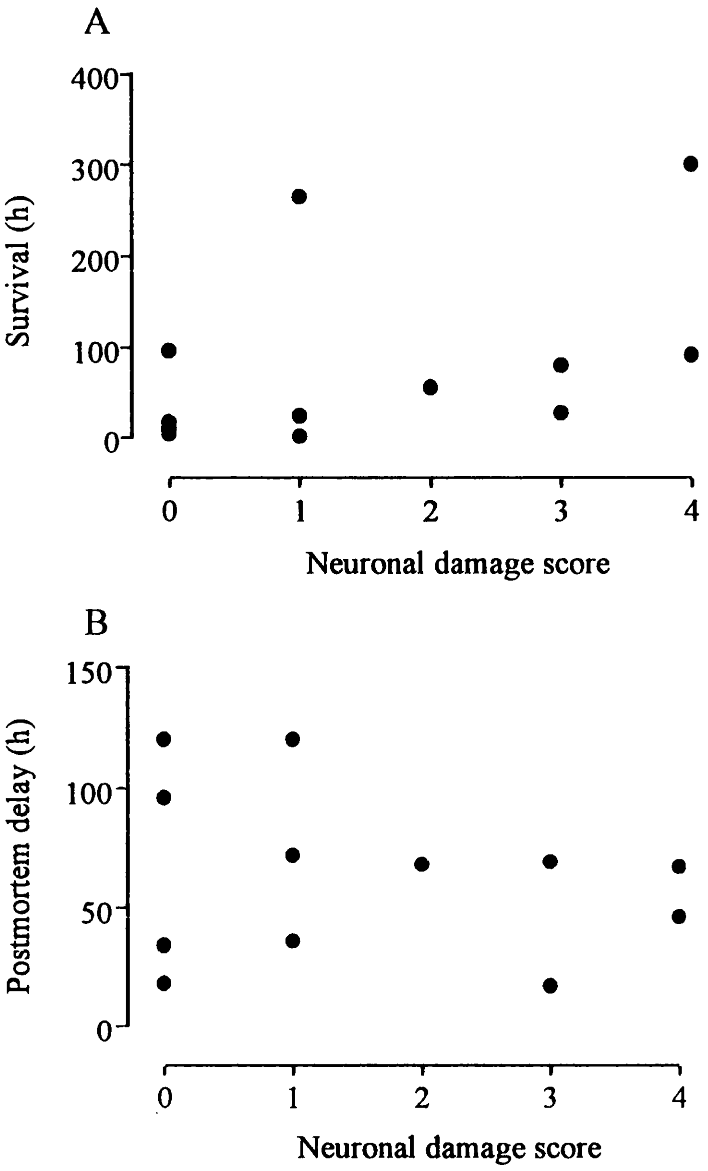


Figure 37

Plot of neuronal damage score in the nbM of head-injured patients and survival (A) or postmortem delay (B). Individual data points correspond to scores attributed to individual head-injured patients. No relationship existed between neuronal damage in the nbM and either of these parameters in head-injured patients.

3.2.2 Correlations between Ch4 neuronal damage and ChAT activity and levels of presynaptic proteins

To determine if damage to Ch4 neurons of the nbM was associated with ChAT activity/immunostaining and presynaptic protein levels in the cortex a number of correlations were performed. ChAT activity in the cingulate gyrus, superior parietal cortex and inferior temporal gyrus for the same head-injured cases and neuronal damage to Ch4 neurons of the nbM were related in that head-injured patients with extensive neuronal damage in the nbM tended to have low levels of ChAT activity, whereas those with no or minimal neuronal damage in the nbM had higher levels of ChAT activity (Figure 38). Association of Ch4 neuronal damage in the nbM did not correlate with the levels of the presynaptic proteins synaptophysin, SNAP-25, syntaxin and synaptotagmin in the cingulate gyrus (Figure 39) and the superior parietal cortex (Figure 40).

3.2.3 ChAT Immunostaining

ChAT immunostaining in controls was prominent in large, circular Ch4 neurons, and in dendritic processes. Immunostaining was absent from the nuclear membrane and nucleolus of controls. In contrast, in head-injured patients ChAT immunostaining was present in shrunken neurons with the nucleolus intensely positive for ChAT (Figure 41). The overall intensity of ChAT immunostaining was reduced in the head-injured patients compared to controls (Figure 41). Localisation of ChAT to shrunken neurons and a reduction in the intensity of staining both imply that the neuronal damage in the human nbM after head injury is to the cholinergic cells.

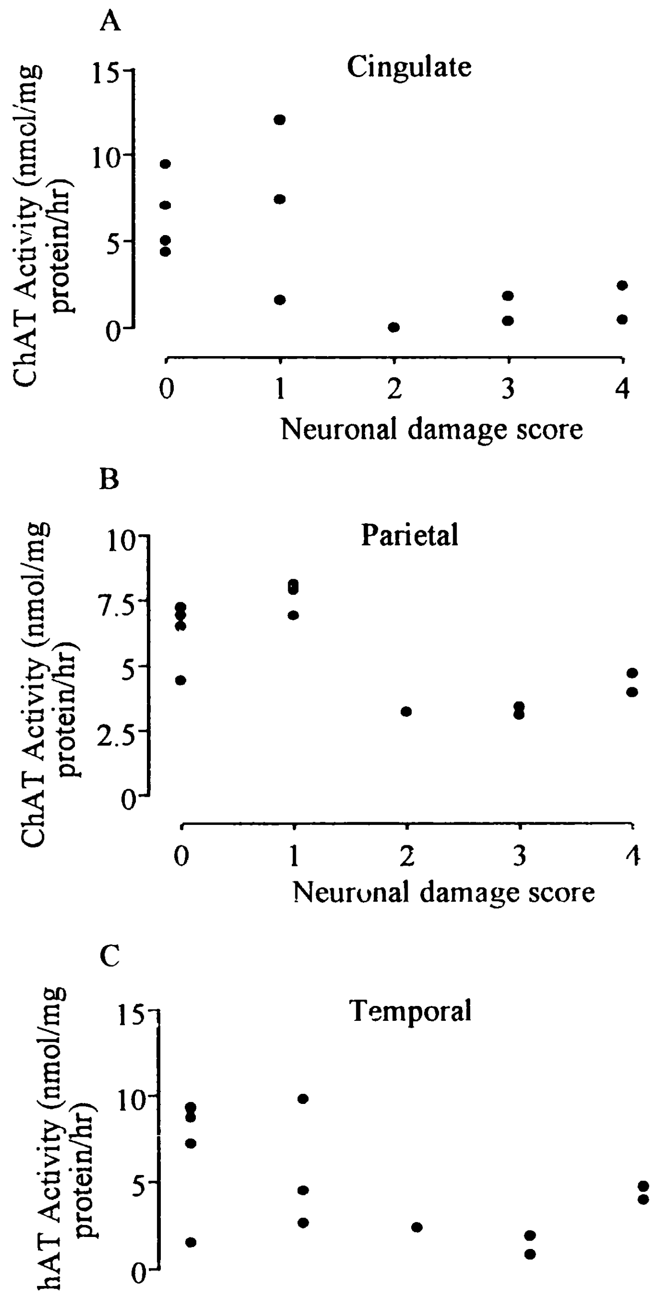


Figure 38

Relationship between neuronal damage in the nbM and ChAT activity (from Murdoch et al., 1998) in the cingulate gyrus (A), superior parietal cortex (B) and inferior temporal gyrus (C) of head-injured patients. Individual data points correspond with individual head-injured cases. In each cortical region, head-injured patients with extensive neuronal damage in the nbM had low levels of ChAT activity, whilst patients with no or minimal neuronal damage in the nbM had higher levels of cortical ChAT activity.

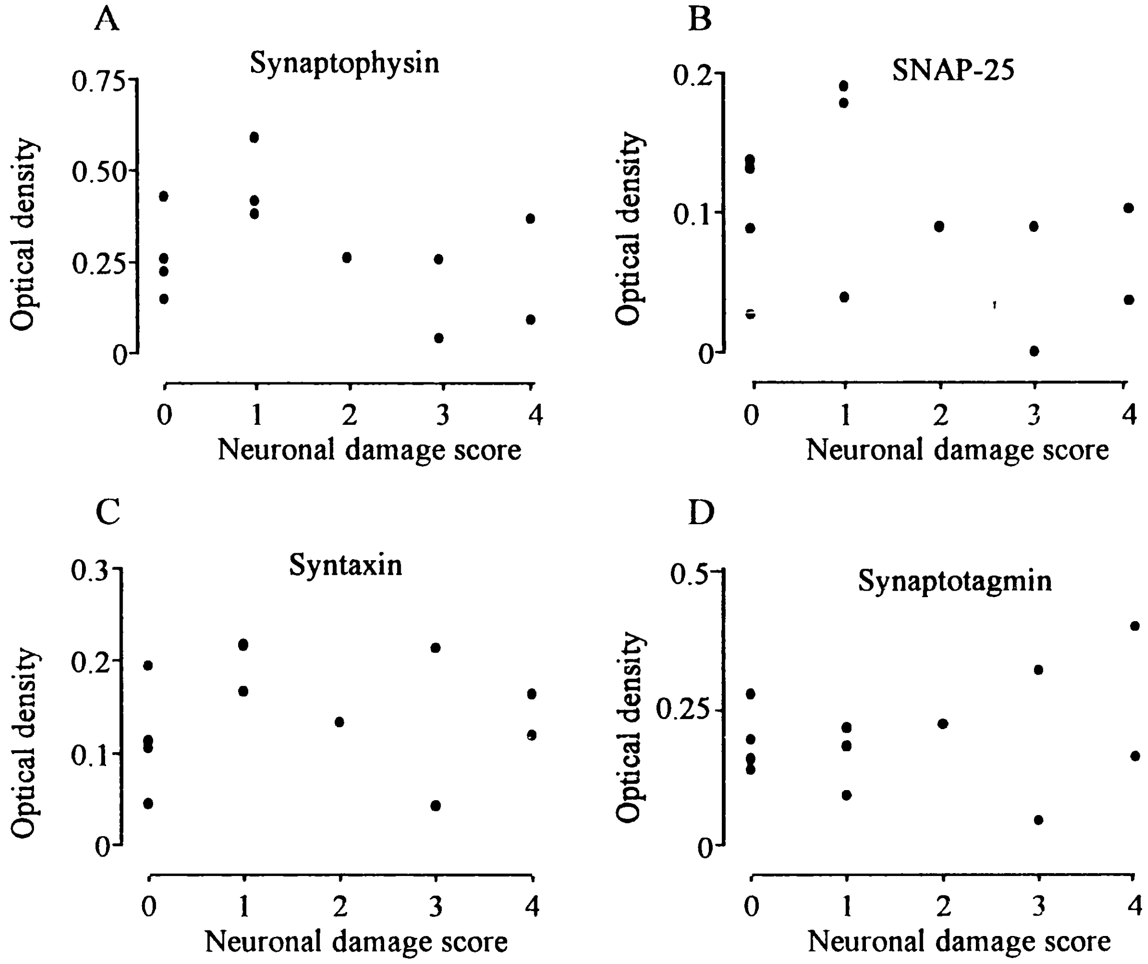


Figure 39

Relationship between neuronal damage in the nbM and the levels of synaptophysin (A), SNAP-25 (B), Syntaxin (C) and Synaptotagmin (D) immunoreactivities in the cingulate gyrus of head-injured patients. Individual data points correspond with individual head-injured cases. No association existed between the extent of neuronal damage in the nbM and the levels of immunoreactivity for each of these presynaptic proteins in the cingulate gyrus of head-injured patients.

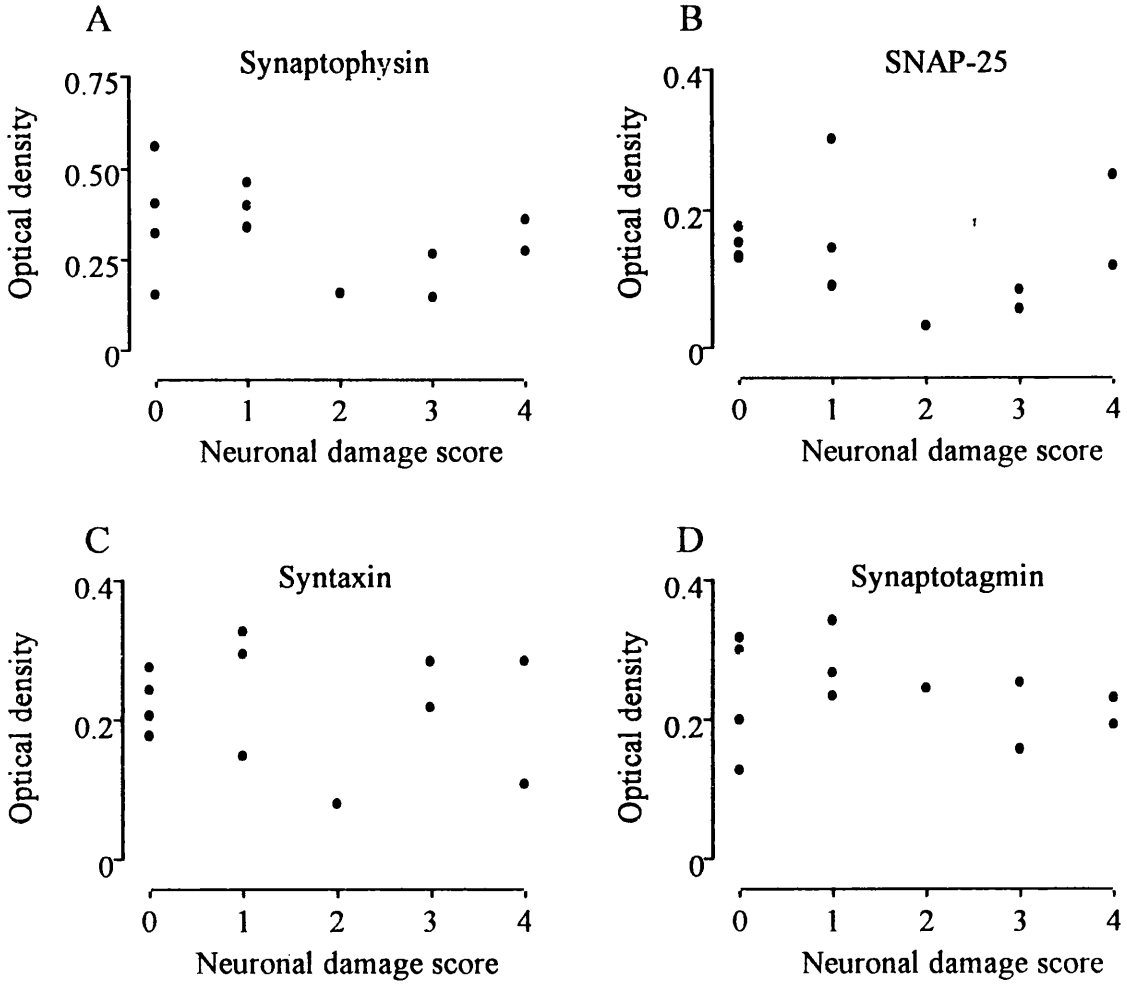
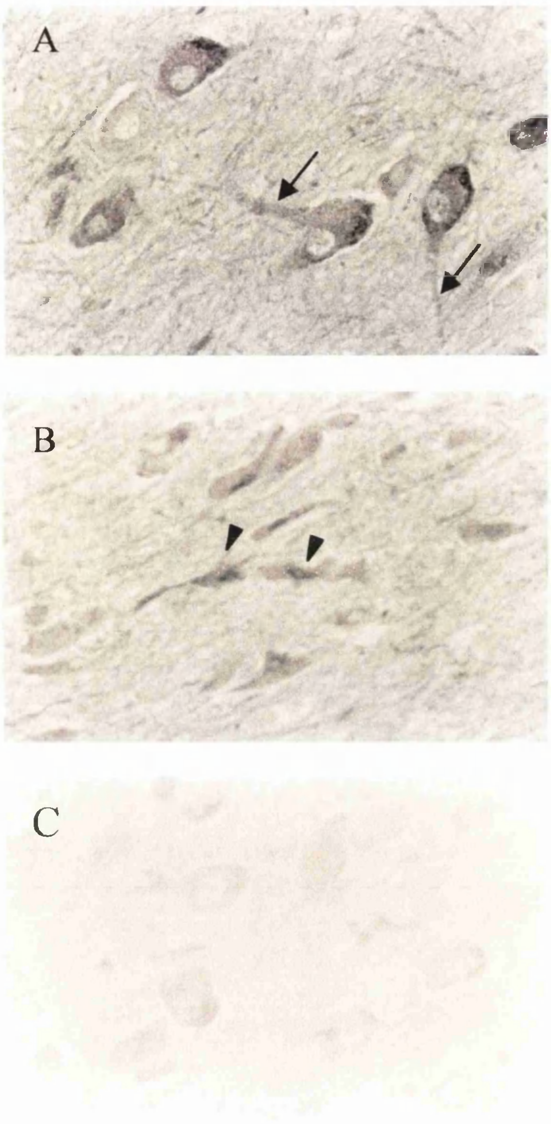


Figure 40

Relationship between neuronal damage in the nbM and the levels of synaptophysin (A), SNAP-25 (B), Syntaxin (C) and Synaptotagmin (D) immunoreactivities in the superior parietal cortex of head-injured patients. Individual data points correspond with individual head-injured cases. No association existed between the extent of neuronal damage in the nbM and the levels of immunoreactivity for each of these presynaptic proteins in the superior parietal cortex of head-injured patients.



Choline Acetyltransferase

Figure 41

Photomicrographs of choline acetyltransferase immunostaining in Ch4 neurons of the nbM in a control (A) and a head-injured patient (B). In the control case, ChAT immunostaining was intense in perikaya and dendritic processes (arrows) of large, circular Ch4 neurons (A). In the head-injured case, intense ChAT immunostaining was present in the nucleolus of shrunken, irregular nbM neurons (arrowheads) (B). No ChAT immunostaining was detected in the negative control (no primary antibody) (C). Magnification X400.

3.3 Presynaptic pathology in animal models of brain injury

The pathophysiology of human head injury is extremely complex involving a wide variety of different mechanisms. Ischaemic damage is one of the most common neuropathological features after head injury (Graham et al., 1978). In order to determine if ischaemia per se, in the absence of other pathologies, affected presynaptic proteins, the proteins examined in human material were studied in a number of animal models of cerebral ischaemia. Techniques used were Western blotting and immunohistochemistry followed by densitometric analysis.

3.3.1 Permanent middle cerebral artery occlusion in the rat: Intraluminal thread model

Occlusion of the MCA by insertion of an intraluminal thread for 24h produced an area of ischaemia damage easily delineated using H&E staining (Figure 42). The area of in the ipsilateral hemisphere corresponds to those brain regions supplied by the MCA. In the ipsilateral hemisphere, the cingulate gyrus was unaffected with staining comparable to the contralateral hemisphere.

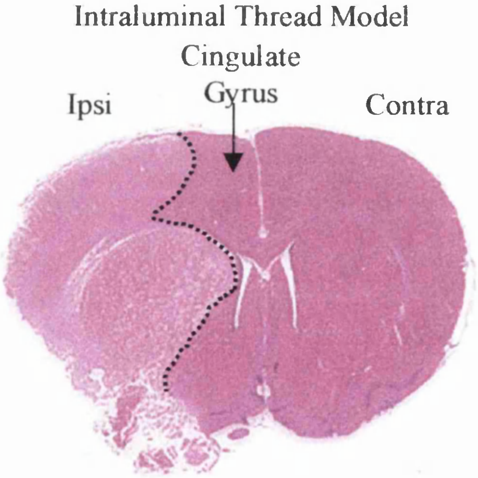


Figure 42

Representative H&E stained coronal section at the level of the caudate nucleus after 24h permanent MCAo using the intraluminal thread model. The dotted line delineates the area of palor corresponding to the zone of ischaemic brain damage as a consequence of compromised blood flow. Note the H&E staining of the ipsilateral cingulate cortex is normal (arrow) and a zone of tissue degradation exists as a consequence of severe ischaemic damage. Brain tissue was dissected from the ipsilateral cortex (area of palor) and the corresponding contralateral cortex. The cingulate cortex was dissected from both cerebral hemispheres.

Western blotting of homogenates from the cingulate gyrus and the MCA territory from the contralateral and ipsilateral hemisphere was performed for synaptophysin, syntaxin and SNAP-25 immunoreactivity (Figure 43). For each of these presynaptic proteins, the level of immunoreactivity in the MCA territory and cingulate gyrus were no different in the ipsilateral hemisphere compared with the contralateral. This suggests that presynaptic proteins are not degraded after 24h permanent MCAo using the intraluminal thread model.

3.3.2 Permanent middle cerebral artery occlusion in the rat: Diathermy model

24h after permanent MCAo, the brains were removed, fixed and processed for immunohistochemistry. Sections were immunostained for the presynaptic proteins synaptophysin, SNAP-25 and syntaxin. In the contralateral cortex, immunostaining for each of these proteins was diffuse and restricted to the neuropil. Neuronal perikarya were counterstained with haematoxylin and none of the presynaptic protein immunostaining was localised to the large, circular cell bodies. (Figure 44). In the ipsilateral cortex neuronal perikarya were shrunken and triangular with vacuolation of the neuropil. The pattern of immunohistochemical staining in the ipsilateral cortex was different to the contralateral cortex for each of the presynaptic proteins. SNAP-25 and syntaxin immunoreactivity appeared to be reduced, although this could not be quantified due to severe oedema of the tissue (Figure 44). In the contralateral caudate, synaptophysin and syntaxin immunoreactivity were restricted to the neuropil, being completely absent from axon fibre tracts and healthy neuronal perikarya (Figure 45). SNAP-25 immunostaining was present in both the neuropil and the axonal tracts, but not the neuronal perikarya in the contralateral caudate. In the ipsilateral hemisphere, widespread neuronal damage existed after 24h permanent MCAo. Synaptophysin immunostaining was similar to the contralateral caudate, however, SNAP-25 and to a lesser extent syntaxin immunoreactivity had accumulated as intensely staining globules in the axon fibre tracts (Figure 45). A diffuse pattern of immunostaining for each of the presynaptic proteins was preserved in the neuropil.

3.3.3 Transient bilateral common carotid artery occlusion (BCCAo) in the mouse.

Presynaptic proteins were not altered in brain tissue which had been rendered permanently ischaemic. In head-injured patients there may be transient periods of ischaemia followed by reperfusion of the tissue. These events can be modeled in

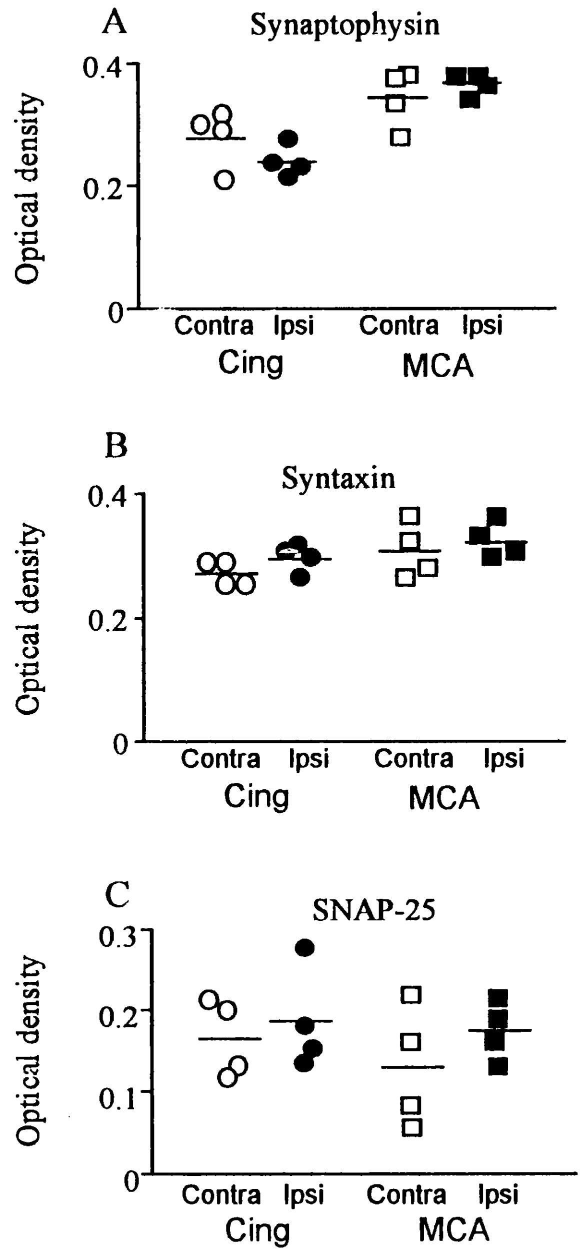


Figure 43

Quantitative data for Western blots of the presynaptic proteins synaptophysin (A), syntaxin (B) and SNAP-25 (C) in cingulate (Cing) cortex (circles) and middle cerebral artery (MCA) territory (squares) after 24h permanent MCAo using the intraluminal thread model in the rat (n=4). Open (contralateral) and closed (ipsilateral) shapes show optical densities for the presynaptic proteins in the contralateral and ipsilateral hemispheres respectively.

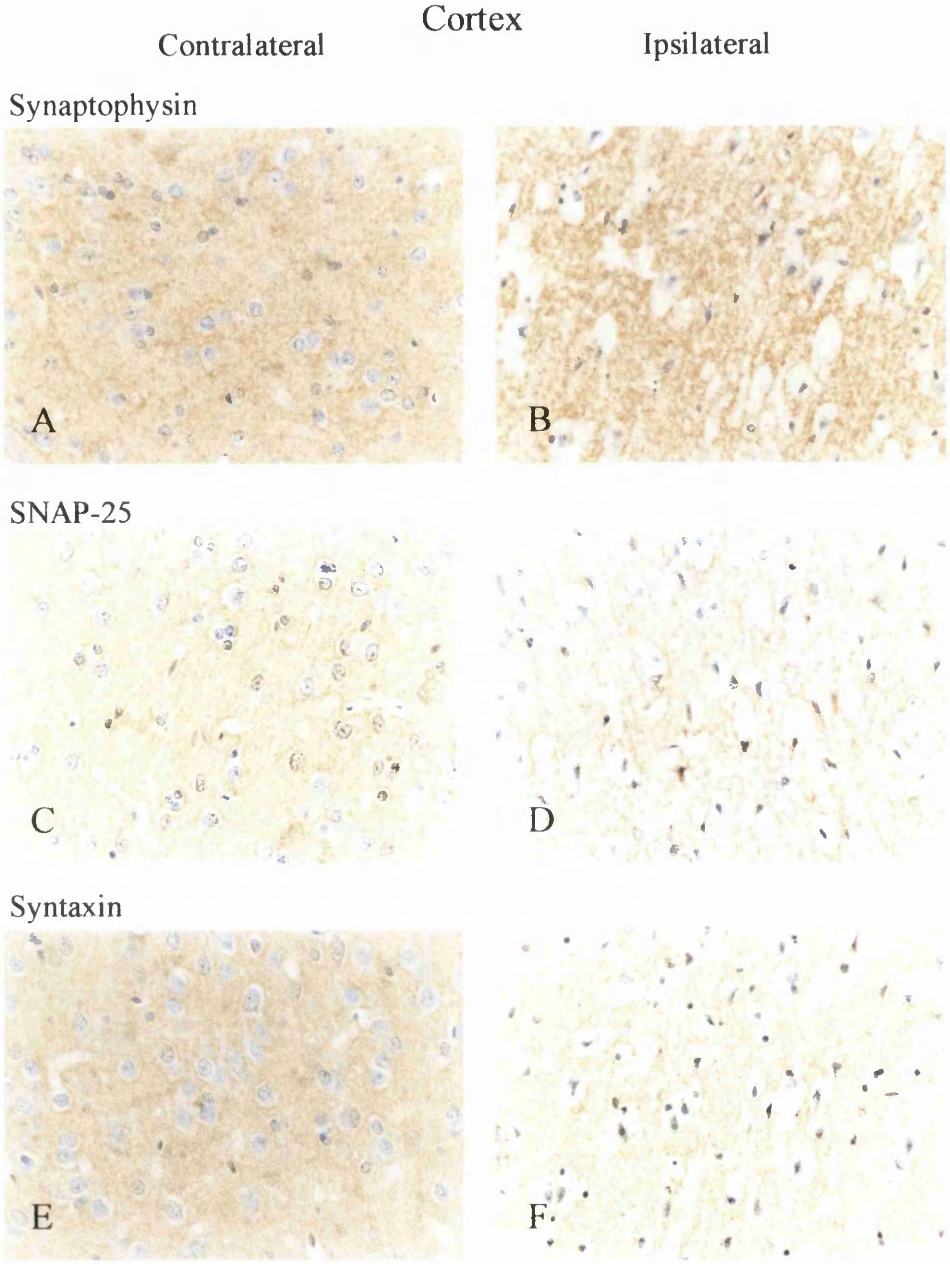


Figure 44

Photomicrographs of synaptophysin (A, B), SNAP-25 (C, D) and syntaxin (E, F) immunostaining in the contralateral (A, C, E) and ipsilateral cortex (B, D, F) after 24h permanent MCAo in the rat. Immunostaining for each of the presynaptic proteins was restricted to and diffuse in the cortical neuropil in the contralateral hemisphere. After MCAo, extensive neuronal loss existed in the ipsilateral cortex and tissue damage as a consequence of oedema was observed. Immunostaining for the presynaptic proteins was present but difficult to quantify due to tissue damage induced by oedema.

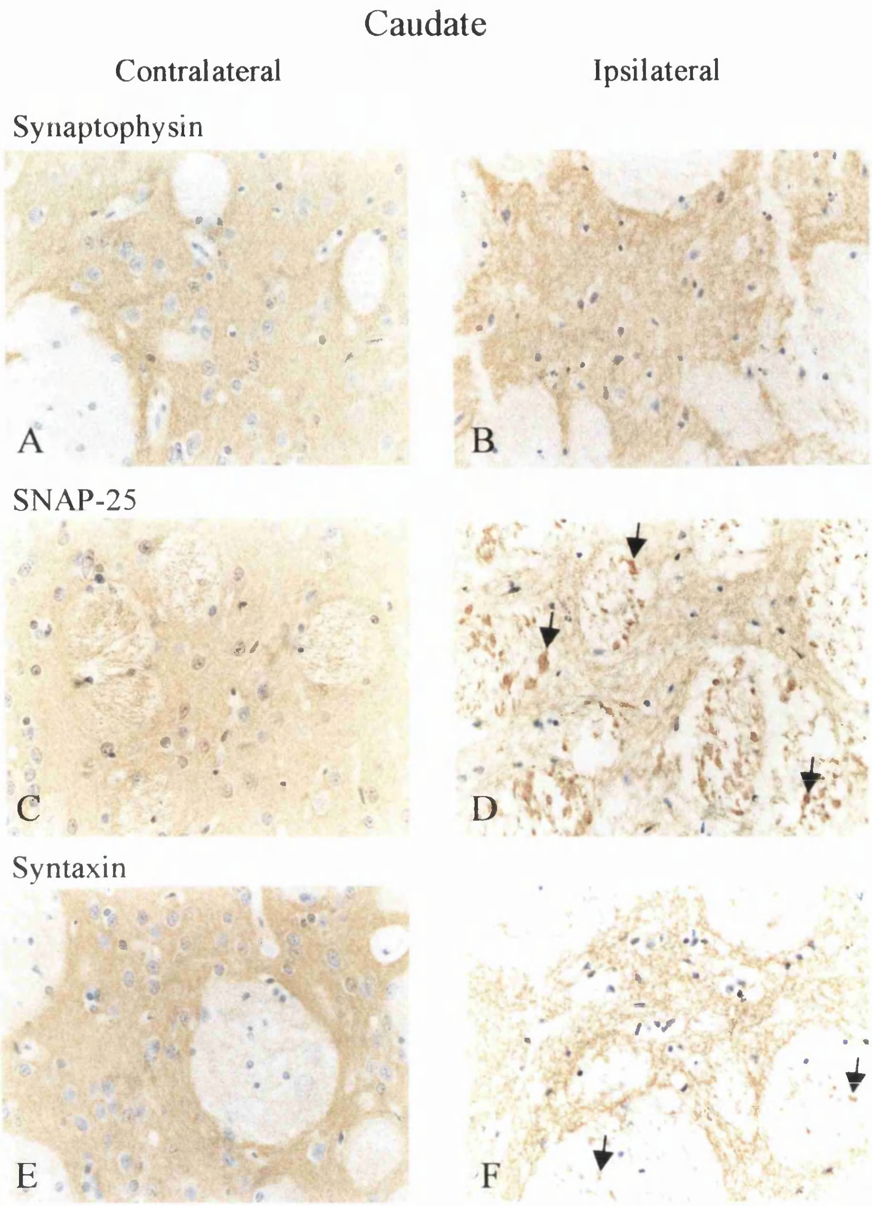


Figure 45

Representative photomicrographs of synaptophysin (A, B), SNAP-25 (C, D) and syntaxin (E, F) immunostaining in the contralateral (A, C, E) and ipsilateral caudate (B, D, F) after 24h permanent MCAo in the rat. Immunostaining for each of the presynaptic proteins was diffuse in the neuropil in the contralateral hemisphere. Subtle SNAP-25 immunoreactivity was also localised in axon fibre tracts in the contralateral hemisphere. Widespread neuronal loss existed after MCAo, but immunostaining for the presynaptic proteins persisted. Accumulation of SNAP-25 and syntaxin immunoreactivities were observed in the fibre tracts in the ipsilateral caudate (arrows).

rodents: to obtain an understanding of the timescale of possible presynaptic changes after transient ischaemia a time-course study was performed in mice. Animals were allowed to survive 1, 3 or 10 days after 17min of transient bilateral common carotid artery occlusion. Shams had an identical surgical procedure with the exception of applying the clips to the carotid arteries. After the appropriate survival period animals were fixed and the brains processed for histology and immunohistochemistry. Histological and immunohistochemical assessments were performed at the level of the caudate and the dorsal hippocampus, regions where ischaemic damage to neuronal perikarya is well charcterised in this animal model.

Caudate: Histology and Immunohistochemistry

Neuronal damage was semi-quantitatively assessed in the caudate using H&E stained sections. In shams, neuronal perikarya were large and circular, intensely staining for haematoxylin and negative for eosin. The neuropil and axon fibre tracts were stained with eosin. (Figure 46). One day after BCCAo, neuronal perikarya were no longer circular, but shrunken and triangular with an intensely eosinophilic neuronal cytoplasm (Figure 46). At 3 and 10 days after BCCAo there was widespread neuronal loss and vacuolation of the neuropil. Immunohistochemistry for synaptophysin in the caudate of shams was restricted to the neuropil and absent from haematoxylin counterstained round neuronal perikarya. From 1 through to 10 days after BCCAo, extensive neuronal loss was evident with preservation of synaptophysin immunostaining in the neuropil (Figure 47). Similarly, SNAP-25 immunostaining was present in the neuropil of shams and was preserved 1, 3 and 10 days after BCCAo in the caudate. (Figure 48). The relative optical density of the immunostaining for both synaptophysin and SNAP-25 in the neuropil was quantified for shams, 1, 3 and 10 days survival after BCCAo. No difference in the level of immunostaining for either synaptophysin and SNAP-25 were detected over the time-course examined (Figure 49).

Hippocampus: Histology

The extent of neuronal damage was semi-quantitatively assessed in the CA1, CA2, CA3, hilus and the dentate gyrus of shams and animals allowed to survive 1, 3 and 10 days after BCCAo. Minimal/no neuronal damage was detected in any of the hippocampal layers of sham animals. At each of the survival timepoints, maximal neuronal damage was in the CA2 and CA1 sectors with minimal neuronal loss in the

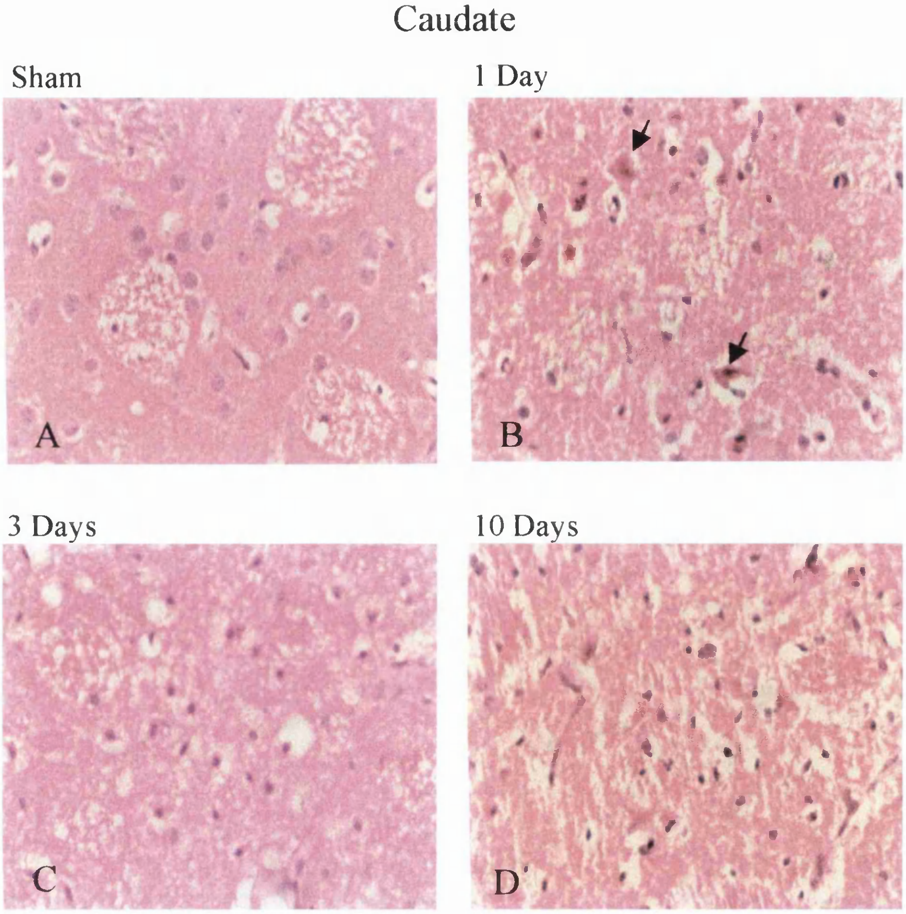


Figure 46

Photomicrographs of histological assessment of neuronal damage in the caudate of sham animals (A), and 1day (B), 3 days (C) and 10 days (D) after BCCAo in the mouse. In Sham animals neurons are large and circular, and positive for haematoxylin. 1 day after global ischaemia, cell bodies are shrunken and the neuronal cytoplasm eosinophilic (arrow) (B). Widespread neuronal loss existed at both 3 days (C) and 10 days after global ischaemia. Magnification X400.

Caudate: Synaptophysin

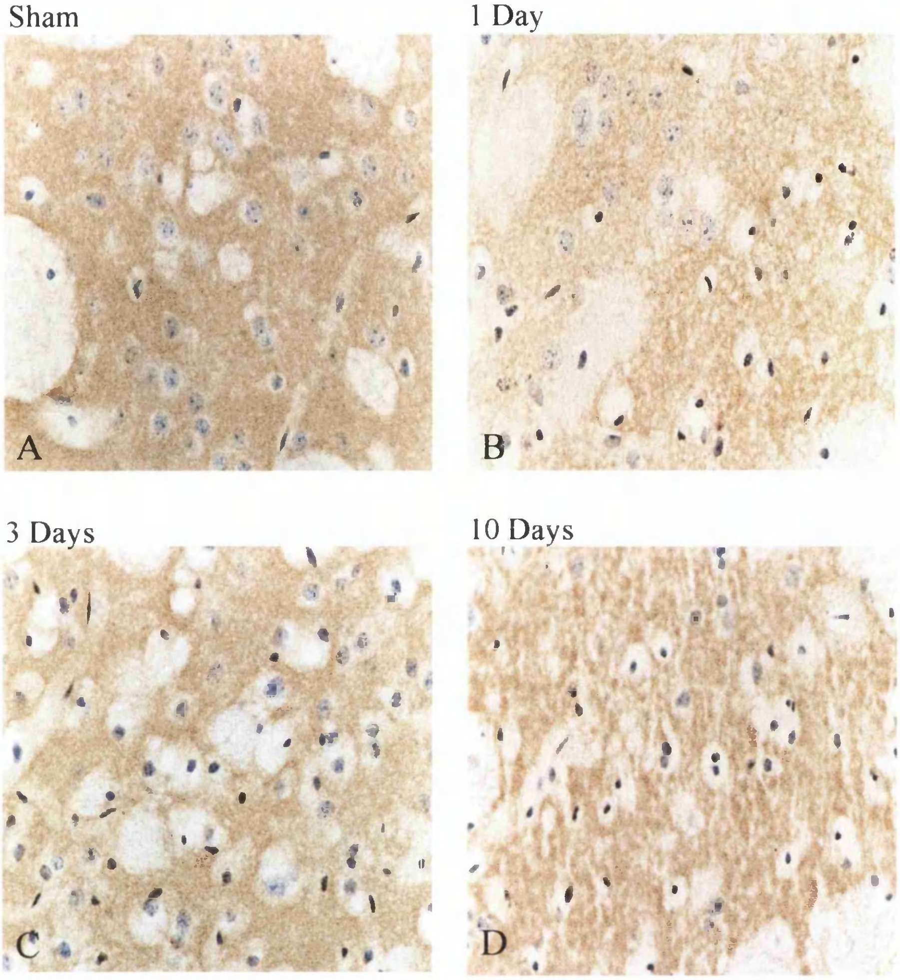
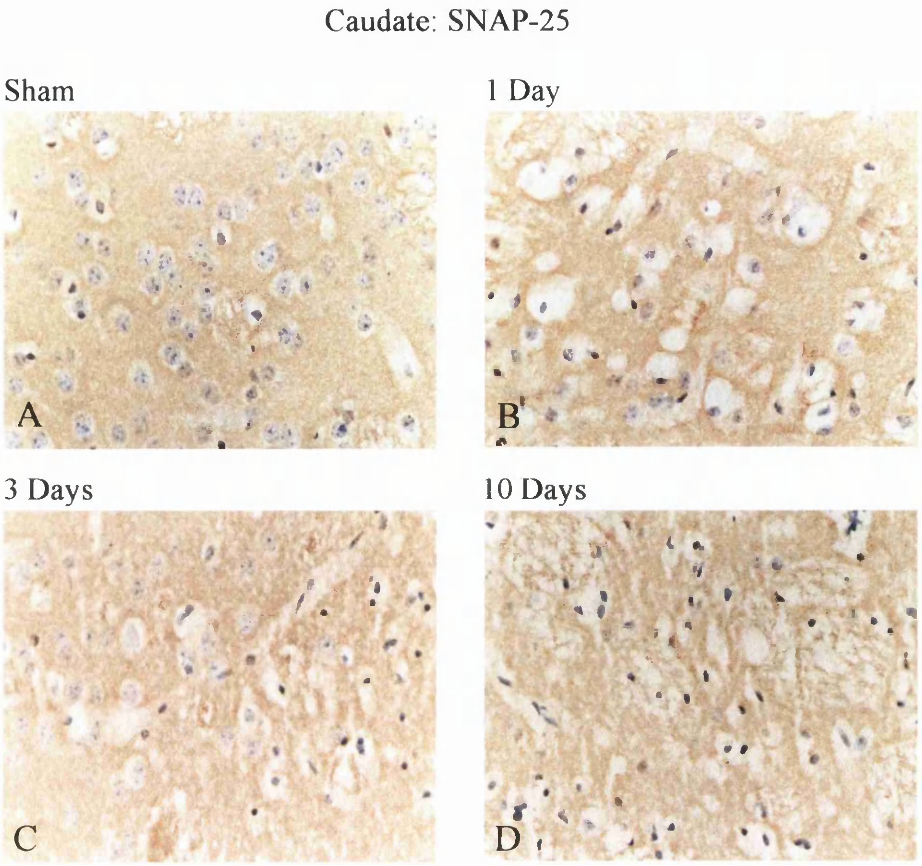


Figure 47

Photomicrographs of synaptophysin immunostaining in the caudate of sham animals (A) and 1day (B), 3 days (C) and 10 days (D) after BCCAo in the mouse. Neuronal loss in the caudate was observed from 1day after global ischaemia (A). Synaptophysin immunostaining in the neuropil persisted up to 10 days after global ischaemia in the mouse. Magnification X400.


Caudate: SNAP-25

Sham

1 Day

3 Days

10 Days

Figure 48

SNAP-25 immunostaining in the caudate of sham animals (A) and 1day (B), 3 days (C) and 10 days (D) after BCCAo in the mouse. Neuronal damage in the caudate was observed from 1day after global ischaemia (A). SNAP-25 immunostaining in the neuropil persisted at 1, 3, and 10 days after global ischaemia in the mouse. Magnification X400.

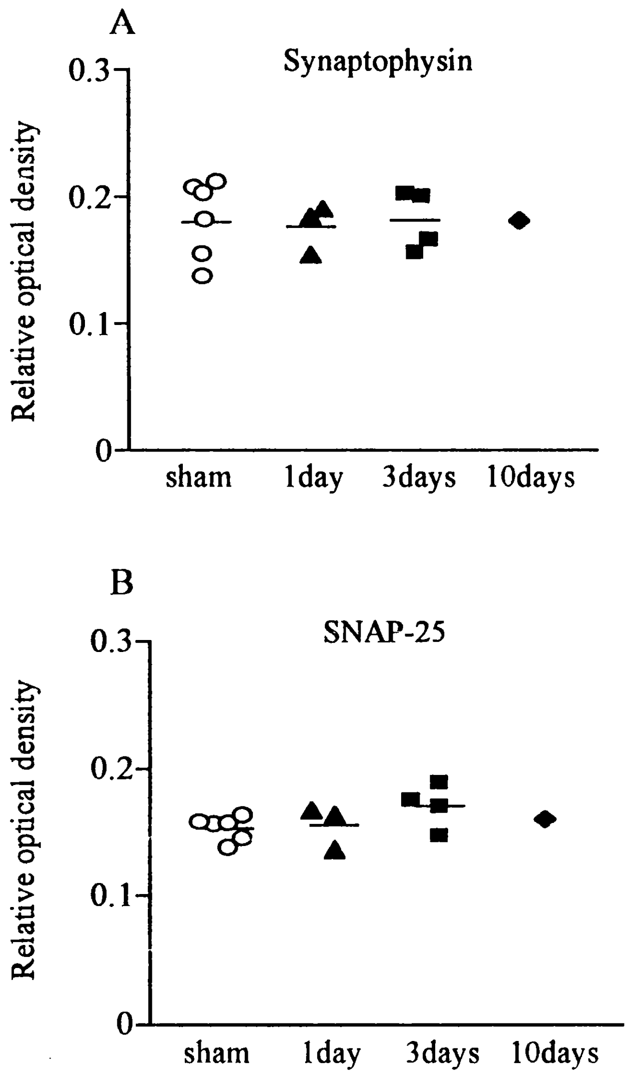


Figure 49

Relative optical densities of synaptophysin (A) and SNAP-25 (B) immunoreactivities in the caudate of sham animals (open circles) and mice allowed to survive 1, 3 and 10 days after BCCAo (closed shapes). The levels of immunostaining for both synaptophysin and SNAP-25 were no different between shams and animals allowed to survive either timepoint after surgery.

CA3 region (Figure 50). Haematoxylin and eosin staining of the CA2 region of the hippocampus demonstrated large, circular neurons in sham animals. CA2 neurons in the hippocampus of animals allowed to survive 1 and 3 days after BCCAo were shrunken and irregular with an eosinophilic neuronal cytoplasm (Figure 51). After 10 days survival, eosinophilic neuronal cytoplasms were still visible amidst a necrotic CA2 layer. The CA1 pyramidal neurons in sham animals were large and regular, being positive for haematoxylin. In animals allowed to survive 1 and 3 days after BCCAo, a portion of the CA1 neurons were triangular and shrunken, with an intensely eosinophilic neuronal cytoplasm (Figure 52). The pattern of CA1 H&E staining at 10 days after BCCAo was similar to that observed in the CA2 10 days after BCCAo (Figure 51).

Hippocampus: Immunohistochemistry

To address the level of presynaptic protein immunostaining in the hippocampus at each timepoint after BCCAo, the relative optical density of presynaptic staining was determined in regions of the hippocampus adjacent to neuronal layers in both hemispheres: the stratum oriens and stratum lacunosum of the CA1, the presynaptic innervation of the CA3, and the outer, middle and inner molecular layers of the dentate gyrus. Levels of synaptophysin immunoreactivity were broadly similar between hemispheres and periods of survival after BCCAo in all six of the hippocampal layers measured, indicating that synaptophysin immunostaining is preserved up to 10 days in the caudate (Figure 47) and in the hippocampus (Figure 53, 54) after global ischaemia. SNAP-25 immunoreactivity was similar between hemispheres and there was a trend towards a reduction in the relative optical density of SNAP-25 immunostaining in all hippocampal layers from 1 through to 10 days survival compared with sham animals (Figure 55, 56). Levels of relative optical density of syntaxin immunostaining was similar in the stratum oriens, stratum lacunosum and the CA3 sector of the hippocampus (Figure 57). In each of the molecular layers of the dentate gyrus there was a trend towards a reduction in syntaxin immunostaining from 1 through to 10 days survival after BCCAo compared to sham animals (Figure 58).

Cortex: Immunohistochemistry

In the cortex of sham animals, synaptophysin immunostaining was diffuse in the neuropil and absent from neuronal perikarya. At 1, 3 and 10 days after BCCAo, widespread neuronal loss exists although synaptophysin immunostaining was preserved

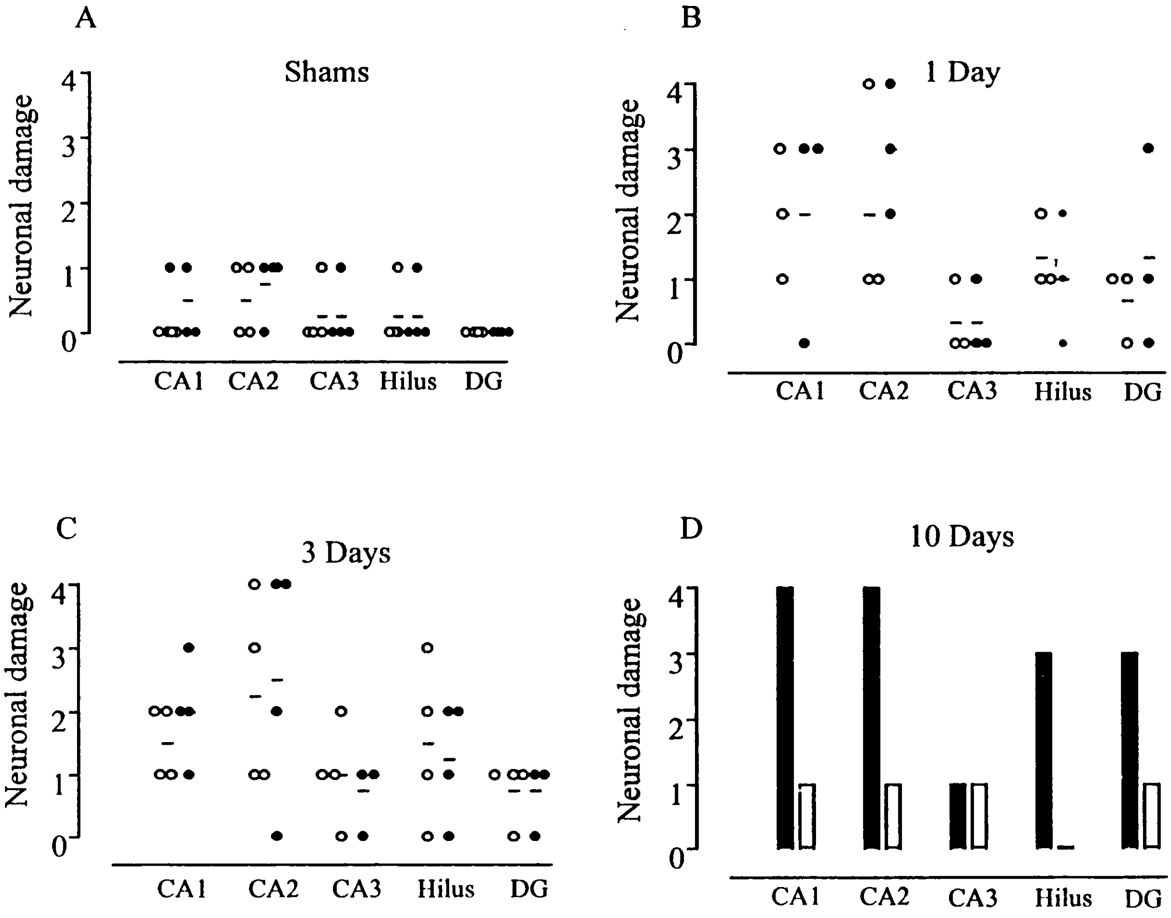


Figure 50

Semi-quantitative assessment of neuronal damage in anatomical layers of the hippocampus of sham animals (n=4) (A) and mice allowed to survive 1 (n=3) (B), 3 (n=4) (C) or 10 days (n=1) (D) after BCCAo. Data is shown for both the left (open shapes) and right hemisphere (closed shapes) and represents individual animal scores. No or minimal neuronal damage existed in any of the hippocampal layers in sham animals. CA2 and CA1 layers were most vulnerable to ischaemic damage at each timepoint.

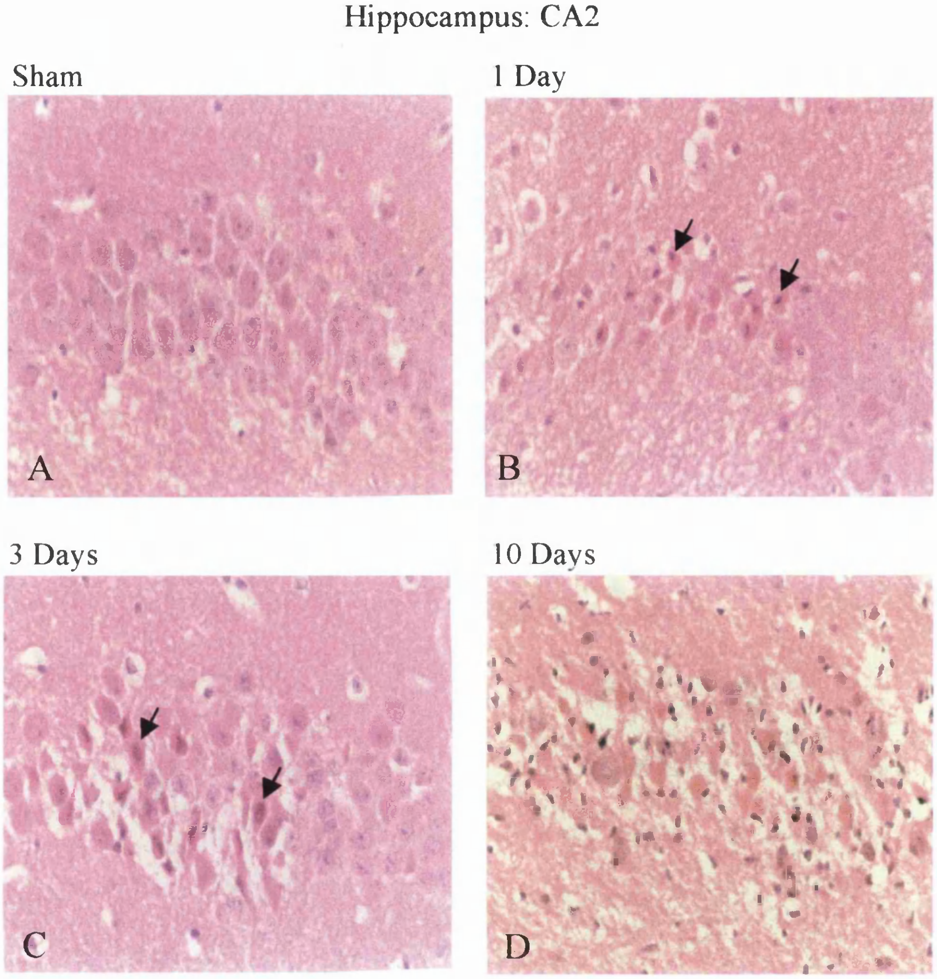


Figure 51

Photomicrographs of histological assessment of neuronal damage in the CA2 sector of the hippocampus of sham animals (A), and 1day (B), 3 days (C) and 10 days (D) after BCCAo in the mouse. In sham animals, CA2 neurons were large and regular, with Nissl substances visible (A). At 1day and again at 3 days after global ischaemia, a portion of the CA2 neuronal perikarya was shrunken with an intensely eosinophilic cytoplasm (arrows) (B, C). The majority of neurons in the CA2 sector of the hippocampus were shrunken 10 days after global ischaemia (D). Magnification X400.

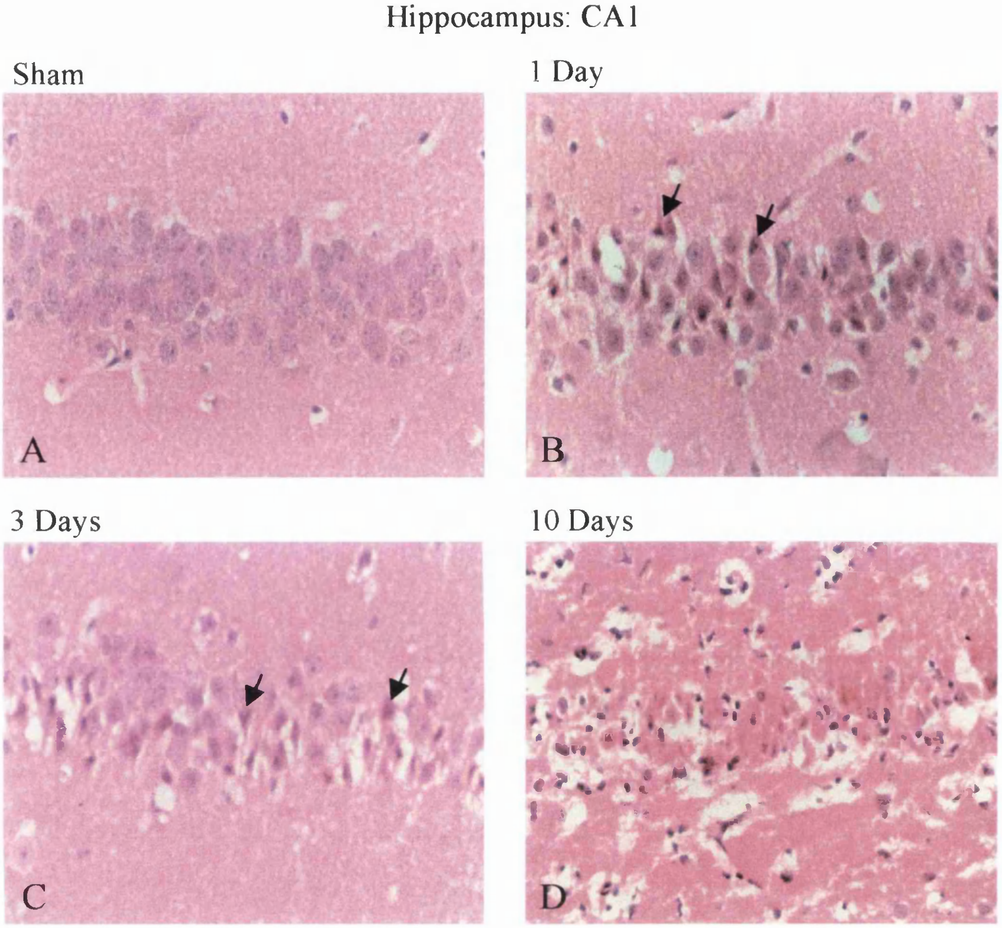


Figure 52

Photomicrographs of histological assessment of neuronal damage in the CA1 sector of the hippocampus of sham animals (A), and 1day (B), 3 days (C) and 10 days (D) after BCCAo in the mouse. At 1 and 3 days after global ischaemia, a proportion of CA1 neurons possessed shrunken cell bodies and eosinophilic neuronal cytoplasms (arrows) (B, C). Complete destruction of CA1 neurons and disruption the overall tissue structure was evident 10 days after global ischaemia. Magnification X400.

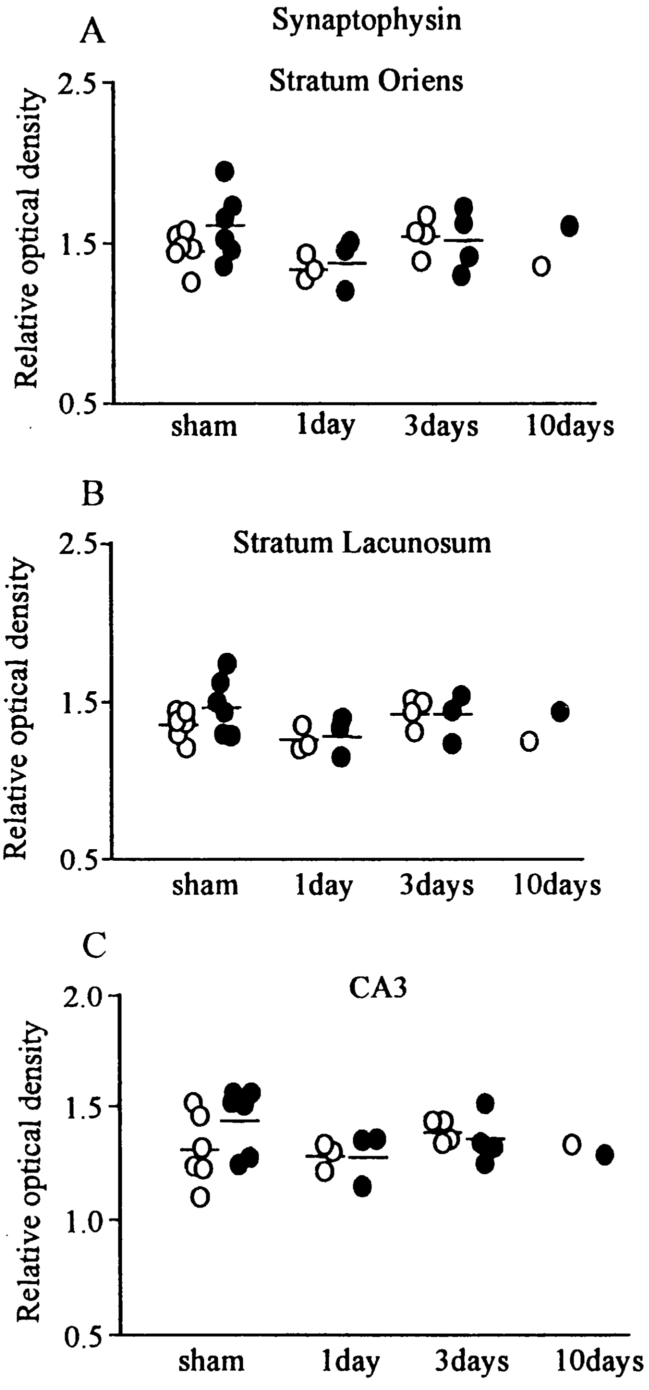


Figure 53

Relative optical density of synaptophysin immunoreactivity in the stratum oriens (A), stratum lacunosum (B) and CA3 (C) of sham animals (n=6) and mice allowed to survive 1 (n=3), 3 (n=4) or 10 days (n=1) after BCCAo. Data is shown for both the left (open circles) and right hemisphere (closed circles). Bars represent the group means.

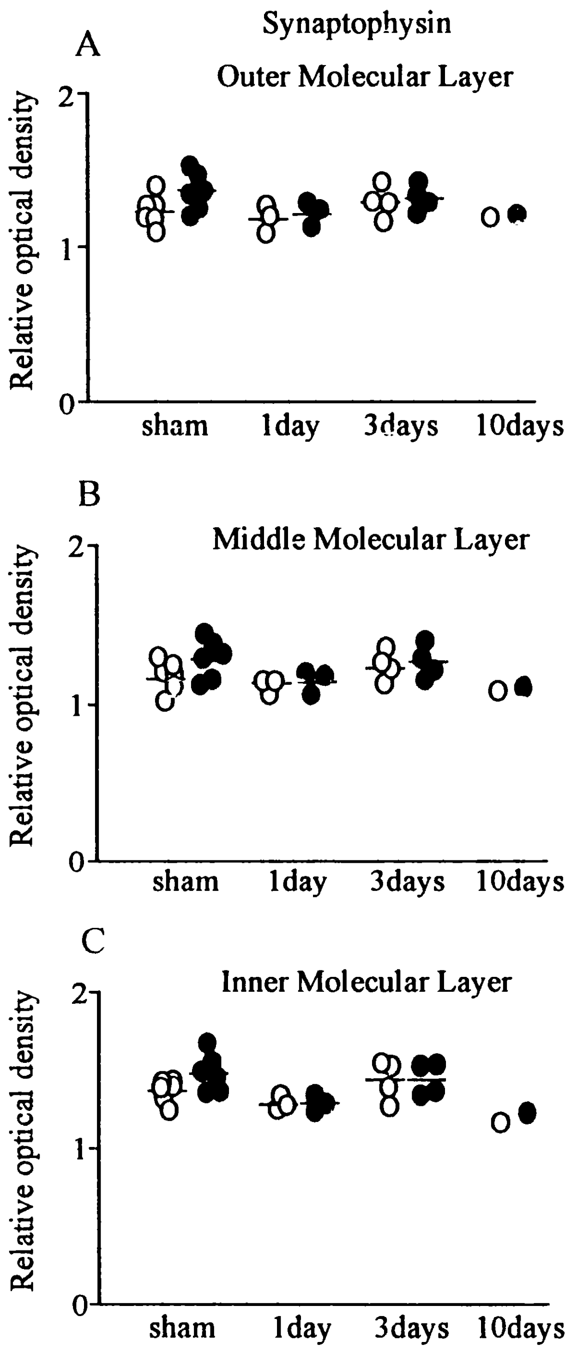


Figure 54

Relative optical density of synaptophysin immunoreactivity in the outer (A), middle (B) and inner molecular layer (C) of sham animals (n=6) and mice allowed to survive 1 (n=3), 3 (n=4) or 10 days (n=1) after BCCAo. Data is shown for both the left (open circles) and right hemisphere (closed circles). Bars represent the group means.

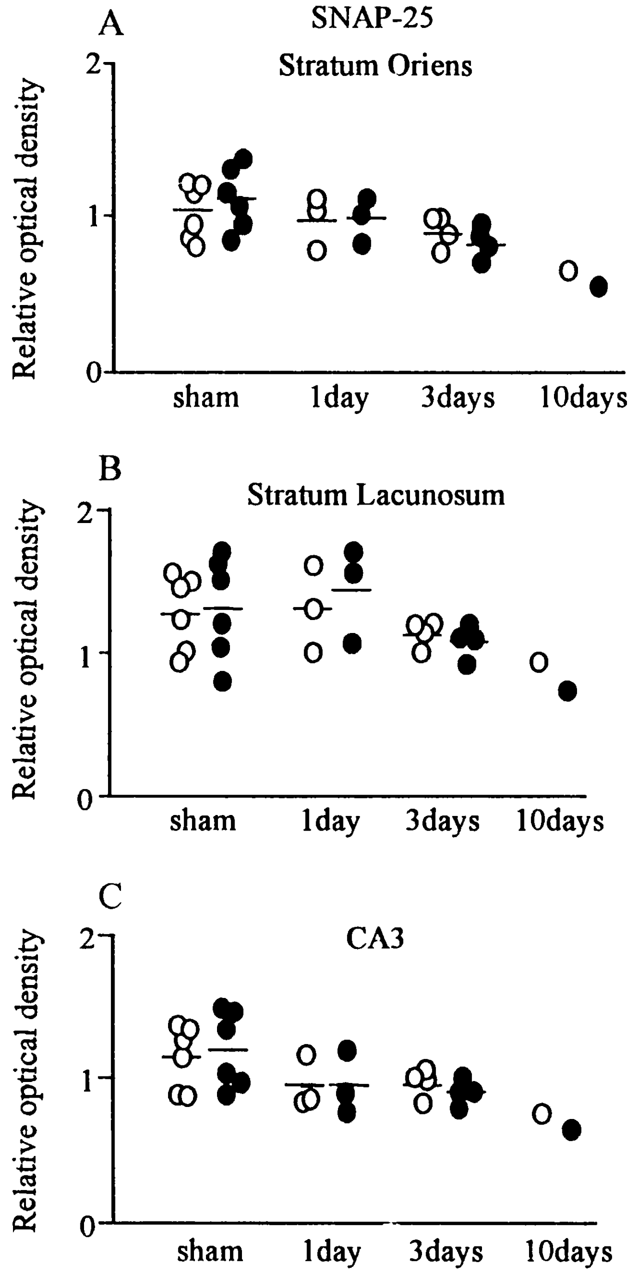


Figure 55

Relative optical density of SNAP-25 immunoreactivity in the stratum oriens (A), stratum lacunosum (B) and CA3 (C) of sham animals (n=6) and mice allowed to survive 1 (n=3), 3 (n=4) or 10 days (n=1) after BCCAo. Data is shown for both the left (open circles) and right hemisphere (closed circles). Bars represent the group means.

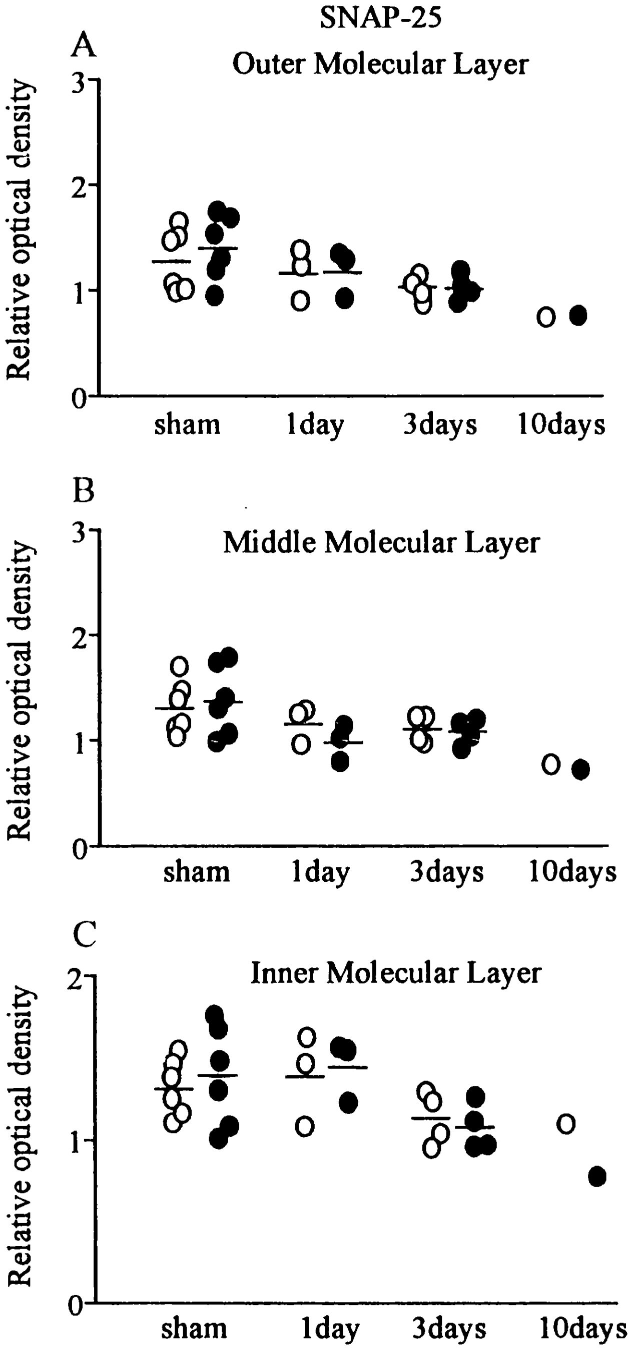


Figure 56

Relative optical density of SNAP-25 immunoreactivity in the outer (A), middle (B) and inner molecular layer (C) of sham animals (n=6) and mice allowed to survive 1 (n=3), 3 (n=4) or 10 days (n=1) after BCCAo. Data is shown for both the left (open circles) and right hemisphere (closed circles). Bars represent the group means.

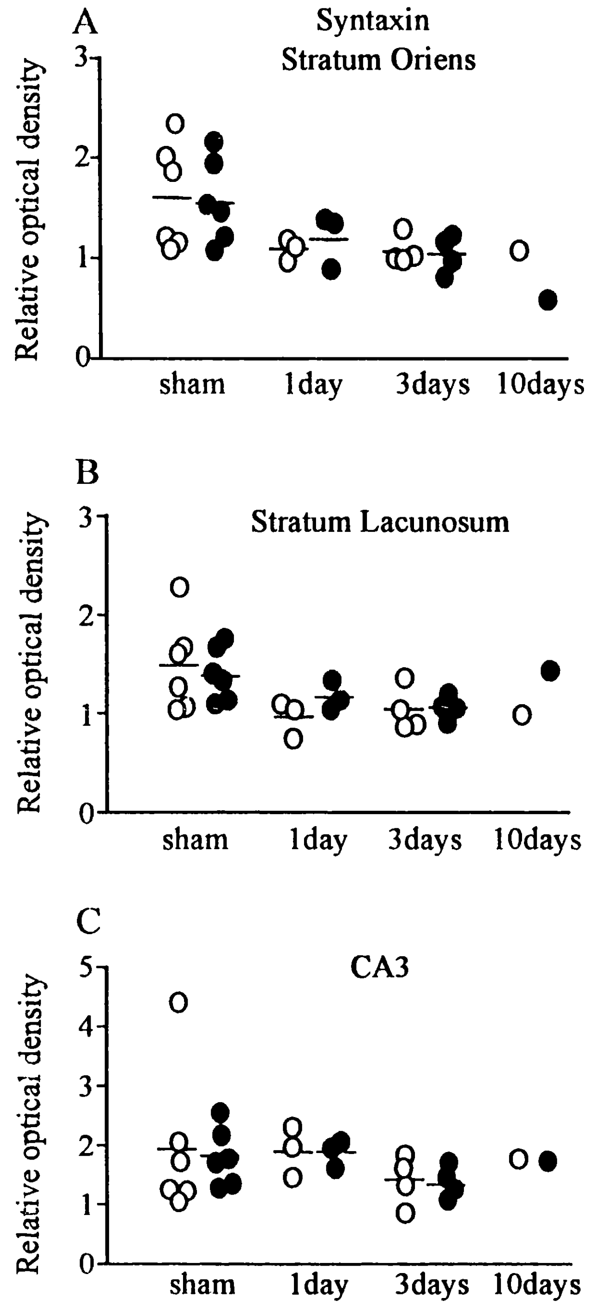


Figure 57

Relative optical density of syntaxin immunoreactivity in the stratum oriens (A), stratum lacunosum (B) and CA3 (C) of sham animals (n=6) and mice allowed to survive 1 (n=3), 3 (n=4) or 10 days (n=1) after BCCAo. Data is shown for both the left (open circles) and right hemisphere (closed circles). Bars represent the group means.

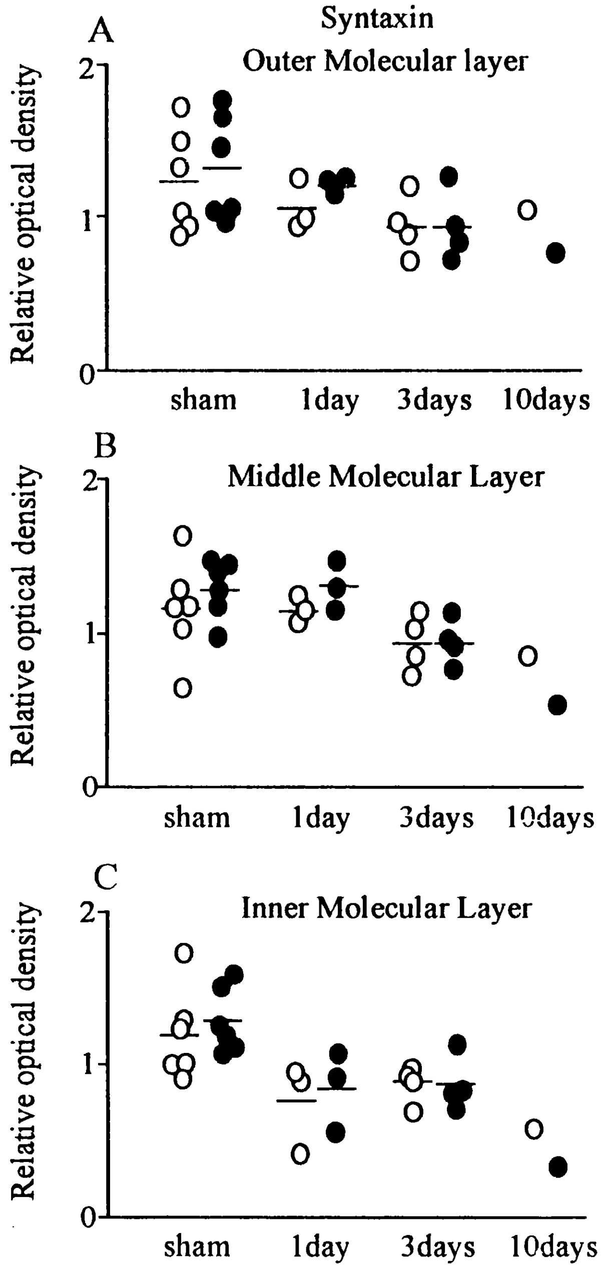


Figure 58

Relative optical density of syntaxin immunoreactivity in the outer (A), middle (B) and inner molecular layer (C) of sham animals (n=6) and mice allowed to survive 1 (n=3), 3 (n=4) or 10 days (=1) after BCCAo. Data is shown for both the left (open circles) and right hemisphere (closed circles). Bars represent the group means.

in the neuropil (Figure 59). Similarly, an identical pattern of neuronal loss but preservation of neuropil immunostaining was observed with SNAP-25 (Figure 60). These data imply that although there might be subtle changes in the level of these presynaptic proteins in specific brain regions, these alterations are small and presynaptic proteins are preserved in a number of animal models of acute brain injury.

3.3.4 MAP-2 immunostaining after transient bilateral common carotid artery in the mouse

To compare the sensitivity of presynaptic proteins with that of postsynaptic markers, MAP-2 immunostaining was performed in sections of the caudate and hippocampus after BCCAo in the mouse. In sham animals, MAP-2 immunostaining was diffuse labeling dendritic processes in both the caudate and hippocampus. At 3 and 10 days after BCCAo, low power pictures demonstrated a loss of MAP-2 in the caudate and in the CA2 and CA1 sectors of the hippocampus (Figure 61). High power magnification of MAP-2 immunostaining in the CA1 shows labeling of dendritic processes and around pyramidal neuron cell bodies. In contrast, after BCCAo MAP-2 immunostaining was absent from dendritic processes and shrunken CA1 neurons, with residual MAP-2 immunostaining associated with a few remaining morphologically normal neurons (Figure 62). Likewise, in the caudate of sham animals MAP-2 immunostaining was diffuse in the neuropil, but not accumulated around cell bodies. One day after BCCAo, MAP-2 immunostaining accumulated around a few cell bodies but was reduced in the neuropil. By 3 days, widespread depletion of MAP-2 in the caudate was observed (Figure 63). In the cortex of sham animals, MAP-2 immunostaining was present in parallel strands of dendritic process but absent from cell bodies. One day after BCCAo, MAP-2 immunostaining was redistributed from dendritic processes and accumulated around neuronal perikarya. At 3 days after BCCAo, MAP-2 immunostaining was reduced from the neuropil and dendritic processes, but remained around cell bodies (Figure 64). In each of the anatomical brain regions examined, MAP-2 immunoreactivity was disrupted at the early survival times after BCCAo. In contrast, immunostaining for each of the presynaptic proteins was preserved in all of the brain regions assessed up to 10 days after BCCAo. These morphological findings suggest that the major pathological changes following brain injury are postsynaptic.

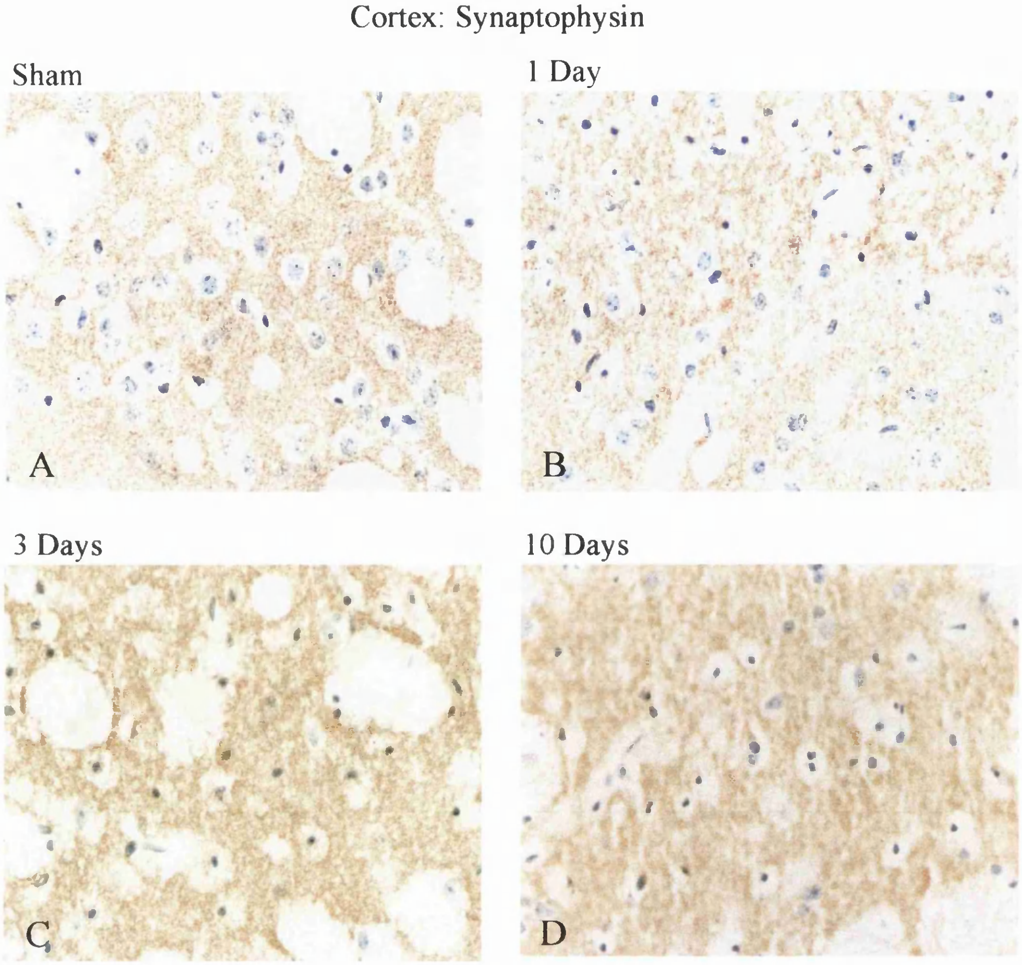
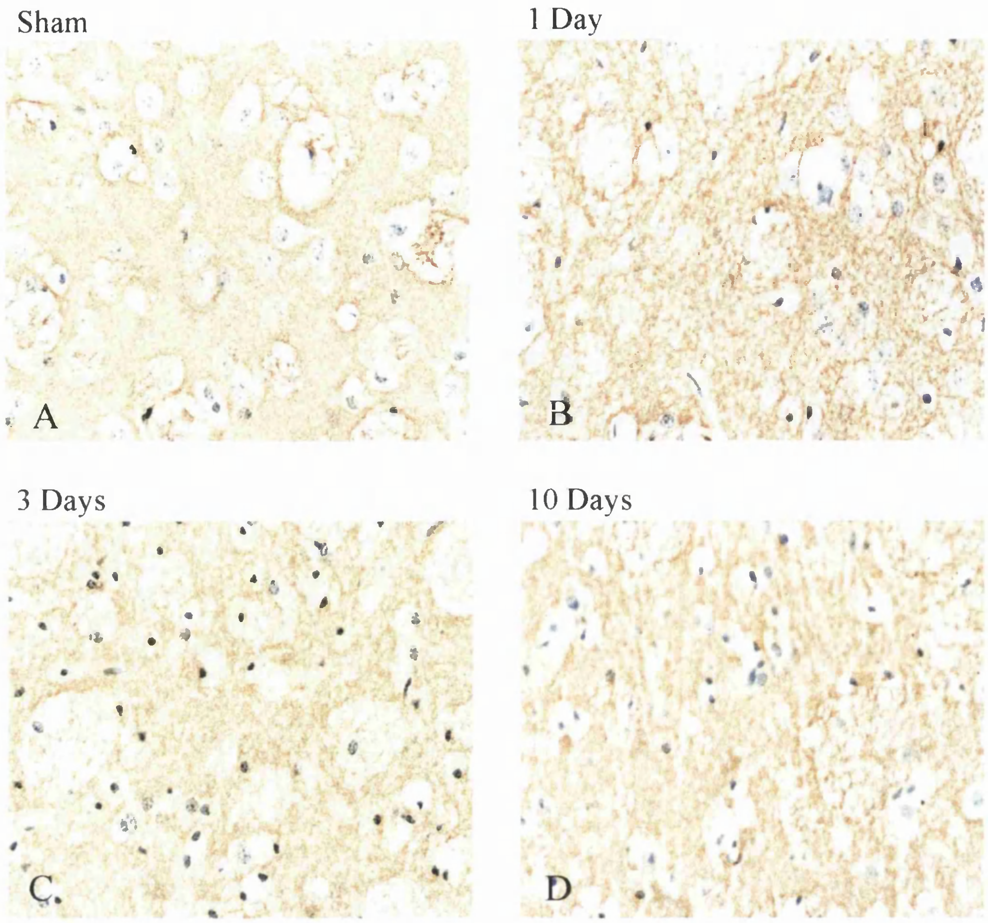


Figure 59

Representative photomicrographs of synaptophysin immunostaining in the cortex of sham animals (A) and 1day (B), 3 days (C) and 10 days (D) after BCCAo in the mouse. Despite neuronal damage in the cortex 1 day after global ischaemia (B), synaptophysin immunoreactivity persisted in the cortex at 3 and 10 days after global ischaemia in the mouse. Magnification X400.

Cortex: SNAP-25



Sham

1 Day

3 Days

10 Days

Figure 60

Photomicrographs of SNAP-25 immunostaining in the cortex of sham animals (A) and 1day (B), 3 days (C) and 10 days (D) after BCCAo in the mouse. Despite extensive neuronal damage in the cortex 1day after global ischaemia (B), SNAP-25 immunoreactivity persisted in the cortex at 1, 3 and 10 days after global ischaemia in the mouse. Magnification X400.

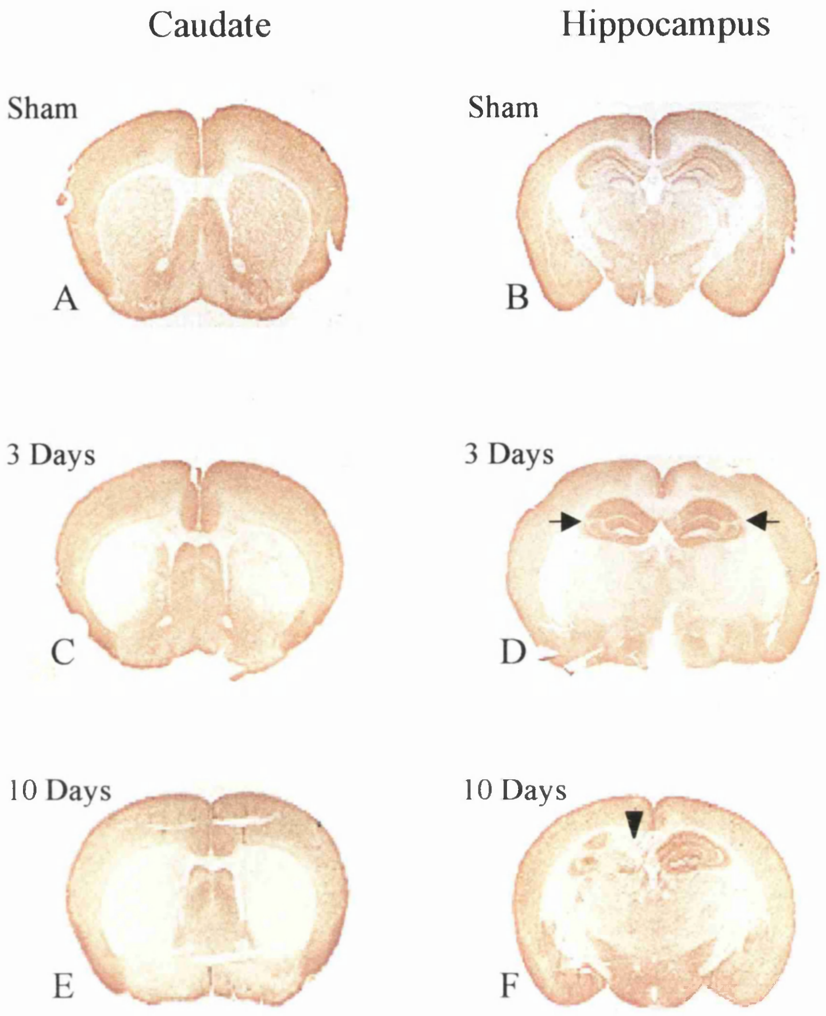


Figure 61

Photomicrograph of loss of MAP-2 immunostaining in coronal sections of the caudate (A, C, E) and hippocampus (B, D, F) in shams (A, B) and 1day (C, D) and 3days (E, F) after BCCAo in the mouse. Widespread loss of MAP-2 immunostaining was evident in the caudate at both 3 and 10 days after global ischaemia. Disappearance of MAP-2 immunostaining was restricted to the CA2 sector (arrows) of the hippocampus at 3days after global ischaemia, and absent from the CA2 (arrows) and CA1 (arrowheads) neuronal layers of the hippocampus 10 days after global ischaemia. Magnification X400.

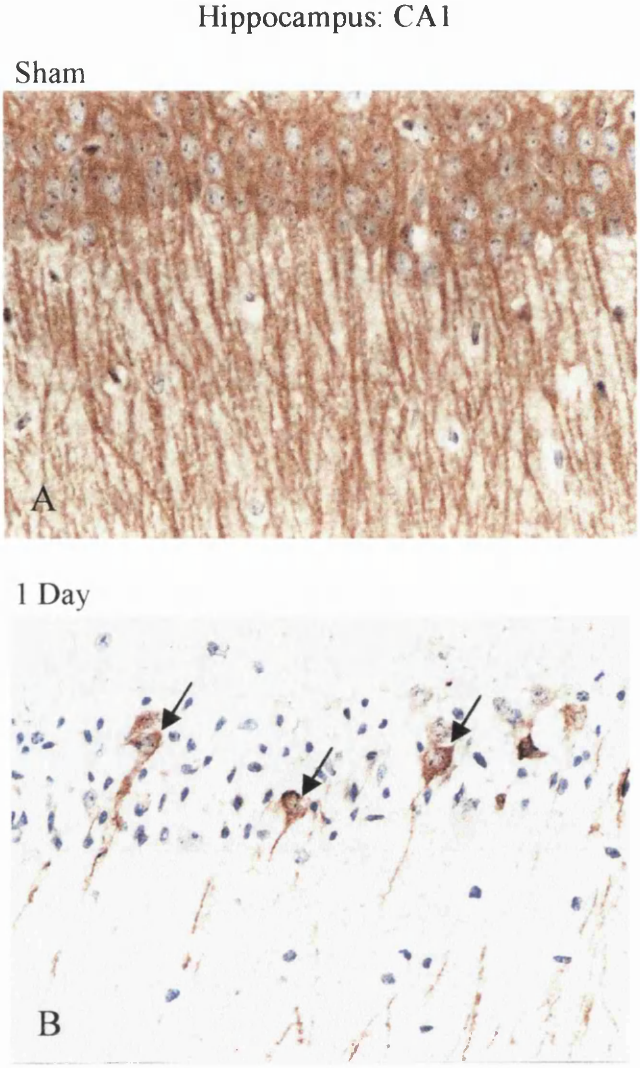


Figure 62

Photomicrograph showing MAP-2 immunostaining in the CA1 sector of the hippocampus in sham animals (A) and 1day after BCCAo in the mouse (B). In shams intense MAP-2 immunostaining was localised to dendrites and around the neuronal cell bodies. Widespread loss of dendritic and neuronal staining was evident at 1 day after global ischaemia, although preservation of MAP-2 immunostaining was restricted to a few morphologically normal CA1 neurons (arrows). Magnification X400.

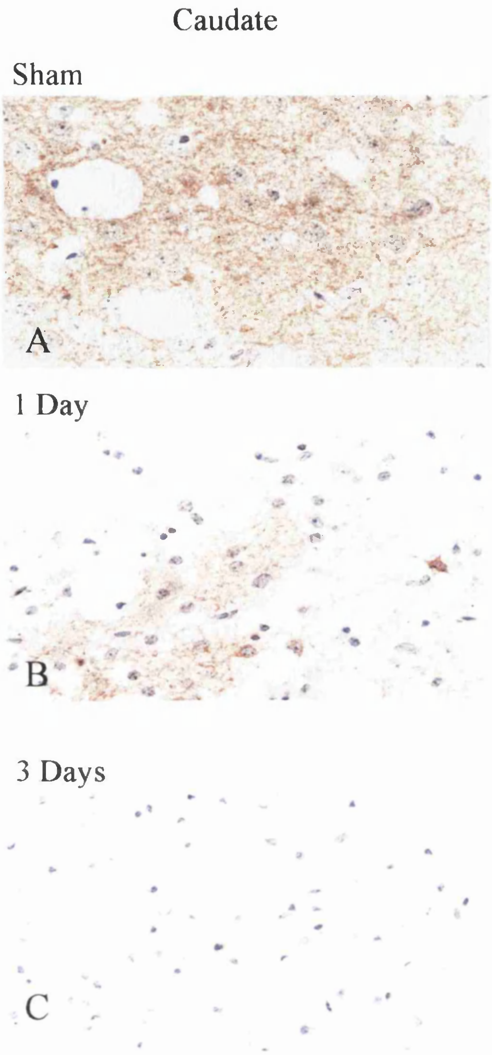


Figure 63

Photomicrographs of MAP-2 immunostaining in the caudate in sham animals (A) and 1day (B) and 3days (C) after BCCAo in the mouse. The diffuse pattern of MAP-2 immunostaining in the caudate of shams was replaced by a gradual (B) and almost complete loss of MAP-2 immunostaining (C) 1day and 3days after global ischaemia in the mouse. Magnification X400.

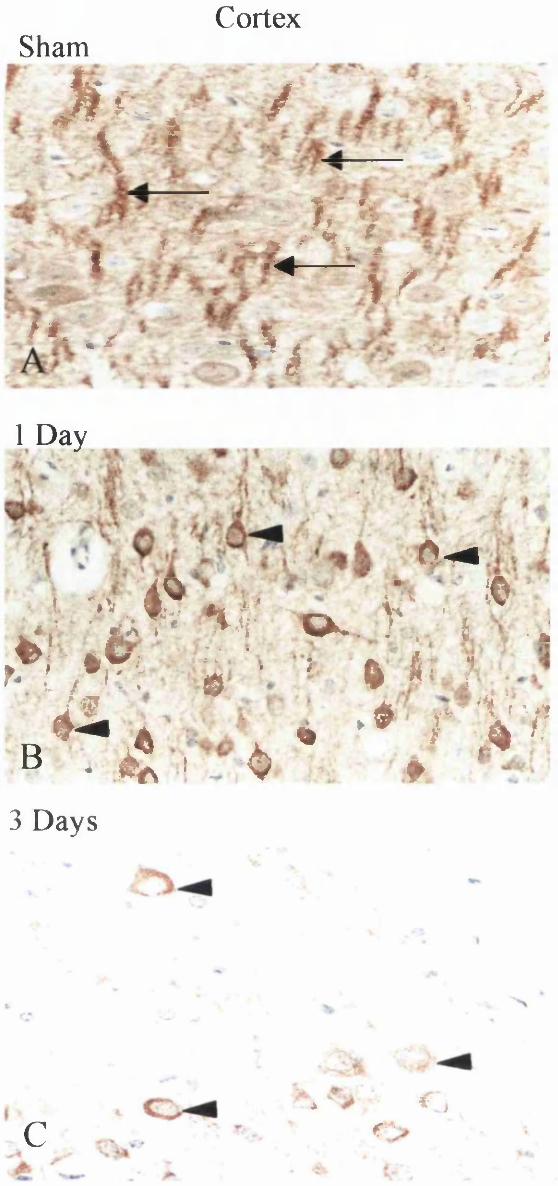


Figure 64

Photomicrographs of disrupted MAP-2 immunostaining in the cortex in sham animals (A) and 1day (B) and 3 days (C) after BCCAo in the mouse. An even pattern of dendritic staining in the shams (arrows) was replaced by redistribution and accumulation of MAP-2 immunostaining around neurons (arrowheads) and loss of dendritic MAP-2 immunostaining 1day and 3days after global ischaemia. Magnification X400.

3.4 GGF-2 after acute brain injury

A number of growth factor systems have been proposed to influence outcome after ischaemic and traumatic brain injury. One growth factor signaling system receiving recent attention is that involving the neuregulins. The neuregulins have multiple actions in the nervous system, including cell survival, proliferation and differentiation. (Carraway and Burden, 1995). The neuregulin, GGF-2 is involved in a spectrum of neurobiological processes (Marchionni et al., 1997). The aim of the study was to elucidate if GGF-2 exists in adult human postmortem brain and to determine both the level and distribution of GGF-2 in the cerebral cortex by Western blotting and immunohistochemistry of patients who had sustained a fatal head injury. The level of GGF-2 was also investigated by Western blotting in homogenates of brain tissue from animal models of acute brain injury.

3.4.1 GGF-2 and human head injury

Five primary antibodies to GGF-2 (51210, 13366, 19937, CN16 and 51211), each directed against a distinct epitope on the protein detected a single band on the immunoblot for both the control and head-injured patient. (Figure 65). The band detected by each antibody ran at the predicted molecular weight of 55kDa, and this pattern was repeated using an additional 2 controls and 2 head-injured patients in an identical experimental design.

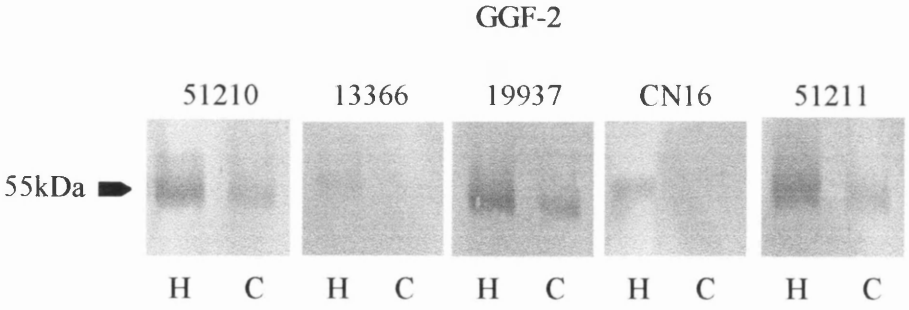


Figure 65

Western blots showing GGF-2 immunoreactivity in homogenates from a control (C) and a head-injured (H) case (A). The immmunoreactive bands detected by 5 primary antibodies to GGF-2 are shown. Similar protein bands were detected using additional controls (n=3) and head-injured cases (n=3) examined with the panel of 5 primary antibodies.

In the cingulate gyrus, the levels of GGF-2 immunoreactivity detected with CN16 were significantly increased in the head-injured group compared to controls (*$p<0.05$), equivalent to a 60% increase in the head-injured group over controls (Figure 66). There was considerable heterogeneity in the level of GGF-2 immunoreactivity in the cingulate gyrus of head-injured patients, with 9 out of the 16 head-injured patients having a GGF-2 immunoreactivity higher than the maximum value in the control group. Immunohistochemical staining using CN16 revealed minimal labeling of GGF-2 in the neuronal perikarya and neuropil in the cingulate gyrus of controls. In sections from this brain area in the head-injured patients, increased GGF-2 immunostaining was observed (Figure 67). Staining was localised to the neuronal cytoplasm of shrunken perikarya. GGF-2 immunostaining was also prominent in glia of head-injured patients.

In the superior parietal cortex there was no difference in the level of GGF-2 in head-injured patients compared with controls (Figure 68). There was no association between the level of GGF-2 immunoreactivity in the cingulate gyrus of the superior parietal cortex of head-injured patients (Figure 69). The heterogeneity in the GGF-2 response was not associated with the presence or absence of ischaemic brain damage in either the cingulate gyrus or the superior parietal cortex (Figure 70). There was no correlation of GGF-2 immunoreactivity in the cingulate gyrus and neuropathological evidence of a SDH in head-injured compared with those head-injured patients with no SDH (Figure 71). Association of GGF-2 immunoreactivity in head-injured patients with a left SDH with survival time after injury suggested that the GGF-2 response is maximal over the first 24 h after brain injury (acute), followed by a reduction to lower levels of GGF-2 immunoreactivity over longer periods (chronic) of survival after head injury (Figure 71).

3.4.2 GGF-2 immunoreactivity in white matter and Alzheimer's disease

GGF-2 is known to not only to regulate glial cell survival, but has also been implicated in neuronal-glial interaction via an indirect trophic support loop. GGF-2 immunoreactivity was no different in subcortical white matter from the corpus callosum of the same group of controls and head-injured patients (Figure 72). To further confirm that the increase in GGF-2 immunoreactivity detected is an acute pathophysiological response, the level of GGF-2 immunoreactivity was determined in the cingulate gyrus

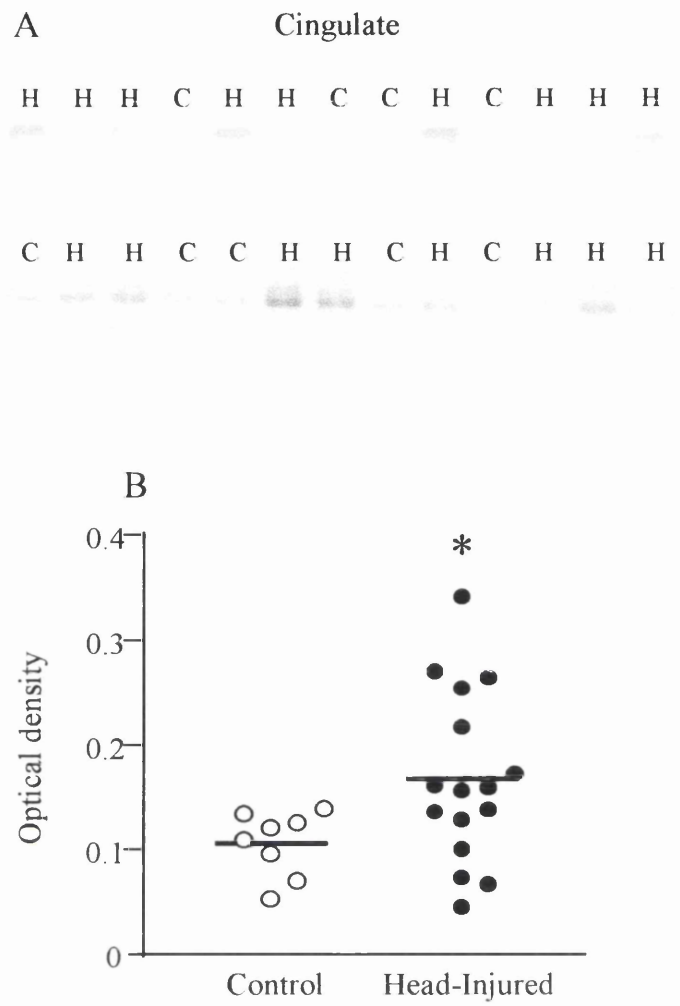


Figure 66

GGF-2 immunoreactivity in the cingulate gyrus is increased in head-injured patients compared to controls. A: Western blots showing the level of CN16 immunoreactivity in 8 controls (C) and 16 head-injured patients (H). A control and a head-injured case were loaded on both gels accounting for 26 bands detected. B: Optical density values of the 55kDa band in immunoblot (A). The mean value of GGF-2 immunoreactivity for the head-injured group was 60% higher than the mean of the controls, with *$p<0.05$, by unpaired two-tailed Student's t test.

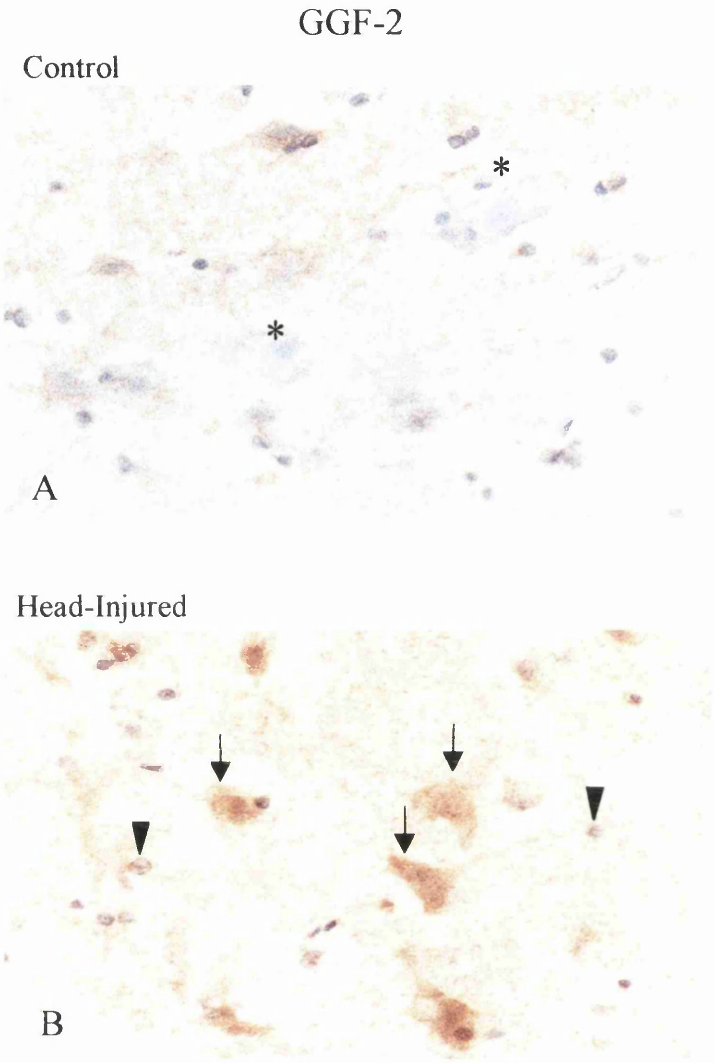


Figure 67

Representative GGF-2 immunostaining in the cingulate gyrus of controls (A) and head-injured patients (B). There was minimal GGF-2 immunostaining within the cytoplasm of neurons in controls (*) and faint GGF-2 immunostaining was present in the neuropil.

In head-injured cases, GGF-2 immunoreactivity accumulated in the neuronal cytoplasm of shrunken neuronal cell bodies (arrows) and was prominent in glia (arrowheads). Magnification X400.

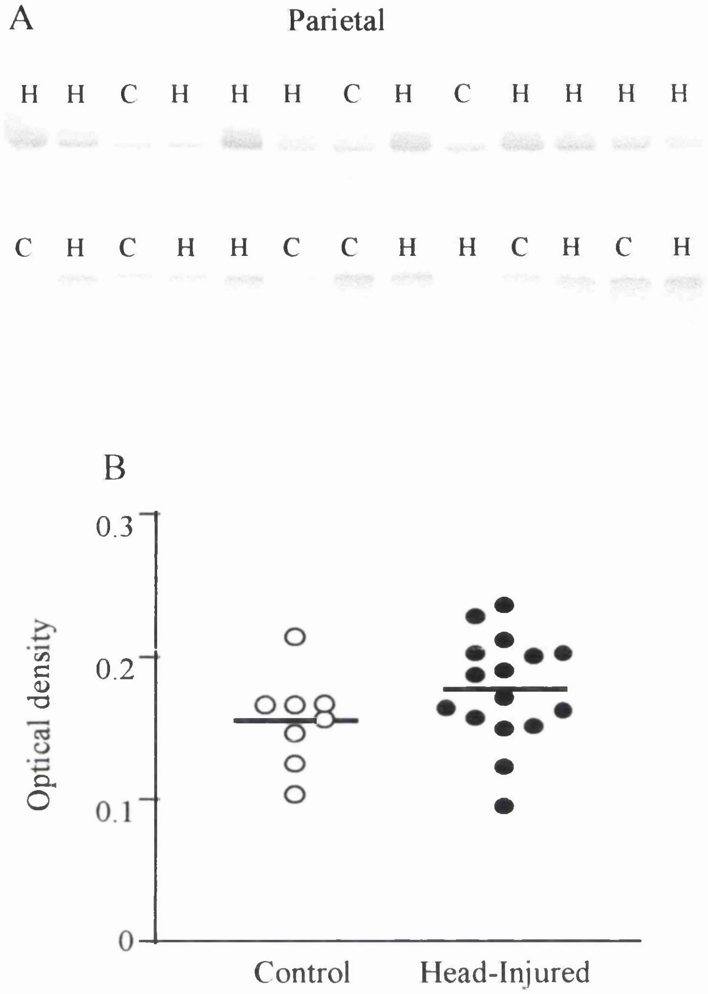


Figure 68

GGF-2 immunoreactivity in the superior parietal cortex of controls and head-injured patients. A: Western blots showing the level of CN16 immunoreactivity in 8 controls (C) and 16 head-injured patients (H). B: Optical density values of the GGF-2 immunoreactive bands in (A). No statistically significant difference existed in the level of GGF-2 immunoreactivity between head-injured patients and controls.

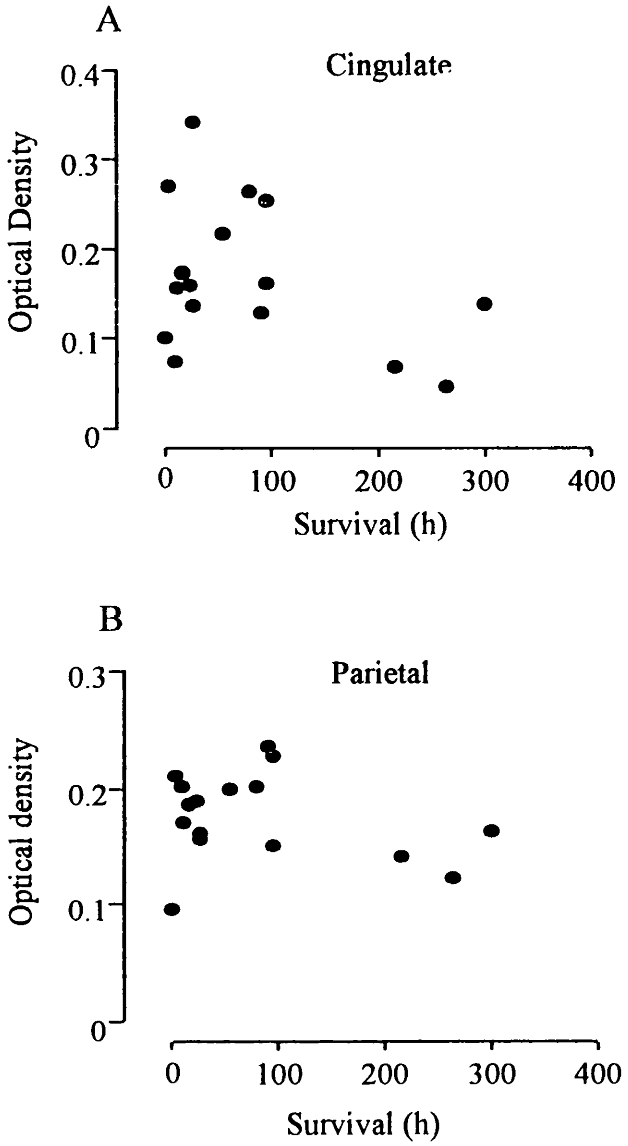


Figure 69

Relationship between the optical density of GGF-2 immunoreactivity in the cingulate gyrus (A) and the superior parietal cortex (B) and the survival time prior to death of head-injured patients. Individual data points represent individual head-injured patients.

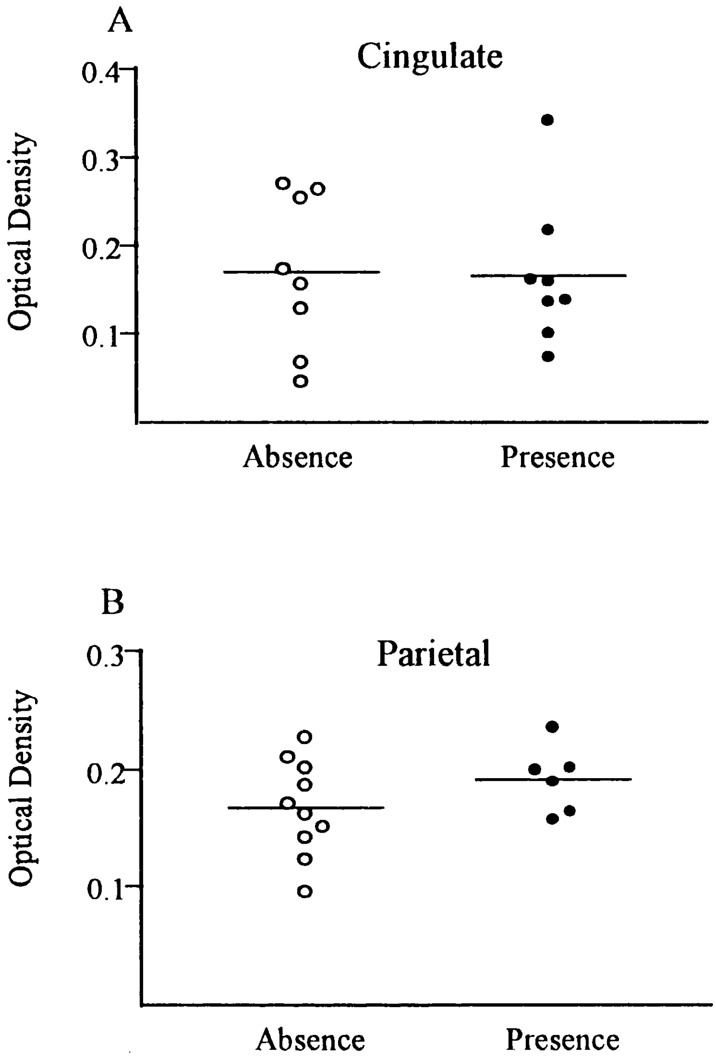


Figure 70

Relationship between GGF-2 immunoreactivity in the cingulate gyrus (A) and the superior parietal cortex (B) and the presence (open circles) or absence (closed circles) of ischaemic brain damage histologically assessed in blocks adjacent to those used for Western blotting. The bars represent the group means.

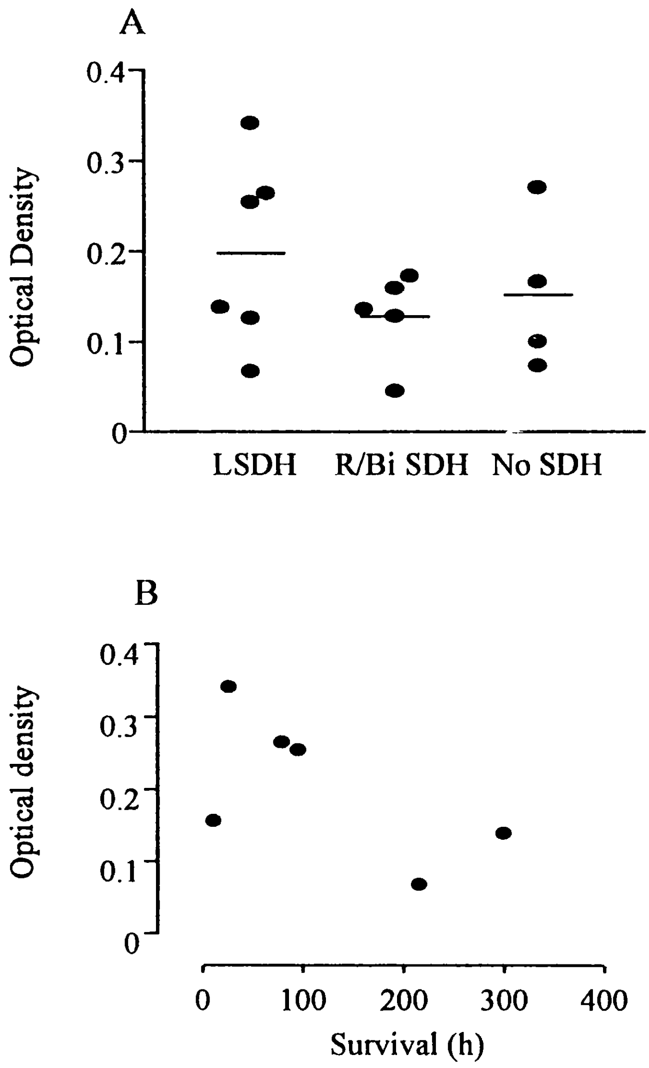


Figure 71

A: Relationship between GGF-2 immunoreactivity in the cingulate gyrus and neuropathological identification of a left (LSDH) or right/bilateral (R/bi SDH), or no subdural haematoma (no SDH). Bars represent the group means. B: Relationship between GGF-2 immunoreactivity in the cingulate gyrus of head-injured patients with a left subdural haematoma and survival time after injury prior to death.

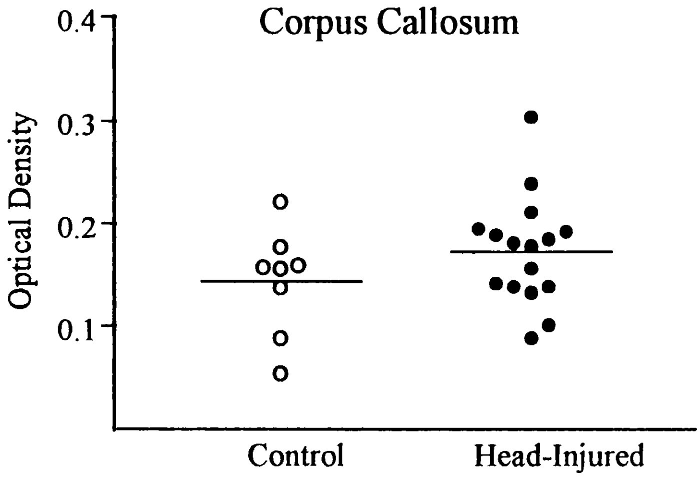


Figure 72

Quantitative data for GGF-2 immunoreactivity detected in the subcortical white matter of the corpus callosum in controls (open circles) and head-injured patients (closed circles). Bars represent the group means. Unpaired two-tailed Student's *t* test revealed there was no difference in the level of GGF-2 immunoreactivity between groups.

from a small cohort of Alzheimer's disease patients and age-matched controls (Figure 73). No difference in the level of GGF-2 immunoreactivity existed between groups.

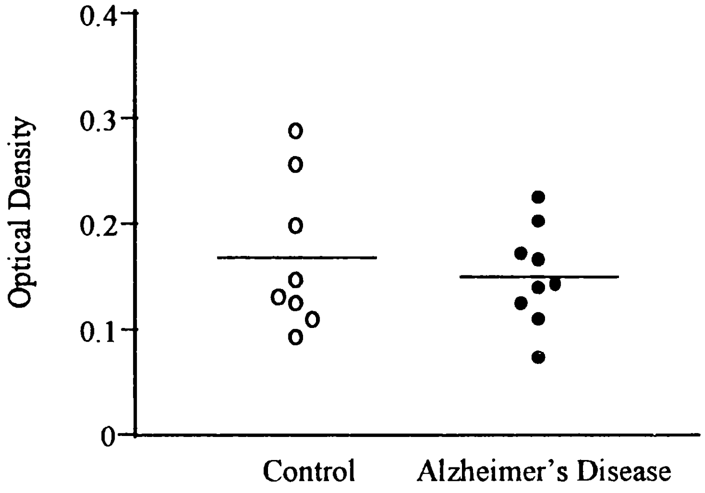


Figure 73

Quantitative data for GGF-2 immunoreactivity in homogenates from the cingulate gyrus of controls (open circles) and Alzheimer's disease patients (closed circles). There was no difference in the level of GGF-2 immunoreactivity in Alzheimer's disease patients compared with aged-matched controls.

3.4.3 GGF-2 in animal models of acute brain injury

Ischaemic brain damage and presence of a subdural haematoma are common neuropathological findings after head injury. To determine whether these components of brain damage contribute to the elevated levels of GGF-2, these features were examined in animal models of acute brain injury. After 24h permanent MCAo in the rat, the 55kDa band of GGF-2 immunoreactivity accumulated primarily in the ipsilateral caudate. In comparison, in both sham animals and the contralateral hemisphere of experimental animals a low level the 55kDa band of GGF-2 immunoreactivity was detected (Figure 74). The level of GGF-2 was determined in the cortex underlying a SDH 4h after surgery in the rat. In Western blots of rat brain homogenates, two bands of GGF-2 immunoreactivity were detected: an 80kDa band and the previously observed 55kDa band. In a Western blot, the 80 kDa band was predominantly present in sham animals, but after SDH it was reduced and replaced by an accumulation of the 55kDa band in the cortex both ipsilateral and contralateral to the SDH (Figure 75).

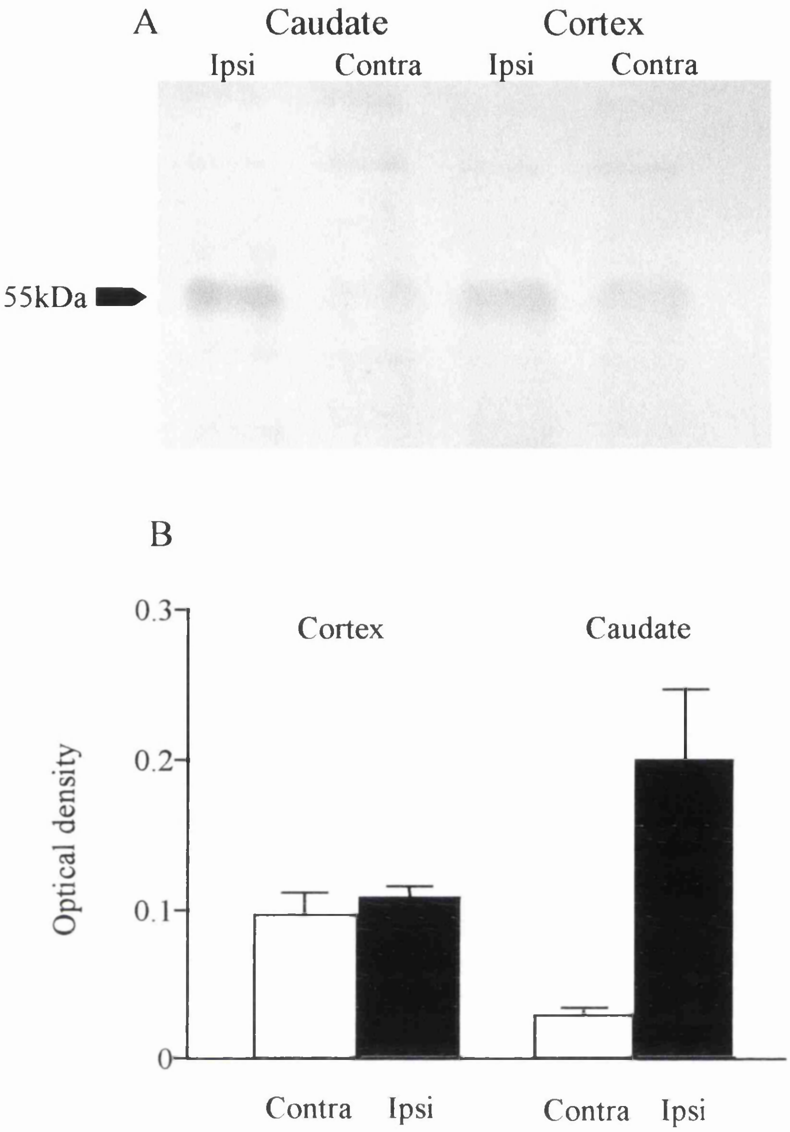


Figure 74

GGF-2 immunoreactivity in brain homogenates after 24h permanent MCAo in the rat. A: Representative Western blot of GGF-2 immunoreactivity in the contralateral (contra) and ipsilateral (ipsi) cortex and caudate and 24h after MCAo in the rat (n=2). B: Quantitative data for GGF-2 immunoreactivity detected by Western blotting.Immunoreactivity for the 55kDa band was increased in the ipsilateral and caudate after MCAo compared with the contralateral hemisphere.

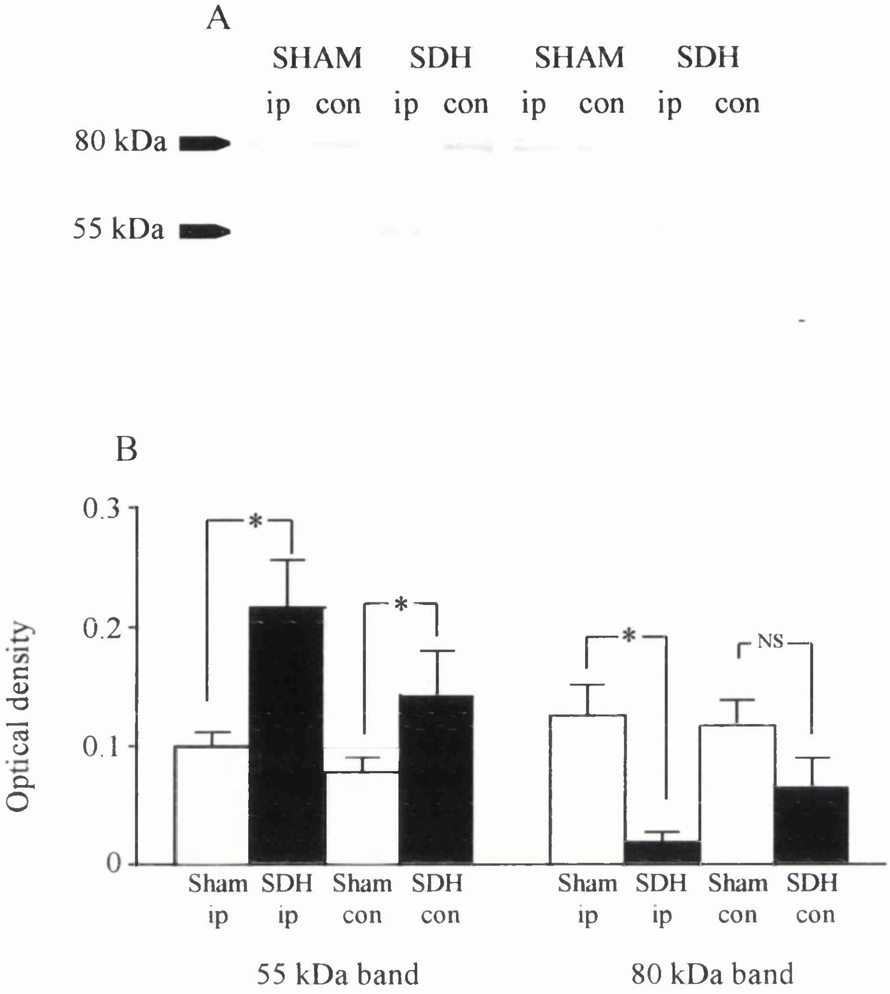


Figure 75

GGF-2 immunoreactivity in cortical homogenates 4h after subdural haematoma (SDH) in the rat. A: Representative Western blot showing detection of a 80kDa and 55kDa bands of GGF-2 immunoreactivity in the ipsilateral (ip) and contralateral (con) cortex in sham animals and after 4h SDH. B: Quantitative data for GGF-2 immunoreactivity detected in Western blots. GGF-2 immunoreactivity of the 55kDa band was significantly increased, $^*p<0.05$, in both the ipsilateral and contralateral cortex after SDH (filled bars) compared to SHAM animals (open bars). The 80kDa band of GGF-2 immunoreactivity was significantly reduced, $^*p<0.05$, in the ipsilateral cortex after subdural haematoma compared to sham animals.

CHAPTER 4: DISCUSSION

4.1 Human Postmortem Studies

4.1.1 Nicotinic receptor binding

Mnemonic and cognitive deficits existing after head injury are attributable to deficits in the central cholinergic system, on the basis of TBI in experimental animals (Leonard et al., 1994) and improvement with cholinomimetic therapeutic strategies in both rodents (Pike et al., 1997; Chen et al., 1998a, 1998b) and man (Levin et al., 1986; Cardenas et al., 1994). Abnormalities of cholinergic neurotransmission in human head injury could occur either by changes in receptors or a loss of presynaptic inputs. The aim of this initial study was to determine the integrity of high affinity nAChRs after human head injury.

The status of brain nAChRs after head injury is related to the question of potential cholinergic therapy. A proportion of high affinity nAChRs are postulated to have a presynaptic distribution (Abdulla et al., 1995) and function to modulate synaptic transmission (for review see Langer, 1997). Using brain homogenates, the density of high affinity nAChRs was found to be unaltered in each of the cortical areas examined from head-injured patients compared with controls. In addition, autoradiographic analyses of [^{3}H]-nicotine and [^{125}I]-bungarotoxin binding were no different in the cingulate gyrus from the same group of head-injured patients and controls (Murdoch et al., 1998). No relationship existed between age or postmortem delay and the density of cortical nicotine binding sites. The latter observation is not surprising given that previous studies in models of the human autopsy process, have indicated that the binding components of receptors are relatively stable postmortem (Whitehouse et al., 1984). [^{3}H]-Nicotine, at the concentration used in the present study, predominantly binds to the high affinity $\alpha_4\beta_2$ nAChR, whereas [^{125}I]-α-bungarotoxin binds to nAChRs containing the α7 subunit. The results therefore indicate that neither binding site is reduced in the cortex after head injury.

The preservation of cortical nAChRs contrasts with the widespread deficits in cortical ChAT activity reported in head-injured patients (Dewar and Graham, 1996; Murdoch et al., 1998). Similarly, experimental brain injury in rats produced a time-dependent loss of both ChAT activity (Gorman et al., 1996) and ChAT immunohistochemical staining (Leonard et al., 1994; Sinson et al., 1995). Moreover, lesioning of the nbM using either high frequency electrical lesioning (Smith et al., 1989) or the cholinergic specific immunotoxin ^{192}IgG-saporin (Bednar et al., 1998) produced no apparent changes in cortical nAChR density despite extensive reductions in ChAT activity. This implies that ChAT activity and nAChRs in the cortex are differentially affected in acute brain injury. This view is supported by Court and Perry, 1991, who speculated that even where nAChRs and ChAT share locations, it is unlikely that they will be modulated by the same control mechanisms. In addition to investigations of nAChRs, alterations in muscarinic cholinergic receptors have been assessed after experimentally-induced brain injury in animals. Selective labeling of M_1 and M_2 muscarinic receptor subtypes revealed a differential response, in that M_2 sites were reduced at 24h but not 3h after injury, while M_1 sites were unaltered at both timepoints compared to controls (DeAngelis et al., 1994). The M_2 receptor subtype is believed to be expressed on presynaptic cholinergic neurons (Rouse et al., 1997). Controlled cortical impact injury in rats resulted in a loss of M_2 receptors in the hippocampus 2-4weeks post-injury (Ciallella et al., 1998; Shao et al., 1999). Likewise, Jiang and colleagues, 1994, reported alterations in muscarinic receptor binding sites in the neocortex 15 days after TBI. However, in human postmortem material neither M_1 or M_2 muscarinic receptor binding were altered in the temporal cortex of head-injured patients compared to controls (Dewar and Graham, 1996), indicating a difference between man and animals and a need for further characterisation of cholinergic receptor status after brain injury.

There is increasing appreciation that the postmortem pathological correlates of a head injury share some similarities with those of AD. In AD, loss of cortical ChAT activity is paralleled by an approximate 50% reduction in cortical (Whitehouse et al., 1986) and hippocampal nAChRs (Perry, 1986) compared with aged-matched controls. In contrast to the loss of nAChRs in AD, no or only modest changes in binding to muscarinic and α-bungarotoxin cholinergic receptors has been reported in the neocortex of AD patients

(Davies and Feisullin, 1981; Sugaya et al., 1990; Nordberg, 1992). The preservation of nAChRs and α-bungarotoxin receptors after head injury, may reflect a consequence of the pathophysiological processes associated with acute brain injury rather than the chronic degeneration of AD in elderly patients.

Although use of tritiated agonists has the advantage of recognising only fully assembled receptors (Whiteaker et al., 1998), binding studies using homogenates do not distinguish between pre- and postsynaptic nAChRs (Miyai et al., 1990). The principal receptor subunit combination that comprises the high affinity [^{3}H]-nicotine binding site consists of the subunits α_4 and β_2 (Whiting et al., 1991; Flores et al., 1992). Nicotine has approximately 50-fold higher affinity for the $\alpha_4\beta_2$ subtype found in the brain than non-$\alpha_4\beta_2$ subtypes found in the adrenal gland (Flores et al., 1997). The precise cellular location of the $\alpha_4\beta_2$ subunit comprised nAChRs in the neocortex is unclear. The parallel reduction in nicotine binding sites and ChAT activity in AD suggests that these receptors are presynaptic (Whitehouse et al., 1986). Nevertheless, the extent to which high affinity nAChRs reside presynaptically on cholinergic terminals remains controversial with the possibility that a major portion of nAChRs are located either postsynaptically or on non-cholinergic terminals (Wonnacott, 1997; Bednar et al., 1998). The initial conclusion that cholinergic presynaptic terminals are preserved after head injury, by way of measurement of high affinity nAChRs, might be incorrect given that this method may not solely detect presynaptic nAChRs, since high-affinity nAChRs may have been detected postsynaptically and/or non-cholinergic terminals in addition to presynaptic sites.

One environmental factor that may have confounded the nAChR data in the head-injured patients and controls is chronic nicotine intake via tobacco smoking. Administration of nicotine to rodents either in their drinking water or by direct infusion into the brain (Kellar et al., 1989; Marks et al., 1992; Collins et al., 1996) increased the binding of [^{3}H]-nicotine by as much as threefold. More importantly, studies using human postmortem brain tissue demonstrated a significant increase in the density of [^{3}H]-nicotine binding in cerebral cortex, hippocampus and midbrain obtained from cigarette smokers compared with that in non-smoking controls (Benwell et al., 1988; Perry et al., 1996). Moreover, in the brains of mice subjected to chronic nicotine administration, the most significant increase in [^{3}H]-nicotine binding was detected in the cortex (Pietela et al., 1998), and this increase was considered to be due mainly to an upregulation in $\alpha_4\beta_2$ nAChR number (Peng et al., 1994b) but not affinity for the ligand. Details of smoking habits were not available for either the head-injured patients or controls in this study. However, if a significant number of the head-injured patients were heavy smokers, the concurrent upregulation of the $\alpha_4\beta_2$ subtype comprising nAChR would mask any change in [^{3}H]-nicotine binding in this group compared with controls.

4.1.2. Presynaptic pathology after head injury

Over recent years identification and biochemical characterisation of numerous synaptic vesicle and presynaptic plasma membrane proteins has provided fundamental insight into the molecular mechanisms underlying neurotransmitter release (for review see Bennett and Scheller, 1994). A plethora of protein interactions have been identified that could function in the different steps of neurotransmitter release (see Figure 6B). This potentially provides a number of potential protein interactions that could be interrupted or dysfunctional in acute brain injury. As understanding of the brain mechanisms of neurotransmitter release has advanced, attention has shifted to how these proteins are modified to produce changes in synaptic efficacy (Bajjalieh and Scheller, 1995).

Using a range of methods, a number of synaptic proteins have been routinely employed as reliable measures of synaptic density in human postmortem brain tissue (Hamos et al., 1989; Alford et al., 1994; Akulinin et al., 1998). To obtain a better understanding of the individual molecular alterations in synapses after head injury, a number of distinct presynaptic proteins were examined in the cortex after head injury. Levels of synaptophysin, SNAP-25 and syntaxin, as detected by Western blotting analyses, were significantly reduced in the cingulate gyrus of head-injured patients compared with controls. Synaptophysin is the major integral membrane protein of synaptic vesicles, accounting for approximately 7% of total vesicle protein (Wiedenmann and Franke, 1985), and is estimated to be present in more than 95% of cortical presynaptic terminals (Navone et al., 1986). This observation was supported by immunohistochemical staining of sections with an antibody to synaptophysin. Synaptophysin immunoreactivity was significantly reduced by 30% in head-injured patients compared with controls, consistent with a loss of cortical presynaptic terminals in the neuropil after head injury. This is in line with Akulinin and co-workers, 1998, who reported that synaptophysin immunoreactivity was reduced by 35% in the neocortex 1week after cardiac arrest, presumably resulting in an episode/s of transient cerebral ischaemia comparable to head injury.

SNAP-25 and syntaxin are localised to the membrane of the presynaptic nerve terminal (Li et al., 1996) and share homology not only in their distribution but also their function as t-SNAREs (Bauerfiend et al., 1996). In addition to the plasmalemmal localisation, significant pools of both proteins are also present on the membrane of synaptic vesicles and other synaptic trafficking organelles (Walch-Solimena et al., 1995). These findings suggest proteins located on both the synaptic vesicle and the nerve terminal membranes are reduced after head injury, implying there is an overall loss of presynaptic terminals rather than site or protein-specific changes. The abundance of synaptophysin on synaptic vesicles and the localisation of SNAP-25 and syntaxin on multiple sites might contribute to their loss, whilst other presynaptic marker proteins are preserved after head injury. Synaptophysin, SNAP-25 and syntaxin have a common link in that they are all believed to interact with the synaptic vesicle protein synaptobrevin (VAMP), see figure 6A (McMahon and Sudhof, 1995). Although the level of synaptobrevin was not examined in the present study, it seems possible that if synaptobrevin or its function is particularly susceptible to trauma, this would contribute to the loss of these particular presynaptic proteins after head injury.

Lipid modification, attachment of palmitic acid, is responsible for anchoring the intrinsically hydrophilic protein SNAP-25 to the nerve terminal membrane (Oyler et al., 1989; Hess et al., 1992). Free radical production is implicated in brain injury on the basis of a number of studies in experimental animals (Werns and Luccechi, 1990; Doppenberg et al., 1998). There is mounting evidence that oxygen radical-induced lipid peroxidation plays a pathological role in acute brain injury (Braughler and Hall, 1989; Siesjo et al., 1989; Lewen and Hillered, 1998). SNAP-25 is known to be rapidly palmitoylated and depalmitoylated, a process which has a role in establishing, maintaining and strengthening synaptic connection (Lane and Yiu, 1997). Deregulation of this process as a consequence of head injury, could explain the loss of SNAP-25 immunoreactivity after head injury.

Similarly, syntaxin present complexed to SNAP-25 would be lost as a result of this process.

The results obtained for Western blotting analysis of SNAP-25 were not supported by immunohistochemical staining of sections of cingulate gyrus for SNAP-25. This may be a reflection of the methodology used to quantify SNAP-25 immunoreactivity. Relative optical density measurements were calculated as the mean grey matter reading minus the mean white matter reading taken from the subcortical white matter of the corpus callosum. Thus a confounding factor may have been the presence of SNAP-25 immunostaining in the white matter tracts of the corpus callosum. By contrast, minimal synaptophysin immunostaining was detected in the corpus callosum of either group. SNAP-25 is conveyed to the presynaptic terminals by fast anterograde axonal transport (Hess et al., 1992; Li et al., 1996). Relative optical density measurement in white matter potentially could have masked deficits in SNAP-25 immunoreactivity in grey matter from the cingulate gyrus of head-injured patients.

Although three of the synaptic proteins examined in the cingulate gyrus were significantly reduced after head injury, there were only modest changes in the levels of the synaptic vesicle proteins synaptotagmin and synapsin I, suggesting a differential involvement of synaptic proteins in head injury. Levels of synaptotagmin immuoreactivity were decreased by 18%, whereas synapsin Ia and Ib immunoreactivities were increased by 12 and 20% respectively. This differential response may be a consequence of the properties of the presynaptic proteins. Synaptotagmin is the putative Ca^{2+} sensor for neurotransmitter release (Littleton et al., 1994). Although levels of synaptotagmin have been reported to be reduced in AD (Davidsson and Blennow, 1998), there is no evidence of loss of synaptotagmin after acute brain injury. Synapsin I is the most abundant of all neuronal phosphoproteins (Thiel, 1993), and unlike the other presynaptic proteins is enriched on the cytoplasmic surface of synaptic vesicles (Huttner et al., 1983). In animal models of plasticity, phosphorylation-state specific alterations in synapsin I have been detected, without changes in the levels of total synapsin I (Yamagata et al., 1995). Specifically, reactivity at phospho-site 3 and to a lesser extent phospho-site 1 antibodies were increased in the hippocampus and neocortex of kindled animals (Yamagata et al., 1995). The antibody used to detect synapsin I in the present study recognised all phosphorylation states of the protein, therefore we were unable to examine possible phosphorylation-state specific changes in synapsin I in the cortex after head injury.

Despite selective loss of several synaptic proteins in the cingulate gyrus, no significant reductions in any of the synaptic proteins was detected in the superior parietal cortex from the same group of head-injured patients and controls (see Table 6). Anatomical and inter-patient heterogeneity in the level of presynaptic proteins in the head-injured patients may be a reflection of the multiple types and severity of neuropathological features present after head injury (Graham et al., 1978). Peculiarities in findings in the parietal cortex have been noted previously. Although ChAT activity has been found to be reduced throughout the cortex after head injury, downregulation of ChAT activity was of a smaller magnitude in the parietal cortex than other cortical regions (Murdoch et al., 1998). Similarly, fluid percussion injury in the rat reduced ChAT activity in the frontal and temporal cortices, but increased ChAT activity in the parietal cortex (Gorman et al., 1996). Excitotoxic lesions of the rat nbM resulted in a significant loss of [^{3}H]-cytisine binding to nAChRs in the parietal cortex only (Bednar et al., 1998). The present data add to the catalogue of inconsistencies observed in the parietal cortex compared with other anatomical brain regions after experimental brain damage and human head injury.

This investigation into the integrity of presynaptic terminals after head injury clearly illustrates protein and anatomically specific changes. Evidence of synaptic pathology in both the cortex (DeKosky and Scheff, 1990; Terry et al., 1991) and hippocampus (Hamos et al., 1989; Davidsson and Blennow, 1998) has been frequently reported in postmortem brain tissue from AD patients. Moreover, Shimohama and colleagues, 1997, provided evidence of differential involvement of presynaptic proteins in AD, in that synaptophysin and synaptotagmin only were significantly reduced in the temporal cortex of AD patients compared with aged-matched controls. SNAP-25, syntaxin and synaptotagmin were only modestly different between groups. This suggests that synaptic protein loss is another pathological correlate of AD and head injury. Therefore, the findings in the present study imply that there is a differential involvement of presynaptic proteins, and that there is loss of presynaptic terminals after head injury. The positive association of ChAT activity and synaptophysin immunoreactivity (detected by Western blot analyses) in both the cingulate gyrus and to a greater extent in the superior parietal cortex, suggests that the loss of ChAT activity is associated to some extent with physical destruction of presynaptic terminals.

4.1.3 Postsynaptic alterations after human head injury

To compare the cortical presynaptic deficits detected after head injury to the sensitivity of equivalent postsynaptic equivalents, MAP-2 immunoreactivity was detected in the cingulate gyrus of the same group of controls and head-injured patients. MAP-2 has been routinely used in immunostaining and immunoblotting techniques as an early and sensitive marker to examine the cytoarchitecture of postsynaptic structures after brain injury (Kitagawa et al., 1989; Kudo et al., 1990; Yanagihara et al., 1990). MAP-2 is confined to neurons, its concentration higher in dendrites than in perikarya (Bernhardt and Marcus, 1984; De Camilli et al., 1984) and within dendrites it is present both in dendritic shaft and spine synapses (Caceres et al., 1984). MAP-2 consists of a high molecular weight doublet (280kDa) and a low molecular mass protein (70kDa) which is much more abundant in neurons of the developing brain rather than in adult tissue (Garner and Matus, 1988). Assembly and stability of neurofilaments and microtubules is thought to be regulated by MAP-2 (Bernhardt and Matus, 1984).

In controls, MAP-2 immunoreactivity was localised to dendrites, appearing as long, branching processes. However in the cingulate gyrus from head-injured patients who survived 72h and 96h respectively, this continuous MAP-2 staining pattern was replaced by dendritic swelling and fragmentation, and accumulation in perikarya. Postmantur et al., 1996, identified fragmented dendrites in layers of the cerebral cortex after cortical impact in rats. Moreover, the same group speculated that the MAP-2 immunostaining pattern suggested shearing of dendrites as a consequence of TBI. Whether head injury in humans results in shearing of dendrites (i.e. in a similar manner to axons, Graham and Gennarelli, 1997) could not be determined in the current study. Nevertheless, this study provides direct evidence of dendritic damage in the cortex after human head injury. Further characterisation of the distribution and timescale of postsynaptic changes, compared with presynaptic alterations, as a consequence of acute brain injury are described in section 4.2.4.

4.1.4. Free radical-mediated damage: Nitrotyrosine formation

Deficits in presynaptic proteins and therefore presynaptic terminals after human head injury have been established in this thesis. However, mechanisms of damage contributing to these changes in the human brain are not known. Neuronal damage by oxidative stress has been hypothesised as a critical mechanism of cellular damage in ageing (Berlett and Stadtman, 1997) and neurodegenerative diseases (Good et al., 1996). Although most studies have traditionally focused on the contribution of ROS, recent reports have described neuronal tissue damage mediated by excessive production of the nitric oxide radical ($^{\bullet}NO$) (Mesenge et al., 1996; Spinnewyn et al., 1999). $^{\bullet}NO$ is synthesised from L-arginine and molecular oxygen (Palmer et al., 1999) by the action of a number nitric oxide synthase (NOS) isoforms: namely neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). Constitutive nNOS is postulated to produce relatively small quantities of $^{\bullet}NO$ under basal conditions (Garthwaite, 1991; Faraci and Brian, 1994). However, iNOS identified in neuronal astrocytes and microglia in the brain (Bredt et al., 1990) is responsible for the synthesis of large amounts of $^{\bullet}NO$ in response to stimuli (Kirk et al., 1990). Transient cerebral ischaemia in the rat has been consistently shown to induce augmented tissue levels of $^{\bullet}NO$ (Kader et al., 1993; Sato et al., 1993) and total NOS activity (Hamada et al., 1995). A number of groups have speculated that upregulation of iNOS activity is predominantly responsible for increased $^{\bullet}NO$ in global (Forman et al. 1998) and focal (Hirabayashi et al., 1999) models of transient cerebral ischaemia in the rat. Moreover, administration of NOS inhibitors has been shown to attenuate $^{\bullet}NO$ production (Forman et al., 1998) and afford considerable neuroprotection (Spinnewyn et al., 1999). Transgenic mice deficient in a NOS gene possessed less neurological damage resulting from cerebral ischaemia and reperfusion (Ferriero et al. 1996).

In contrast to other free radicals, $^{\bullet}NO$ is relatively stable with a lifetime of several seconds (Koppal et al., 1999). However, when produced in an oxidising environment it reacts with oxygen radicals (particularly superoxide) at a diffusion-controlled rate leading to the formation of more reactive nitrogen oxides: the peroxynitrite anion, $^{\bullet}NO_2$ and N_2O_3 (Beckman and Koppenol, 1996). Peroxynitrite is not a radical, nevertheless, it is a far more potent oxidant than its radical precursors (Beckman et al., 1990), with a half-life of less than 1 second (Pryor and Squadrito, 1995). The association between peroxynitrite and some neurodegenerative disorders has been inferred from increased levels of 3-nitrotyrosine in pathologic tissue (Beal et al., 1997; Ara et al., 1998) Immunohistochemical and dot-blotting techniques have been used to measure peroxynitrite production, via nitrotyrosine formation, following TBI in mice (Mesenge et al., 1996) and focal ischaemia in rats (Takizawa et al., 1999). In the present study, immunoblotting techniques were used to assess whether there was evidence of peroxynitrite-mediated damage in the cortex after human head injury.

Nitrotyrosine immunoreactivity identified by western blotting detected a number of proteins of various molecular weights in control and head-injured patients. This is in agreement with Di Stasi et al., 1999, who reported substrates of a range of molecular masses nitrated by peroxynitrite in synaptosomes after peroxynitrite treatment. This is equivalent to nitration of tyrosine residues on proteins of varying mass. Although the mean nitrotyrosine immunoreactivity was no different between the controls and head-injured patients, the vast majority of head-injured cases had a nitrotyrosine immunoreactivity above that of the controls. The dot-blot analysis provides, for the first time, a suggestion that peroxynitrite-mediated damage may have a role in human head injury.

Peroxynitrite-mediated nitration of proteins has been shown to occur in tyrosine, tryptophan, methionine and alanine residues (Ischiropoulos et al., 1992; Beckman, 1996). To date several proteins have been found to be preferential targets of peroxynitrite-mediated nitration: these include manganese superoxide dismutase (MacMillan-Crow et al., 1998), low molecular weight subunit of neurofilament triplet (Crow et al., 1997), tyrosine hydroxylase (Ara et al., 1998) and prostacyclin synthase (Zou et al., 1998). With regard to synaptic proteins as potential targets, Di Stasi and co-workers, 1999, identified synaptophysin as a major protein target modified by peroxynitrite. In view of the reduction in synaptophysin and trend towards an increase in nitrotyrosine immunoreactivities, it seems possible that peroxynitrite-mediated damage may have contributed to the presynaptic deficit existing after head injury. Given the abundance of synaptophysin, approximately 7% of vesicle protein, it is not unforseeable that this is a major target for peroxynitrite, although other abundant synaptic proteins such as synapsin I and synaptotagmin have been reported not to be nitrated (Di Stasi et al., 1999). In agreement with this, in this thesis there was no evidence of loss of either synaptotagmin or synapsin I after head injury. A possible explanation is the presence of 10 copies of the tyrosine rich pentapeptide repeat in the cytoplasmic carboxyl terminus of synaptophysin (Sudhof et al., 1987), providing a rich environment for potential peroxynitrite-mediated nitration. Phosphorylation of key tyrosine residues plays a crucial role in signal transduction. Nitration of these tyrosine residues is believed to downregulate tyrosine-dependent signaling (Kong et al., 1996; Mallozi et al., 1997). In agreement, Di Stasi et al., 1999, identified pp60^{src} kinase as one of the major protein targets modified by peroxynitrite.

Additional evidence of synaptic dysfunction and degeneration induced by oxidative insults has been demonstrated by the use of synaptosomes. Synaptosomes prepared after cortical contusion in the rat demonstrated a biphasic increase in lipid peroxidation in the ipsilateral cortex and hippocampus (Sullivan et al., 1998). A number of previous studies have shown that the aldehyde product of membrane lipid peroxidation, 4-hydroxynonenal, can disrupt synaptic homeostasis *in vitro* (Keller et al., 1997; Subramaniam et al., 1997) and *in vivo* (Azbill et al., 1997). Moreover, increased protein-protein interactions and increased lipid fluidity observed in physical state synaptosomal membranes at 1h reperfusion following a 10min period of ischaemia was prevented with the spin-trap agent *N*-tert-butyl-α-phenylnitrone (Hall et al., 1995).

The loss of synaptic proteins along with a trend towards an increase in nitrotyrosine levels in the cortex of head-injured patients compared with controls is consistent with the possibility of peroxynitrite causing oxidative stress-induced conformational and structural changes. These observations suggest that head injury promotes the release of $^{\bullet}$NO, and that inhibition of NOS activity and synthesis of $^{\bullet}$NO may contribute to prevent presynaptic pathology after human head injury.

4.1.5 nbM pathology after head injury

Deficits in cortical ChAT activity present in human postmortem brain after a head injury (Dewar and Graham, 1996; Murdoch et al., 1997; 1998) may reflect damage to presynaptic terminals or alternatively damage to Ch4 neurons in the nbM. In the present study, histological and immunohistochemical analyses provided evidence of ischaemic damage to Ch4 neurons in the nbM of two-thirds of the head-injured patients. The cause/s of this damage after head injury are not known; however, macroscopic and microscopic analyses identified a number of mechanisms that may be involved. In several head-injured cases (n=5), there was neuropathological evidence of midline shift of brain tissue and internal herniation as a consequence of raised ICP (Graham et al., 1987). This mechanical distortion of the brain may account for the features consistent with plastic creep (Langfitt et al., 1964), first described in monkeys by experimental obstruction of the tentorial incisura using a saline injection into an inflatable balloon, resulting in transformation of neuronal perikarya from a round to elongated shape. Ischaemic damage to nbM neurons was also observed in some of the head-injured patients. Ischaemia within the nbM may have arisen as a consequence of anterior choroidal artery occlusion. This in turn may have been a consequence of raised intracranial pressure. It is interesting to note that in a previous study, patients who had a SDH had lower levels of cortical ChAT activity than those patients without this secondary insult (Murdoch et al., 1998). In that study we speculated that since SDH is associated with raised ICP and shift of midline structures, neurons within the nbM may be damaged.

Damage to Ch4 neurons in the nbM of head-injured patients is in agreement with results obtained using the fluid percussion model in rats (Schmidt and Grady, 1995; Sinson et al., 1997), where midline injury produced an average 41% bilateral loss of Ch4 neurons (Schmidt and Grady, 1995). Loss of nbM neurons after experimental TBI in rats was attenuated by intracerebral infusion of NGF (Sinson et al., 1997). Similarly, NGF treatment has been reported to reverse the loss of cholinergic septal neurons after TBI in rats (Fischer et al., 1987; Hagg et al., 1988). Aldbeck and co-workers, 1999, reported that acute application of NGF increased the firing rate of aged rat basal forebrain neurons. In view of the cytoprotective effect of NGF on cholinergic cells and its ability to elevate ChAT activity *in vitro* and *in vivo* (Whittlemore and Sieger, 1987), it is possible that shrinkage of nbM neurons after human head injury might be a consequence of compromised retrograde transport of NGF due to disruption or degeneration of cholinergic axons projecting to target zones in the cerebral cortex (Vogels et al., 1989). In the present study, ChAT immunoreactivity was reduced in Ch4 neurons in the nbM of head-injured patients. Although there was a concominant loss of ChAT and NGF receptor immunoreactive cells in the rat basal forebrain after fluid percussion injury (Leonard et al., 1994), it is not known whether NGF receptor immunohistochemistry is reduced in Ch4 neurons after human head injury. This pathological process may play a key role given the importance and frequency of diffuse axonal injury after human head injury (Graham and Gennarelli, 1997).

Morphological abnormalities observed in nbM neurons in head-injured patients differed from those previously reported in AD. Several kinds of cell degeneration in the nbM have been observed in AD, including swelling (Arendt et al., 1985) and increased nuclear size (Iraizoz et al., 1999), the latter regarded as a plastic response due to elevated cellular activity (Buschmann and Lavelle, 1983; Donselaar et al., 1986). The neuropathological differences in nbM neurons in AD compared with head injury may be a reflection of the underlying mechanisms involved in chronic neurodegenerative versus acute brain injury. In the majority of studies of nbM neurons in AD, investigators have relied on the large size and hyperchromatic staining pattern seen with classic histological techniques for identification of Ch4 neurons in the nbM. Mapping studies have demonstrated these large, hyperchromatic neurons to be putative cholinergic neurons (Mesulam et al., 1983a; McGeer et al., 1984). In the present study, detection of ChAT immunostaining in Ch4 neurons in the nbM verified that the neurons with histological evidence of damage or death were cholinergic in origin. In contrast, using stereological analyses of immunostaining for the cholinergic specific markers ChAT and VAChT in the nbM of AD patients, Gilmor and colleagues, 1999, reported a non-significant 15% reduction in the number of cholinergic neurons compared with aged-matched controls. This is in agreement with other human postmortem studies (Pearson etal., 1983; Vogels et al., 1989), suggesting that cholinergic neurons in the nbM shrink during the pathophysiological processes of AD. The alteration in the pattern of ChAT immunostaining in the nbM of head-injured patients suggests that the shrunken neurons are cholinergic, which we speculate contributes to the reduction of cortical ChAT activity previously reported for this same group of head-injured patients (Murdoch et al., 1998).

4.1.6 Correlation of nbM damage and ChAT activity

Correlation analyses of nbM neuronal damage and cortical ChAT activity in the cingulate gyrus, superior parietal cortex and temporal gyrus from head-injured patients (Murdoch et al., 1998), revealed a positive association between these two parameters, such that lower levels of cortical ChAT activity were associated with greater degrees of neuronal damage in the nbM. Likewise, stereotactic lesioning of the nbM in rats produced a loss of Ch4 neurons with a concominant reduction in cortical ChAT activity (Wenk et al., 1980; Bednar et al., 1999). Moreover, electrolytic lesioning of the nbM in the rat produced significant reductions in glucose utilisation in 10 of 17 cortical regions examined, including the cingulate and parietal cortices (Browne et al., 1998). The trajectories of connections between the nbM and cerebral cortex have been traced in rodents (Eckenstein et al., 1988), monkeys (Mesulam et al., 1983a; Satoh and Fibiger, 1985; Everitt et al., 1988) and in man (Selden et al., 1998). Using injections of tritiated amino acids into the midportion of the nbM and surrounding structures, Kitt et al., 1987, described a medial projection to the cingulate cortex that travelled within the cingulum, a lateral projection to the frontoparietal and insular cortices that travelled within the external and extreme capsules and a ventral projection to the temporal cortex and amygdyla that travelled within the uncinate fasciculus. Although this was performed in the rhesus monkey a similar pattern of cholinergic innervation has been described in the human brain (Selden et al., 1998). The fact that ChAT activity in each of the cortical regions examined correlated strongly with the neuronal damage score in the nbM of head-injured patients, suggests that Ch4 nbM neurons contributing to the medial, lateral and ventral projections were all damaged as a consequence of head injury. Interestingly, association of ChAT deficits in cerebral cortex and extent of disappearence of cholinergic soma in the basal forebrain of AD patients produced no correlation (Perry et al., 1982; Allen et al., 1990).

4.1.7 Correlation of nbM damage and levels of presynaptic proteins

No relationship existed upon correlation of the neuronal damage score in the nbM with levels of any of the presynaptic proteins detected (by Western blotting) in either the cingulate gyrus or the superior parietal cortex. This suggests that although there was Western blot and immunohistochemical evidence of presynaptic terminal loss after head injury, degeneration of cholinergic terminals is not the primary pathology responsible for reductions in cortical ChAT activity after head injury. Alternatively, the presynaptic proteins examined are not cholinergic-specific, but are located on all presynaptic terminals, for all neurotransmitter systems within the cerebral cortex. Therefore, we cannot rule out a presynaptic contribution to loss of cortical ChAT activity after head injury, given that there was a striking correlation between ChAT activity in the cingulate gyrus and the superior parietal cortex, and the level of synaptophysin immunoreactivity, as detected by Western blot analyses.

4.2 Presynaptic pathology in animal models of acute brain injury

Synaptic transmission is regarded the currency of information exchange in the brain (Sudhof, 1995). Alteration in strength of synaptic transmission, referred to as synaptic plasticity, is believed to have both presynaptic and postsynaptic origins. Dissection of the subtle molecular alterations of the synapses and of plasticity might yield clues as to the mechanism/s of cognitive and mnemonic deficits existing after head injury. The existence of CNS plasticity after head injury is a controversial issue (Cotman and Nieto-Sampedro, 1982), however, alterations in the levels of a number of presynaptic proteins in the neocortex of head-injured patients reported earlier in this thesis provides an indication that alterations in synaptic strength does exist after head injury. The aim of the present study was to further characterise the integrity of presynaptic terminals, by analysing the levels of the three proteins significantly reduced in the cingulate gyrus of head-injured patients, in a number of models (permanent and transient) of cerebral ischaemia.

4.2.1 Presynaptic pathology in permanent models of cerebral ischaemia

Two models of 24h permanent MCAo were used, namely the intraluminal thread model (Zea Longa et al., 1989) and the diathermy model (Tamura et al., 1981). Using the intraluminal thread model and Western blot analyses, no change in the levels of synaptophysin, SNAP-25 or syntaxin immunoreactivities were observed in the ipsilateral MCA territory compared with the contralateral hemisphere. The cingulate gyrus receives its blood supply from the anterior cerebral artery, explaining why optical densities in the ipsilateral cingulate gyrus are unchanged compared with the contralateral hemisphere. This implies that although the H/E staining indicates an area of neuronal damage in the ipsilateral hemisphere, presynaptic terminals are preserved 24h after permanent MCAo using this model. This was comparable with the immunohistochemical findings obtained for the diathermy model of 24h permanent MCAo. Despite widespread neuronal damage in the ipsilateral cortex and caudate, immunostaining for synaptophysin, SNAP-25 and syntaxin was preserved. In agreement with Korematsu et al., 1993, immunostaining for the presynaptic proteins in the ipsilateral cortex and caudate had a more coarse staining pattern compared with the contralateral hemisphere. The accumulation of SNAP-25 and syntaxin in the axonal fibre tracts of the ipsilateral caudate is presumably a consequence of these proteins being fast anterogradely transported as part of pleiotrophic organelles from the cell body to their appropriate targets (Li et al., 1996). Whether these accumulations reflect axotomy of axons, or increased demand for these synaptic proteins as a consequence of neuronal remodelling and plasticity is not known. In view of the extent of oedema and resultant disintegration of tissue it was not possible to measure the relative optical densities of the immunostaining for these presynaptic proteins in this model of cerebral ischaemia. The permanent models of focal cerebral ischaemia used did not support a presynaptic deficit after this type of insult per se. A murine model of transient forebrain ischaemia was therefore employed to investigate whether temporal alterations in presynaptic inputs existed over longer timepoints, and if transient ischaemia with reperfusion rather than permanent ischaemia could be involved.

4.2.2 Neuronal damage after transient forebrain ischaemia

One of the major pathophysiological mechanisms involved in human head injury is ischaemic damage. Transient global ischaemia in rodents is known to injure discrete populations of neurons. Specifically, neurons in the caudate nucleus (Yang et al., 1997; Horsburgh et al., 1999) and the hippocampus (Petito et al., 1987) are among the most vulnerable in the CNS to an ischaemic insult. Within the hippocampus, the CA1 pyramidal neurons have been shown to be the most susceptible to ischaemic damage following transient forebrain ischaemia (Kirino, 1982; Pulsinelli et al., 1982; Onodera et al., 1986) in rodents. In the present study, neuronal damage was evident in the caudate at 1day, and widespread 3 and 10 days after 17min transient forebrain ischaemia. Within the hippocampal formation, the CA1 and CA2 sectors and the hilar region proved most susceptible to ischaemic damage at each of the survival periods. CA3 pyramidal cells and dentate gyrus neurons possessed less extensive damage at each of the timepoints examined. Previous studies have suggested that CA3 pyramidal cells and dentate gyrus neurons are relatively resistant to ischaemic damage following transient forebrain ischaemia in gerbils (Marti et al., 1998) and rats (Francis and Pulsinelli, 1982). This study has provided evidence of selective neuronal depletion caused by transient forebrain ischaemia.

4.2.3 Presynaptic pathology in transient forebrain ischaemia

Synaptic plasticity in the brain after injury has been studied in a number of species and experimental models. Consequently, a diverse collection of results exists adding controversy to the existence of CNS plasticity after injury. The aim of the present study was to investigate the temporal profile of presynaptic pathology in the caudate and various presynaptic inputs of the hippocampus after transient forebrain ischaemia in the mouse. Immunostaining and relative optical density measurements were used to ascertain the level of the three synaptic proteins that were reduced in the cingulate gyrus in head-injured patients. Immunostaining for synaptophysin and SNAP-25 was preserved in the neuropil of the caudate in mice allowed to survive each of the timepoints after global ischaemia. In addition, relative optical density measurements confirmed both synaptophysin and SNAP-25 immunostaining were preserved in the caudate of ischaemic compared with sham animals. A similar pattern of results were obtained in the various hippocampal layers examined. There was minimal change in the relative optical density of synaptophysin immunostaining in all of the hippocampal layers measured. Similarly, relative optical densities of SNAP-25 and syntaxin immunostaining were relatively unaltered in all hippocampal layers at each timepoint after global ischaemia, although there was a trend towards a decrease in the outer, middle and inner molecular layers of the dentate gyrus with prolonged survival time after global ischaemia. Synaptophysin and SNAP-25 immunostaining in the cortical neuropil was preserved in ischaemic brains compared with sham animals. Therefore, despite extensive neuronal damage in the caudate, cortex and each of the hippocampal sectors, presynaptic proteins appeared to be preserved, implying that presynaptic terminals remain intact after cerebral ischaemia.

A number of groups have investigated the integrity of presynaptic terminals in animal models of cerebral ischaemia, producing a divergent set of results. Synaptophysin has been reported to be increased in the neocortex between 14-60 days after permanent MCAo (Stroemer et al., 1995). In this thesis, synaptophysin levels were no different in ischaemic compared with sham animals. The difference in results presumably reflects the timepoints considered, since Stroemer and colleagues, 1995, found no change in synaptophysin immunoreactivity 3 or 7 days after the ischaemic episode. Similarly synaptophysin immunoreactivity has been shown to be increased in the caudate (Korematsu et al., 1993) and the stratum lacunosum molecular layer and stratum oriens of the CA1 sector of the hippocampus after transient forebrain ischaemia in rats. A decline in the immunoreactivities for syntaxin and SNAP-25 (Marti et al., 1998), and synapsin I and synaptophysin (Marti et al., 1999) have been detected in the inner molecular layer of the dentate gyrus after global ischaemia in gerbils. The variability in the changes observed is likely to be a consequence of variations in severity of ischaemia, recovery times, species and the experimental models of acute brain injury employed.

Despite these reports of presynaptic pathology after acute brain injury, the overwhelming consensus is that presynaptic terminals are relatively resistant to ischaemic damage. Synaptophysin immunoreactivity has been reported to be preserved in the hippocampal CA1 sector 7 days after transient forebrain ischaemia in the rat (Rischke et al., 1990). Moreover, immunoblots of SVP-38, another synaptic vesicle-specific protein, did not show significant changes in extracts from the CA1 sector after traumatic forebrain ischaemia in the gerbil (Arai et al., 1991). This is in agreement with results obtained in this thesis, since immunoreactivity for each of the presynaptic proteins examined in the stratum oriens and stratum lacunosum was unchanged up to 10 days after transient forebrain ischaemia. Kitagawa et al., 1992, reported synapsin I immunoreactivity was unchanged after 2 months in the degenerated CA1 layer. In addition, electron microscopy studies revealed intact presynaptic terminals, rich in synaptic vesicles, twelve months after an ischaemic insult (Bonnekoh et al., 1990; Kirino et al., 1990).

The results obtained using both permanent and transient models of cerebral ischaemia are in agreement with the majority of studies, in that presynaptic terminals appear to be relatively resistant to ischaemic damage. Moreover, in studies where presynaptic deficits have been reported, the loss was always smaller than that of the postsynaptic elements and confined to areas of overt infarction (Kitagawa et al., 1992). The preservation of immunoreactivity for each of the synaptic proteins might represent preservation of the specific epitopes rather than preservation of the synaptic proteins themselves. However, Kitagawa and colleagues, 1992, have previously reported loss of not only antigenicity but also breakdown of presynaptic proteins after cerebral ischaemia. It is unknown how and why presynaptic terminals were persistently preserved. A possible explanation is that reactive astrocytes present in the ischaemic territory (Hatakeyama et al., 1989; Kirino et al., 1990) may contribute to the maintenance of presynaptic terminals by supplying neurotrophic factors required for the survival of detached terminals and their original neurons (Kromer, 1996). Alternatively, postsynaptic remnants have been hypothesised as having a role in chronic maintenance of presynaptic terminals (Kirino et al., 1990).

4.2.4 Postsynaptic alterations after transient forebrain ischaemia

Presynaptic terminals appeared to be preserved in the cortex, caudate and various hippocampal layers after transient forebrain ischaemia in the mouse. To contrast this with the status of equivalent postsynaptic structures, the pattern of MAP-2 immunostaining was assessed in the same tissue. The low power magnification images indicate the early and anatomically-specific loss of MAP-2 after global ischaemia, the most extensive depletion of MAP-2 being present in the hippocampus and caudate. High power magnification images highlighted the extent and rapidity of loss of MAP-2 from the neuronal perikarya and dendrites after transient forebrain ischaemia, particularly in the caudate, and the CA1 and CA2 sectors of the hippocampus.

MAP-2 has been used as a reliable and sensitive postsynaptic marker in a number of models of cerebral ischaemia (Kitagawa et al., 1989; Inuzuka et al., 1990; Yoshimi et al., 1991) and TBI (Taft et al., 1992; Kudo et al., 1993; Folkerts et al., 1998). In the present study, MAP-2 immunostaining was disrupted in the caudate, cortex and the hippocampus after transient forebrain ischaemia. Similarly, previous studies have detected early changes in MAP-2 expression in each of these anatomical brain regions. For example, MAP-2 immunostaining was reduced in the CA1 sector neonatal (Malinak and Silvestein, 1996) and mature rodents (Matesic and Lin, 1994) after cerebral ischaemia. Ultrastructural investigation of the stratum oriens and stratum lacunosum molecular layer of the CA1 sector, identified early swelling of dendritic structures as early as 5min after transient forebrain ischaemic in the gerbil (Yamamoto et al., 1990). Kitagawa et al., 1992, reported extensive disruption of MAP-2 immunostaining in the caudate of gerbils after global ischaemia. Loss of cortical MAP-2 immunostaining has been reported as early as 3h after direct cortical impact injury (Postmantur et al., 1996) and fluid percussion injury (Taft et al., 1992) in the rat.

Evidence of early loss of postsynaptic elements, by way of disruption of MAP-2 immunostaining has been consistently demonstrated in the cortex of head-injured patients and in several anatomical brain regions after global ischaemia. Although individual deficits in a number of presynaptic proteins was detected in the cortex after head injury, this observation was not replicated after transient forebrain ischaemia in the mouse. Nevertheless, in agreement with the majority of previous studies, the dissociation of MAP-2 and presynaptic protein immunoreactivities suggest that presynaptic terminals are relatively resistant to acute brain injury compared with the postsynaptic sites.

4.3 GGF-2 after human head injury

A number of growth factor systems have been proposed to influence outcome after ischaemic and traumatic brain injury. One major growth factor signaling system is that involving the neuregulin family of peptides. The aim of the present study was to elucidate whether GGF-2, a member of the neuregulin family, existed in the adult human brain and to determine the level of GGF-2 in the cerebral cortex of patients who sustained a fatal head injury.

The present study provides the first demonstration that GGF-2 exists in the human brain. Moreover, Western blotting and immunohistochemical analyses indicated that GGF-2 immunoreactivity was significantly increased in the cingulate gyrus, but not the superior parietal cortex of head-injured patients compared with controls. Although there was no evidence of elevation of GGF-2 in the superior parietal cortex of the head-injured patients, when the group mean values were considered only one control case in the group exceeded the mean level of GGF-2 immunoreactivity in the head-injured group. Therefore, an anatomically widespread elevation of GGF-2 in the cerebral cortex of some head-injured patients cannot be excluded. Anatomical and inter-patient heterogeneity in the elevation of GGF-2 in the head-injured patients examined may be a reflection of the multiple types and severity of neuropathological features after head injury. Association of GGF-2 immunoreactivity with presence of a left SDH in head-injured patients, indicated increased GGF-2 immunoreactivity in patients who survived 24-96h after head injury. Ischaemic brain damage is one of the most common neuropathological features after a head injury (Graham et al., 1987). However, the presence or absence of ischaemic brain damage after human head injury, determined by histological assessment in blocks adjacent to those used for Western blotting, in the present study did not identify those patients with the greatest increase in GGF-2 levels. Thus, while ischaemia may be one, it is not the sole stimulus for the increase in GGF-2 after head injury. Eilam and co-workers, 1998, have shown that neuregulins along with the receptor ErbB4, is induced by three stimuli in the rat brain: activation of excitatory amino acid receptors, increased locomotor activity and tetanic stimulation of the perforant path. Activation of excitatory amino acid receptors has been proposed to be an important pathophysiological mechanism after head injury (Bullock, 1994; Zauner et al., 1996). Similarly, Ozaki and colleagues, 1997, reported that a neuregulin isoform induced expression of NMDA receptor subunits in the cerebellum. The heterogeneity of the GGF-2 response in the cingulate gyrus and the superior parietal cortex may be a consequence of variable levels of receptor activation in the cortex of head-injured patients. Increased expression of additional growth factors have been described acutely after global ischaemia (Takeda et al., 1993; Masamura et al., 1996), middle cerebral artery occlusion (Lin et al., 1998; Plate et al., 1999) and TBI (Logan and Berry, 1993; Hicks et al., 1997). Administration of peptide growth factors have been reported to improve functional outcome (Wen et al., 1995; Saatman et al., 1997) and/or reduced morphological damage (Hayashi et al., 1998; Justicia and Planas, 1999) in models of acute brain injury. It remains to be determined whether GGF-2 has therapeutic efficacy in animal models of ischaemia.

4.3.1 GGF-2 in white matter and in Alzheimer's disease

The GGF-2 response in the human brain was characterised in subcortical white matter from the same group of head-injured patients and controls and in the cingulate gyrus of AD patients and aged-matched controls. The observation that GGF-2 immunoreactivity was not increased in the corpus callosum of head-injured patients may support a primarily neuronal rather that a glial response. Indirect effects of neuronally expressed GGF-2 may be to regulate glial cell number, which in turn may provide trophic support for neurons. Several lines of evidence support the concept that GGF-2 acts as an indirect neurotrophin via induction of neurotrophin synthesis and secretion. For example, neurotrophin-3 was upregulated 40-fold in glial cells treated with GGF-2 and this response was blocked by antibodies to GGF-2 (Verdi et al., 1996). In retinal ganglion cells derived from rats at various stages of embryonic and postnatal development, GGF-2 acted directly to promote neuronal survival and neurite outgrowth (Bermingham-McDonogh et al., 1996). Anton and co-workers, 1997, demonstrated that GGF-2 signaling is involved in interactions between migrating neurons and glial cells in the developing cerebral cortex.

The role of growth factors in chronic neurodegeneration has been the subject of extensive investigation. The levels of NGF has been found to be increased in AD brains (Crutcher et al., 1993; Scott et al., 1995). In contrast, both BDNF and NT-3 are reduced in the cerebral cortex of AD patients compared with aged-matched controls (Narisawa-Saito et al., 1996). The present study provides no support for a role of GGF-2 in the pathophysiology of AD. Thus, the elevation of GGF-2 levels after head injury in non-acute brain injury may be a feature of the pathophysiological processes associated with acute brain injury, rather that the chronic degeneration of AD in elderly patients.

4.3.2 GGF-2 in animal models of acute brain injury

Potent therapeutic effects for NGF (Holtzman et al., 1996), bFGF (Koketsu et al., 1994), BDNF (Kiprianova et al., 1999), CNTF (Wen et al., 1995), HGF (Miyazawa et al., 1998), IGF-1 (Saatman et al., 1997) and TGF-α (Justicia and Planas, 1999) have been described in animal models of acute brain injury. The observation that GGF-2 was increased in both neurons and glial cells after permanent and transient MCAo in rats

(Soriano et al., 1998) is indicative of a possible role for GGF-2 in acute brain injury. Similarly in the present study, Western blotting analyses suggested that GGF-2 was increased in the ipsilateral and contralateral cortices after 4h SDH, and in the ipsilateral caudate and cortex after 24h permanent MCAo in the rat. The findings in both human head injury and animal models of cerebral ischaemia suggest that the GGF-2 response may contribute to a plastic response after acute brain injury, potentially providing an environment for neuronal survival and repair. While peptide growth factors stimulate different signal transduction mechanisms, their actions are likely to converge on common effector proteins. Stoop and Poo, 1996, speculated that these targets are protein kinases, downstream of proteins involved in trafficking, docking and exocytosis of synaptic vesicles. In agreement, some neurotrophic factors have been shown to have the capacity to enhance the efficacy of synaptic transmission (Kim et al., 1994; Kang and Schuman, 1995). Moreover, using rat cortical neurons (Takei et al., 1997) and *xenopus* neuromuscular cultures (Wang et al., 1995), some neurotrophic factors have been demonstrated to upregulate the expression of a number of presynaptic proteins. Whether GGF-2 has the capacity to upregulate synaptic proteins and contribute to presynaptic pathology after acute brain injury was beyond the scope of the present study.

In conclusion, the results from this thesis demonstrate that cortical presynaptic terminals are abnormal after head injury, in that subtle individual presynaptic protein alterations were identified. The fact that some presynaptic proteins remained unaltered may be a consequence of the function or quantity of individual proteins. Cortical cholinergic deficits existing after head injury are primarily due to damage to Ch4 neurons in the nbM, although loss of a proportion of cortical presynaptic terminals cannot be excluded. In contrast to the preservation of presynaptic terminals in animal models of acute brain injury, early and neuroanatomicaly widespread disruption of MAP-2 illustrates the vulnerability of postsynaptic elements compared with the presynaptic equivalents. A number of peptide growth factors are thought to have a presynaptic action. Whether GGF-2 acts at the presynaptic locus to protect presynaptic terminals from damage remains to be determined. However, the expression of neuronal nAChR channels during synaptogenesis in response to GGF-2 (Yang et al., 1998), suggests a role for GGF-2 at the presynaptic cholinergic terminal. Whether this is operational in the mammalian CNS in response to brain injury is not known.

4.4 Future work

This thesis has largely characterised presynaptic pathology after acute brain injury. Although, some evidence was presented providing an insight into a possible role of free radical-mediated damage in presynaptic terminal dysfunction, additional studies would be needed to explore this further. A likely approach would be preparation of cortical synaptosomes from human head injury material and exposing these to free radical generators, to establish whether free radical-mediated damage contributes to presynaptic protein loss after head injury. Exposure of cortical synaptosomes to peroxynitrite, followed by immunoprecipitation studies would identify molecular masses of synaptic proteins that are potential targets for peroxynitrite-mediated nitrosylation. Deficits in the cortical cholinergic system have been frequently reported after head injury and experimentally-induced brain damage. However, the presynaptic proteins examined are not restricted to the cholinergic terminals, but are homologous throughout all neurotransmitter systems. Autoradiographic analysis of [^{3}H]-citalopram binding in the cerebral cortex from controls and head-injured patients would provide another index of presynaptic terminal integrity, via measuring the levels of presynaptic serotonin uptake sites after head injury.

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Appendix 1: Histological assessment of neuronal damage

Haematoxylin and Eosin

Sections were dewaxed in histoclear for 10min and rehydrated in 99% and 95% alcohol for 3 and 2min respectively. After rinsing with water, nuclei were stained with haematoxylin for 10min. The haematoxylin stain was then blued in Scott's Tap Water Substitute for a few seconds followed by washing in water. Staining was examined microscopically and if necessary differentiated in acid alcohol, followed by a few dips in Scotts Tap Water Substitutes. Once adequate differentiation was achieved, sections were washed in water and stained with eosin for a maximum of 5min. Thereafter, sections were washed with water, followed by dehydration of sections in alcohol and cleared in histoclear prior to mounting with DPX.

Luxol Fast Blue / Cresyl Violet

Sections were dewaxed in histoclear, rehydrated in 95% alcohol and stained in 0.1% luxol fast blue for 2h in an oven at 60°C. Remove excess staining with 95% alcohol and rinse sections in water. Staining was differentiated in 0.05% aqueous lithium carbonate and differentiation continued in numerous changes of 70% alcohol until grey matter could be distinguished from white matter. After washing, differentiated sections were stained with cresyl violet for 5min and rinsed in running water. Staining was differentiated in 95% alcohol containing a few drops of acetic acid, prior to dehydration, clearing and mounting with DPX.

Appendix 2: Focal Cerebral Ischaemia in the Rat: Permanent Middle Cerebral Artery Occlusion - Intraluminal Thread Model

Four male Sprague-Dawley rats (270g-330g) were anaesthetised using 4% halothane in a mixture of 70% nitrous oxide and 30% oxygen. Animals were orally intubated and mechanically ventilated with halothane concentration reduced 1.5%-2%. The femoral arteries were cannulated to enable monitoring of arterial blood pressure and sampling for blood gas analysis. Body temperature was maintained at 37°C by means of a rectal temperature probe and heating lamp.

Middle cerebral artery occlusion was performed using a modification of the method used by Zea Longa et al., (1989). Briefly, after coagulation by diathermy of the maxillary, lingual, occipital branches of the carotid arteries, the pterygopalantine was tied off with 6-0 suture and microvascular clips applied at the bifurcation of both the common and internal carotid arteries and the internal and pterygopalantine. A 3-0 silk suture (with rounded tip) was inserted into the external carotid, tied loosely in place, and advanced into the internal carotid until (removing clips as required) until resistance was achieved (approximately 22mm). The filament was tied off and diathermied to secure it in place and the animal was allowed to recover.

Appendix 3: Global Cerebral Ischaemia in the Mouse: Bilateral Common Carotid Artery Occlusion

In thirteen C57BL/6 mice, surgical anaesthesia was induced with 3% and maintained with 1.5% halothane delivered in 70% nitrous oxide / 30% oxygen via a face mask. A rectal probe was inserted and core body temperature was maintained at 37°C using a heating lamp. Bilateral common carotid artery occlusion was achieved using the technique of Barone et al., (1993). A ventral midline incision was made and the common carotid arteries were isolated by clearance of muscle and connective tissue. The carotids were elevated and occluded bilaterally for 17min with microaneurysm clips. The duration of ischaemia was based on previous observations made in a study of neuronal damage following different durations of global ischaemia in C57BL/6 mice. Following occlusion, the clips were removed and the incision was sutured closed using a 6-0 silk suture. The procedure for the SHAM animals (n=5) was identical, with the exception of applying the clips.

Animals were placed in an incubator for 2h and allowed to recover for 1 (n=3), 3 (n=4) and 10 days (n=1) and observed daily. At each end point, mice were reanaesthetised and transcardially perfused via the left ventricle with heparinised 0.9% NaCl followed by 4% paraformaldehyde in phosphate buffer. The brain was then postfixed in the skull in 4% paraformaldehyde for a further 24h. Following this, the brain was removed from the skull and washed in phosphate buffer for 2h.

Appendix 4: Subdural Haematoma in the Rat

Nine adult male Sprague-Dawley rats (weight 350-450g) were anaesthetised using 4% halothane in a mixture of 70% nitrous oxide and 30% oxygen. A tracheotomy was performed and the animals were mechanically ventilated, with halothane reduced to 1%. Both femoral arteries were cannulated to monitor arterial blood pressure and sampling for blood gases analysis. Venous cannulae were placed to allow autologous blood to be drawn. Core body temperature was maintained at 37°C by means of a homothermic blanket controlled by a rectal thermometer.

A subdural haematoma was induced using a method described previously (Horsburgh et al., 1997). Briefly, the animals were placed in a stereotaxic frame, the skull exposed and a burr hole was drilled 2mm to the left and 1mm posterior of Bregma. The dura was incised and a blunt J-shaped needle was inserted into the subdural space. The subdural haematoma was produced by slowly injecting 0.4ml of non-heparinised freshly drawn autologous venous blood into the subdural space over a period of 7min. SHAM-operated animals (n=5) underwent the same surgical procedures, without production of a haematoma.

Appendix 5: Focal Cerebral Ischaemia in the Rat: Middle Cerebral Artery Occlusion - Diathermy Model

Four adult male Fisher 344 rats were allowed food and water ad libitum and maintained under standard conditions. Rats were anaesthetised with 5% halothane in a mixture of nitrous oxide/oxygen (70:30). After non-surgical oral intubation, animals were mechanically ventilated and the halothane concentration was reduced to 1.5%. The femoral artery was cannulated to enable monitoring of blood pressure and measurements of blood pH, pCO_2, pO_2. During surgery, body temperature was maintained at 37°C using a rectal thermometer and heating lamp.

The MCA was permanently occluded using the method of Tamura et al., 1981. Briefly, the left temporalis muscle was dissected and a craniectomy was performed at the level where the middle cerebral artery crosses the lateral olfactory tract. The MCA was occluded with micropolar forceps on a low power setting using continuous saline irrigation (n=2). In an identical surgical procedure, SHAM-operated animals (n=2) had the MCA exposed but not occluded. The wound was sutured and the rats were allowed to recover for 24h after surgery. Rats were then deeply anaesthetised with 5% halothane and decapitated.