CHARACTERISING THE ROLE OF SUBSTANCE P IN ACUTE ISCHAEMIC STROKE

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CHAPTER 1:

INTRODUCTION AND LITERATURE REVIEW

1.0 Introduction

Stroke is a major health problem in Australia and other western nations and is a leading cause of morbidity, mortality and dementia. Specifically, each year a staggering 15 million people will suffer a stroke of which 5 million die and 5 million are left permanently disabled (World Health Organisation, 2002). As a result, the social and economic cost of stroke is enormous. In Australia alone, the cost of hospitalisation, treatment and rehabilitation of stroke patients is estimated at $1.2-1.7 billion per year (Dewey et al., 2001). At present, thrombolysis with tissue plasminogen activator (tPA) within 3 h of symptom onset is the only approved stroke therapy. However, there are concerns over the potential neurotoxicity of tPA. Clinical and experimental research has demonstrated that significant blood brain barrier (BBB) disruption and cerebral oedema occur following stroke (Ayata and Ropper, 2002). Also, the discovery of potentially salvageable tissue, referred to as the penumbra, has been demonstrated in both clinical and experimental stroke. These phenomena have generated much interest and an intense research effort directed at elucidating a therapy that will decrease the amount of brain damage and improve functional outcome following stroke. The current thesis demonstrates that neuropeptides, and in particular substance P (SP), play a central role in the development of BBB disruption, oedema and functional deficits following ischaemic stroke. Moreover, administration of a SP antagonist significantly improved survival and outcome even when administered up to 8 h after stroke onset, and has a synergistic effect with tPA. This introduction will first describe stroke and its known pathophysiology before considering in detail the potential contribution of SP to this process.

1.1 Epidemiology

Stroke is the leading cause of disability, the second most common cause of dementia and the third leading cause of death worldwide behind cancer and ischaemic heart disease (Bakhai, 2004; Warlow, 1998; Williams et al., 1999). The incidence of mortality from stroke in Australia is 53/100 000 (Australian Bureau of Statistics). Specifically, over 53 000 individuals suffer a stroke each year, which equates to one stroke every 10 mins (National Stroke Foundation, 2004). The implications of stroke include long-term disability or death, as well as economic burdens that must be met by family, insurance companies, and local and federal healthcare (Barnett, 1982). Although the incidence of stroke at present appears to be stable and stroke mortality is declining, it is likely that the full magnitude of stroke burden will only be seen in future years as our population ages (Australian Bureau of Statistics; Broderick et al., 1992; Sims and Anderson, 2002). Indeed, if nothing is done to prevent stroke then it is likely that the incidence will increase to over 74 000 strokes per year in Australia by 2017 (National Stroke Foundation, 2004).

1.2 The Cerebral Vasculature

Stroke results from a permanent or transient decrease in cerebral blood flow (CBF), most commonly resulting from the transient or permanent occlusion of an artery within the brain (De Girolami et al., 1999; Sims and Anderson, 2002). Under normal conditions the brain is supplied with oxygenated blood via the common carotid and vertebral arteries. The common carotid artery (CCA) branches into the external carotid artery (ECA) that supplies the face, scalp and neck, and also into the internal carotid artery (ICA) that supplies the anterior portion of the brain. The

vertebral arteries supply the posterior portion of the brain. The anterior and posterior circulation connects in the circle of Willis at the base of the brain. Blood permeates the brain via a network of capillary vessels that delivers oxygen (O_2) and nutrients whilst also removing cellular metabolic waste. Obstruction of arterial flow at any point leads to tissue ischaemia and eventually infarction in the brain area supplied by that vessel (Fitzgerald and Folan-Curran, 2002; Hademenos and Massoud, 1997).

The brain is a highly aerobic tissue that almost completely relies on oxidative phosphorylation for the generation of energy in the form of adenosine triphosphate (ATP) (Ross et al., 2003; Saladin, 2001; Zauner and Muizelaar, 1997). It requires a constant supply of O_2 and glucose, utilising approximately 20 percent of the O_2 available within the body and 20 percent of the cardiac output (Hutchinson and Acheson, 1975; Sherwood, 1997; Zauner and Muizelaar, 1997). CBF rates are estimated to be between 60-70ml/100g/min (Lo et al., 2001). Carbon dioxide produced from aerobic oxidation is easily eliminated from the brain via the BBB, however lactic acid generated in anaerobic glycolysis is toxic to neurons and causes pH to decrease (Brodal, 1998; Nolte, 1999; Zauner and Muizelaar, 1997). Although complex and sophisticated in its structure and function, the brain is highly sensitive to insults and has a limited capacity to undergo repair, such that generally, damaged neurons are lost, which is of major concern following cerebral insults such as stroke (Fitzgerald and Folan-Curran, 2002; Gamache, 1987).

1.3 Types of Stroke

Stroke, sometimes referred to as a "brain attack", is a medical emergency requiring immediate attention. It is a clinical syndrome of rapidly developing symptoms or signs of focal/global loss of cerebral function with no apparent cause apart from vascular origin (Warlow, 1998). By definition, symptoms last more than 24 h or lead to death (Warlow, 1998). These symptoms vary depending upon the specific brain region involved but may include, amongst others (De Girolami et al., 1999; Jankovic, 1982):

- sudden weakness or numbness in face/arm/leg on one side
- sudden loss/blurring/dimness of vision
- mental confusion, loss of memory or sudden loss of consciousness
- slurred speech, loss of speech or problems understanding others
- sudden severe headache with no apparent cause
- unexplained dizziness, drowsiness, incoordination or falls
- nausea and vomiting, especially when associated with any other symptoms

There are 2 broadly defined and clinically recognised types of stroke, ischaemic and haemorrhagic, with lacunar strokes also comprising a small subset (Hademenos and Massoud, 1997).

1.3.1 Ischaemic Stroke

Ischaemic stroke accounts for approximately 85 percent of all strokes and arises from a blockage of a cerebral blood vessel by a thrombus or embolus (Hademenos and Massoud, 1997). The thrombus or embolus impedes CBF and renders the tissue supplied by that vessel ischaemic. Of the 2 causes, thrombosis is the most common.

Thrombosis is the mechanism responsible for normal blood clotting and occurs when a blood clot forms along the wall of a major cerebral artery. It is most likely to occur in arteries narrowed by atherosclerosis as the rough and uneven surface of the plaque provides a perfect site for the formation and growth of blood clots. The thrombus itself is an aggregation of platelets and fibrin formed in response to vessel injury or atherosclerosis, although may also occur in uninjured vessels (Hademenos and Massoud, 1997). In contrast, an embolic stroke is most often attributed to an embolus of cardiac origin (Meyer, 1982) and may comprise of a blood clot, piece of atherosclerotic plaque, an air bubble or even fat from the marrow of a broken bone. The embolus is transported in the bloodstream through the cerebral circulation until it reaches a vessel too small for further propagation, where it remains and obstructs the flow of blood through the vessel (Hademenos and Massoud, 1997). Embolism commonly occurs in the setting of atrial fibrillation (AF), myocardial infarction, heart valve disease, hypertension and diabetes. The territory supplied by the middle cerebral artery (MCA) is where embolism most commonly occurs (Jankovic, 1982).

1.3.2 Haemorrhagic Stroke

Haemorrhagic stroke accounts for approximately 15 percent of all strokes and may be classified into two sub-types: intracerebral haemorrhage (ICH) and subarachnoid haemorrhage (SAH; De Girolami et al., 1999; Hademenos and Massoud, 1997). Cerebral vessel walls may become fatigued and abnormally weak, leading to vessel rupture and the seeping of blood into the surrounding area (Hademenos and Massoud, 1997). Bleeding may either be into the brain itself, as in the case of ICH, or into the space between the brain and the skull, as in the case of SAH (De Girolami et al., 1999; Jankovic, 1982). SAH is most commonly associated with

rupture of a berry aneurysm or arteriovenous malformations, whilst ICH is associated with hypertensive arteriolar disease, amyloid deposition, haemorrhagic transformation of an infarct and arteriovenous malformations.

1.3.3 Lacunar Stroke

Lacunar stroke is characterised by the presence of lacunes, or "lake-like" cystic micro-infarcts less than 2cm in diameter, that occur in the lenticular nucleus, thalamus, internal capsule, deep white matter, caudate nucleus and pons. Depending upon their location they may be clinically silent or cause severe neurologic impairment. Lacunar stroke commonly occurs in the setting of hypertension (De Girolami et al., 1999; Jankovic, 1982).

1.3 Outcome of Stroke

The outcome of stroke depends primarily upon which area(s) of the brain are affected and hence varies in severity from recovery in a day, through incomplete recovery to severe disability to death. In addition, prognosis depends on the type of stroke and is influenced by many neurological, functional and psychosocial factors (Stineman et al., 1997; Warlow, 1998). Generally, the effects of stroke on the individual are devastating and may include impairment to the senses, motor and cognitive skills and behaviour. Williams and colleagues interviewed stroke survivors and found that the factors commonly affected following a stroke were energy, family roles, language, mobility, mood, personality, self care, social roles, thinking, vision, upper extremity function and productivity. Other long-term complications included seizures, impaired concentration, emotional instability, depression, poor judgement, erratic sleep cycles and loss of libido (Williams et al.,

1999). A long hospital stay following stroke may also result in the development of aspiration pneumonia, bedsores, urinary tract infection, deep vein thrombosis, limb contractures and incontinence. Thus, apart from the devastating mortality, the morbidity amongst stroke survivors is substantial (Sacco et al., 1997). In long-term survivors (ie; greater than six months) nearly half have hemiparesis, over one fifth can't walk, over half are completely or partially dependent in regards to daily living, nearly one fifth are aphasic and nearly one third are clinically depressed (Wilkinson et al., 1997). Follow up of stroke units reveal that half of all stroke patients are dead by 5 years and only 10-20 percent are alive at 10 years (Gladman, 2000). These figures clearly emphasise that it is not only stroke survival that is important but also rehabilitation and improvement in quality of life. Thus, timely appropriate medical intervention accompanied by rehabilitation gives patients the best chance of a meaningful recovery.

1.3.1 Mortality

The mortality rate of stroke differs according to stroke type and severity. ICH carries a very high overall mortality rate of 50-70 percent. SAH commonly occurs in the setting of hypertension and has a 1 yr survival rate of 50 percent, much higher than ischaemic stroke. ICH tends to occur without warning, however the haematoma resorbs and patients who survive the acute period may have a surprisingly complete neurologic recovery (Jankovic, 1982). Embolic stroke is associated with a 20-30 percent mortality rate and thrombotic stroke a 30-40 percent mortality rate (Meyer, 1982). African Americans are twice as likely to die of a stroke compared to Caucasians (Sacco et al., 1997), an excess that occurs mainly at young ages (Howard et al., 1994a). Mortality from stroke is greater in men than

women in most countries including Australia, however the differences narrow greatly in the over 75 years age group. In Australia from 1990-1994 the overall stroke mortality rate within the 35-74 years age group was 45/100 000 for men and 31/100 000 for women; in the 75-84 years age group the mortality rate was 807/1000 for men and 690/100 000 for women. Despite this, there has been a decline in most western nations, including Australia, in stroke mortality since the beginning of the 1990s (Sarti et al., 2000). There is evidence suggesting that a decrease in some environmental factors such as dietary salt and saturated fat intake may have contributed to the decrease in stroke mortality. Efforts directed towards the detection and effective control of hypertension may have also contributed to the observed decline in stroke mortality through decreasing the incidence and severity of acute stroke events (Sarti et al., 2000). However, with the incidence of diabetes, abdominal obesity and alcohol consumption increasing, in addition to our aging population, stroke will remain a major heath concern for years to come. Overall, approximately 20 percent of patients having their first stroke are dead within a month and one third of those alive at 6 months are dependent for daily living (Warlow, 1998).

1.4.1 Factors Influencing Outcome Following Stroke

The following factors may influence the natural history of neurologic recovery and mortality in stroke: type and severity of stroke, age of the patient, presence of associated risk factors (discussed in 1.4), associated complicating disorders, and severity and persistence of neurologic deficit. Older patients are less able to recover from stroke as age negatively influences prognosis. This is due to the progressive loss of cerebral neurons that occurs with atrophy of the brain, loss of

neuroplasticity, loss of elasticity, atherosclerotic changes of the cerebral vessels that limit the capacity to develop collateral circulation, shrinking of dendritic arbour with increasing age and decreased synthesis of neurotransmitters with aging. In comparison, younger individuals often make a gratifying recovery. History of transient ischaemic attacks (TIAs), prior stroke, hypertension, diabetes mellitus, hyperlipidaemia, heart disease, obesity and smoking, alone or in combination, adversely affect post-stroke outcome, as do complicating disorders such as pneumonia, pulmonary embolism, heart failure, dehydration and electrolyte imbalances. Those individuals presenting in a coma or decerebrate state have a poor prognosis for survival. Neurological deficits that persist for greater than 6 months are considered permanent. Therefore, emphasis should be on early physical, occupational, rehabilitative and speech therapy and these should be continued for as long as there is improvement in adjustment to daily living (Meyer, 1982).

1.3.2 Rehabilitation

Stroke is often more disabling than fatal, and survivors require rehabilitation and support to re-integrate into the community (Kotila et al., 1998; Sacco et al., 1994). Rehabilitation is the active promotion of recovery and is a labour intensive process (Gladman, 2000). It starts immediately following hospital admission and in the short-term aims to decrease stroke complications such as stiffening of the limbs and deep vein thrombosis. In the long-term, rehabilitation aims to restore mental and physical function, allowing individuals to adapt to their disability and return to an active life whilst also preventing further strokes. Optimal recovery depends upon the patients' determination, the support of family and friends and the integrated efforts of specialists. Exercise can improve the range of motion and strengthen

weak muscles, restoring function as much as possible. Occupational therapy teaches patients new ways to perform day to day activities made difficult because of the disability, such as writing, bathing, cooking and job related activities. Speech pathology helps patients to regain lost swallowing ability and language skills. The patients' house may need to be modified to allow them to live with their disability. At a minimum, stroke survivors need to be able to eat, groom, dress their upper body without help, transfer from bed to chair without physical assistance, propel a wheelchair or walk 50 feet, and manage bowel and bladder functions (Stineman et al., 1997). There is a relatively rapid phase of recovery within the first few weeks of stroke, although it is also during this period that most deaths occur. The majority of recovery occurs within 3 months (Stone et al., 2000).

1.3.3 Depression

Depression is an important consequence of stroke as it influences recovery. Kotila and colleagues (Kotila et al., 1998) found that the incidence of depression is high in stroke survivors and their caregivers. Depression was more prevalent in areas without active after-discharge rehabilitation programs, suggesting that modest after-discharge rehabilitation programs that facilitate patients' return to society and aid in developing new social and professional contacts with people is an effective means of decreasing the burden of depression in patients and their caregivers. Following hospitalisation and rehabilitation, approximately 80 percent of stroke survivors return to the community where they rely on family members' emotional, informational and instrumental support for daily living. Stroke caregivers are faced with the patients' difficulty in mobility, self-care, communication, cognitive impairment, depression and personality changes (Han and Haley, 1999). There is a

need for more tailored programs of treatment and community support services to assist patients in achieving basic self-care and independent living goals (Stineman et al., 1997).

1.3.4 Physiological Predictors of Post-Stroke Outcome

Many physiological factors influence outcome following stroke and the presence or absence of certain conditions may worsen outcome. Hypertension, hyperglycaemia, hyperthermia and increased intracranial pressure (ICP) are all independent indicators of poor prognosis (Bath, 2000; Hill and Hachinski, 1998).

Blood Pressure

Acute rises in blood pressure (BP) occur following both haemorrhagic and ischaemic stroke. Hypertension is present in approximately 75 percent of stroke patients and develops rapidly following stroke onset. It is linked to poor outcome as it promotes early re-infarction, symptomatic haemorrhagic transformation and cerebral oedema. However, hypotension is also linked to poor outcome, although its role is less recognised. Hypotension is commonly a marker of serious co-morbid disease, such as ischaemic heart disease, which may contribute to poor outcome. Cerebral perfusion is also decreased in hypotension and this may cause extension of the infarct (Bath, 2000; Hill and Hachinski, 1998). Generally, trials recommend that BP be left alone unless it reaches extreme levels, as lowering of BP worsens outcome in experimental stroke (Green, 2002). Treatment to lower BP is also recommended in patients with complicating medical problems such as heart failure or ischaemic heart disease, those who have signs of accelerated hypertension and those who have deteriorated secondary to re-infarction or continued bleeding. BP

falls during the first week post-stroke, and approximately 30 percent of patients will become hypertensive. Such hypertension should be treated (Bath, 2000; Hill and Hachinski, 1998).

Blood Glucose Levels

Hyperglycaemia may be seen in all types of stroke and is observed in approximately 20-50 percent of all acute stroke patients. Animal models first suggested that hyperglycaemia worsens outcome, with human studies supporting that high blood glucose levels (BGL) are associated with increased mortality and worsened functional outcome, regardless of stroke type (Green, 2002). The mechanisms by which hyperglycaemia may worsen outcome include increasing infarct size, haemorrhagic transformation, blood brain barrier damage, increased cerebral oedema, decreased regional CBF and decreased oxidative metabolism that increases anaerobic glycolysis and subsequently causes lactic acidosis (Bath, 2000). Such observations indicate that reduction of BGL would improve outcome. However, no large trails have assessed normalisation of BGL with insulin in stroke, despite benefits being demonstrated in several myocardial infarction trials (Bath, 2000; Hill and Hachinski, 1998).

Temperature

Hyperthermia, a temperature above 37.5ºC, is common in acute stroke and is evident in approximately 35-60 percent of patients (Green, 2002). Experimental models have demonstrated that hyperthermia is a critical factor in determining infarct size, with even delayed hyperthermia worsening neuronal damage. Early hyperthermia also appears to be important in human stroke in terms of prognosis

(Bath, 2000; Hill and Hachinski, 1998). Hyperthermia increases cerebral metabolism, thereby increasing O_2 requirements, CBF and ICP, and is thus associated with poor outcome. Also, higher glutamate and glycine concentrations are present in the cerebrospinal fluid of hyperthermic patients, indicating that excitotoxicity may contribute to the adverse effects of hyperthermia (Green, 2002). The benefits of hypothermia are well established in experimental models of both permanent and transient ischaemia, showing that lowering of the core temperature reduces stroke lesion size. However, evidence for the effectiveness of hypothermia in the clinical setting is limited. Despite this, those patients presenting with a lower body temperature upon hospital admission have a favourable outcome compared to those with a higher temperature (Green, 2002). Despite there being no clinical trial data available for hypothermia in stroke, evidence from TBI suggests that lowering systemic temperature to 32-33ºC leads to a reduction in ICP and brain damage, thereby improving outcome. The mechanisms by which hypothermia is believed to be neuroprotective is through reduction of cerebral metabolic rate, excitiatory amino acid levels, ICP and oedema formation as well as stabilisation of the BBB and cellular membranes (Ginsberg, 2003).

Intracranial Pressure

Stroke is associated with both cytotoxic and vasogenic oedema, which produces brain swelling, inducing pressure gradients that may lead to brain herniation. Local pressure of the vasculature onto other parts of the brain can also cause infarct extension. Increased ICP is a common complication following both haemorrhagic and ischaemic stroke, usually secondary to cerebral oedema formation (Hill and Hachinski, 1998). There are many techniques that may be employed for reducing

ICP including head elevation, glycerol, mannitol, diuretics, steroids, hyperventilation, barbituates, hypothermia, muscle relaxants and decompressive craniectomy. Efficacy of the majority of techniques is largely unknown due to the absence of randomised trials (Bath, 2000). Steroids are effective in reducing vasogenic oedema but not cytotoxic oedema (Gamache, 1987; Yatsu et al., 1987). Barbituates have a proven benefit in that they decrease the cerebral metabolic rate and preserve cellular bioenergetic status. There is also a beneficial redistribution of the CBF where blood flows from non-ischaemic zones to areas of ischaemia. However, barbituates have only been shown to be useful in some clinical settings (Gamache, 1987; Yatsu et al., 1987). Generally, such measures are largely ineffective, however this is discussed further in Chapter 1.6.5.

1.3.5 Measures of Post-Stroke Outcome

Measures of post-stroke outcome are essential to assess patients' quality of life but also the efficacy of novel treatments in clinical trials. Examples of clinically used outcome scales include the Modified Rankin Scale (MRS), National Institute of Health Stroke Scale (NIHSS), the Barthel Index (BI) and the Glasgow outcome scale (GOS).

Modified Rankin Scale

The MRS is a 6-point scale that assesses functional disability following stroke. Patients are ranked from a score of 0 indicating death to a score of 5 indicating severe disability. The MRS has established validity and reliability (Foell et al., 2003).

National Institute of Health Stroke Scale

The NIHSS assesses neurological impairment following stroke. The NIHSS is a 15-point scale which can be carried out easily and quickly at the patients bedside and is reported to adequately measure subtle and complex behavioural signs. It is an extensive neurological assessment covering numerous aspects of functional outcome including motor function, language, level of consciousness and vision, and accordingly is regarded to have acceptable validity and reliability (Foell et al., 2003). A score of less than 8 indicates mild stroke, a score of 8-15 indicates moderate to severe stroke, and a score greater than 15 indicates severe stroke (Brott et al., 1989). Outcome at 3 months has been found to correlate well with infarct volume.

Barthel Index

The BI assesses activities of daily living (Panicker et al., 2003). A score of less than 41 indicates severe disability, 41-60 indicated moderate disability and a score greater than 60 indicates mild disability. BI score on day 1 post-stroke has been shown to correlate well with that at 6 months.

Glasgow Outcome Scale

The GOS is a 5-point classification that assesses functional status following brain damage. Individuals are assigned to the categories death, persistent vegetative state, severe disability, moderate disability and good recovery depending upon their dependence for daily living and the presence of disability (Jennett and Bond, 1975).

1.4 Stroke Risk Factors and Prevention

1.4.1 Risk Factors

Many risk factors may increase the likelihood of having a stroke and the risk factors for ischaemic and haemorrhagic stroke differ. Some, but not all, risk factors may be decreased through dietary and lifestyle adjustments or medication.

Non-Modifiable Risk Factors

Age, gender, race and heredity have all been identified as non-modifiable risk factors for stroke. Although they are unable to be modified, recognising them enables identification of those individuals most at risk and more vigorous treatment of those risk factors that can be modified (Sacco et al., 1997).

Age is the single most important non-modifiable risk factor, with stroke risk doubling with every decade over 55 years of age. However, not all strokes occur in older individuals, with nearly 30% of strokes victims are under 65 years of age (Australian Bureau of Statisitics). Also, the risk of stroke is more than 30% greater in men compared to women. Family history greatly increases the chances of having a stroke and has long been recognised as a risk factor (Liao et al., 1997; Rothwell, 2000; Sacco et al., 1997). This increased risk may be due to genetic determination of other risk factors and common familial exposure to environmental and lifestyle factors (Sacco et al., 1997). Finally, the incidence of stroke varies greatly among racial groups. Stroke is more common in African Americans than Caucasians, and in Asian communities compared to western communities (Broderick et al., 1992; Longstreth et al., 1992; Sacco et al., 1997).

Potentially Modifiable Risk Factors

Epidemiological evidence shows that stroke is preventable, and that lifestyle adjustments and medication can significantly decrease stroke risk (Ebrahim, 2000). The risk factors discussed below are potentially modifiable.

Hypertension

Hypertension is the single greatest modifiable risk factor for ischaemic stroke (Fournier et al., 2004; Hansson et al., 1998; Whisnant, 1996). It trebles the risk of stroke and is believed to contribute to up to 70% of all strokes (Venables, 2000; Wolf et al., 1991). Hypertension can be treated with lifestyle adjustments and medication, thereby reducing stroke risk as well as reducing the incidence of other cardiovascular events (Hansson et al., 1998).

Cardiac Abnormalities

Individuals with coronary heart disease are twice as likely to suffer a stroke because heart conditions increase the risk of embolus formation. Cardiac valve abnormalities, such as mitral valve stenosis and mitral annular calcification, are associated with atrial fibrillation (AF; Benjamin et al., 1992) and have been found to be important risk factors for stroke (Sacco et al., 1997; Wolf, 1998). AF is a heart rhythm disorder where the atria beat quickly and non-rhythmically, leading to incomplete emptying of the atria, which may lead to clot formation and therefore stroke (De Girolami et al., 1999; Sherwood, 1997). AF is associated with a 3-5 fold increased stroke risk. Conditions such as previous heart attack, heart failure and valvular disease are also associated with embolism.

Diabetes Mellitus

Diabetics have a 3-fold increased risk of thromboembolic stroke compared to the general population (Burchfiel et al., 1994). The increased risk may be explained by a greater susceptibility to atherosclerosis and an increased prevalence of atherogenic risk factors such as hypertension, obesity and abnormal blood lipids (Burchfiel et al., 1994).

Smoking

Smoking is associated with a 50 percent increased risk of stroke, showing a clear dose-response relationship (Shinton and Beevers, 1989). The risk is increased through acceleration of atherosclerosis, as well as promoting clot formation, causing transient increases in BP and a build up of carbon monoxide that reduces the O_2 carrying capacity of the blood and O_2 delivery to the brain (Kawachi et al., 1993). The increased risk of stroke is not surprising given that smoking is an established cause of atherosclerotic disorders such as myocardial infarction and vascular disease (Shinton and Beevers, 1989). Although the increased risk of stroke remains for 2-4 years, giving up smoking markedly reduces stroke risk (Kawachi et al., 1993; Wolf et al., 1988).

Lipids/Cholesterol

There is evidence that very low cholesterol levels weaken the endothelium of intracerebral vessels and may lead to haemorrhagic stroke in hypertensive individuals (Iso et al., 1989). Moreover, the rate of intracerebral haemorrhage is increased with cholesterol levels less than 4.14 mmol/L (Iso et al., 1989). High density lipoproteins (HDL) cholesterol has been found to be protective for the

development of atherosclerosis, whereas total low density lipoprotein (LDL) cholesterol promotes atherosclerosis (Sacco et al., 1997). High blood cholesterol is also an independent risk factor for ischaemic stroke and a reduction in blood cholesterol through the use of statins produces a reduction in stroke risk (Ebrahim, 2000).

Obesity

Obesity carries an increased risk of stroke as it is associated with higher BP, BGL and atherogenic serum lipids, all of which are independent risk factors. A relative weight which is 30 percent above average significantly contributes to the incidence of brain infarction in men between 35-64 years and women between 65-94 years. Central obesity (ie; abdominal fat) is related to the occurrence of atherosclerotic disease and is more dangerous than other fat deposits. Moderate levels of physical activity are associated with a decrease in the incidence of coronary heart disease (Sacco et al., 1997).

Transient Ischaemic Attacks

A transient ischaemic attack (TIA) is an episode of temporary neurologic dysfunction of vascular origin, with rapid onset followed by a swift and complete resolution, without evidence of acute infarction. Typically, symptoms resolve in an hour, however the clinical definition is resolution within 24 h (Barnett, 1982; Jankovic, 1982). TIAs are a significant independent risk factor for stroke (Howard et al., 1994b; Venables, 2000) and are an important warning sign of impeding stroke (Jankovic, 1982). Those individuals who have previously suffered at least 1 TIA have a 10-fold increased risk of stroke (Johnston and Hill, 2004), with

approximately one third of people suffering a TIA going on to have a stroke within 5 years (Sacco et al., 1997).

Other medical and physical conditions that may increase the risk of stroke include sleep apnoea, pregnancy and sickle cell anaemia, in addition to homocysteine, left ventricular hypertrophy, recent large myocardial infarction, infective endocarditis, sickle cell disease, hypercoaguability, stress, dietary factors, high-oestrogen containing oral contraceptives and alcohol (Sacco et al., 1997; Wolf, 1998).

1.4.2 Prevention

Antiplatelet Therapy: Prevention of Stroke

Aspirin is the most widely used antiplatelet agent (Alberts et al., 2004) and it irreversibly and selectively inhibits cyclo-oxygenase (COX) directed breakdown of arachidonic acid, thereby preventing subsequent thromboxane A2 production which is a potent platelet aggregator and vasoconstrictor (McCabe and Brown, 2000). COX-1 is constitutively expressed in the endoplasmic reticulum of most cells and synthesises prostaglandins related to normal cellular functions such as gastric mucosal production, maintenance of renal blood flow and regulation of platelet activation and aggregation. Inhibition of the latter is how aspirin acts to decrease stroke risk. COX-2 is not routinely expressed in most cells but is rapidly induced by inflammatory stimuli and growth factors leading to prostaglandin production that contributes to the inflammatory immune responses (Wolf, 1998; Awtry, 2000). Aspirin is considered a safe and effective stroke therapy, however it has limited efficacy showing a relative risk reduction of 20-25 percent for ischaemic stroke. The British Physicians Society (Peto et al., 1988) found aspirin to significantly

decrease the incidence of transient ischaemic attacks, but with no reduction in stroke risk and an increased risk of disabling stroke. The Physicians Heath Study, found aspirin to increase stroke incidence, primarily of the haemorrhagic type. Overall, the data regarding the use of aspirin for the primary prevention of stroke is not encouraging (Awtry and Loscalzo, 2000), and there is insufficient evidence to recommend its' widespread regular prophylactic use for the primary prevention of stroke (McCabe and Brown, 2000).

Surgical Intervention

Carotid endarterectomy is the surgical cleaning of the arteries in patients with at least one carotid artery displaying atherosclerotic narrowing. Surgery is not suitable for all patients with arterial narrowing, being most effective in patients with mild to moderate narrowing (Dickerson et al., 2007). It has been criticised by many who believe that the risk of complication doesn't warrant the one percent reduction in absolute stroke risk. Nonetheless, it is effective in carefully selected patients (Wolf, 1998; Ebrahim, 2000).

1.4.3 Recurrent Stroke

As mortality from stroke decreases and life expectancy increases, there will be an increased number of people with recurrent stroke (Dickerson et al., 2007). Recurrent stroke is a frequent contributor to mortality from stroke (Warlow, 1998) with risk of death or recurrence after stroke being substantial and greatly influenced by sex, cardiac co-morbidity and pre-existing hypertension (Sacco et al., 1982). Almost one third of recurrent strokes in a 2 yr follow-up occurred within 30 days. Such early recurrence increases motor weakness scores, early mortality and duration

of hospital stay. The rates of recurrent stroke are between 4-14 percent per year and it has been suggested that hypertension and cardiac disease are risk factors for increased recurrent stroke risk (Sacco et al., 1997). Cumulative 5 yr recurrence rate for infarction was almost doubled in men compared to women. Recurrences are typically of the same type as the initial stroke (Sacco et al., 1982).

1.5 Neuropathology and Pathophysiology of Stroke

The extent of injury to the brain following ischaemia is dependent upon the severity of CBF reduction, the duration of ischaemia and the adequacy of collateral flow. Other factors such as age, body temperature, BGL and atherosclerosis may also affect stroke outcome as discussed earlier (Sacco et al., 1997).

1.5.1 Cerebral Blood Flow and Perfusion Reserve

The CNS vasculature is a unique high flow-low pressure perfusion system that preserves blood flow to critical structures and contains networks of microvessels that allow for reversal of flow should obstructions occur. There is also redundancy in the cerebral arterial supply that serves a protective role, however the adequacy of the collateral flow depends upon absence of injury within the arterial supply and target microvessels. The CBF is normally maintained relatively constant by autoregulation (del Zoppo and Hallenbeck, 2000; Fitzgerald and Folan-Curran). Autoregulation is the capacity for the cerebral circulation to maintain constant blood flow under conditions of changing pressure. This phenomenon is observed by vasoconstriction in response to raised intravascular pressure, and vasodilation in response to lowered intravascular pressure. Obviously, there are upper and lower limits of distal BP beyond which autoregulation is no longer effective (Symon,

1987). Under conditions of reduced CBF, the brain is also able to alter the amount of O_2 extracted from the circulation. Normally, the brain extracts approximately 35 percent of the delivered O_2, however it is able to extract up to 70 percent as hypoxia develops. This mechanism is referred to as perfusion reserve. A drop in distal BP or CBF is therefore compensated for by increased O_2 extraction from the blood to meet the tissues metabolic needs. These aforementioned compensatory mechanisms are not unlimited. When distal BP falls below 50 mmHg, or CBF drops below 8-10ml/100g/min, then autoregulation and perfusion reserve are insufficient to prevent the failure of O_2 and substrate delivery to tissue. Although in its strictest sense, this is the ischaemic threshold, the ischaemic threshold is generally accepted as the CBF below which neuronal function is compromised, which is 23ml/100g/min (Fitzgerald and Folan-Curran, 2002). Neurons are heterogeneously vulnerable to ischaemia and gradual silencing of neurons occurs. For example, some regions are more vulnerable to ischaemia such as the CA neurons of the hippocampus.

1.5.2 Cerebral Infarction

Collateral flow can provide a degree of protection against reduced CBF, however this protection depends upon the severity in the reduction of CBF. Decreased blood flow to any area of the brain, either through vascular occlusion or haemorrhage, leads to neuronal damage and the potential development of infarction. Infarction refers to an area of tissue that is irreversibly damaged as a direct result of CBF falling below critical levels (De Girolami et al., 1999; Fitzgerald and Folan-Curran, 2002). It is generally accepted that the infarct may be divided into 2 regions: the core and the penumbra.

The Infarct Core

The core of the infarct is most severely affected by the O_2 depletion, such that only very fast and effective strategies can reverse the reduction in CBF, increase blood flow above the critical threshold and potentially salvage neurons (Heiss et al., 1999; Lassen and Astrup, 1987). When CBF drops below the critical level of 8-10ml/100g/min, ATP synthesis fails to meet the enormous demands of the cell and membrane failure ensues with massive potassium (K^+) efflux and the influx of calcium (Ca^{2+}), sodium (Na^+), chloride (Cl^-) and water, causing membrane depolarisation. Tissue in such regions is generally not salvageable (del Zoppo and Hallenbeck, 2000), and cells die rapidly by necrosis due to severe O_2 deprivation.

The Penumbra

Surrounding the infarct core is the penumbra, derived from the Greek word meaning "almost shadow". The penumbra is the region that lies between the lethally damaged core and the normal brain tissue. In this region CBF is moderately reduced but remains at levels sufficient to sustain basic neuronal function, including ATP production. However, synaptic failure and severe lactic acidosis occur (Heiss et al., 1999; Small and Buchan, 2000). Cells are compromised but still viable, and are referred to as reversibly injured. Should ischaemia continue, or the decrease in CBF become more severe, neurons within the penumbra may progress to become irreversibly damaged, thereby increasing infarct size. However, with adequate and timely reperfusion, neuronal loss may be reduced and functional outcome improved. Thus, the lethally damaged core is effectively of no clinical interest and therapeutic intervention targets the penumbra.

Progression of the penumbra may be accelerated by failure to restore blood flow, poor collateral circulation and hyperglycaemia (Gladstone et al., 2002). Neurons within the penumbra depolarise intermittently but at the expense of energy consumption. Within the penumbra there is also increased O_2 extraction, selective gene expression, lactic acidosis and a decrease in protein synthesis. Cells may survive for many hours to days after the initial ischaemic insult, but eventually die by apoptosis. As such, the penumbra is of most clinical interest. However, some researchers are now placing less emphasis of salvaging penumbral tissue per se, instead focussing on the functional capacity and synaptic plasticity of the peri-lesion area.

The penumbra may be readily identified with neuroimaging (Heiss et al., 1994; Quast et al., 1993; Rafalowska, 2002; Weinstein et al., 2004). PET studies show that the penumbra makes up approximately 18 percent of the final infarct volume. This implies that neuroprotective agents may only be able to salvage a small amount of brain. However, this is highly varied due to the individual nature of the penumbra. Potentially salvageable tissue is present up to 6 h following stroke onset in some patients as confirmed by MRI studies (Gladstone et al., 2002). Numerous experimental studies have investigated the evolution of the penumbra under various CBF levels, demonstrating that recovery of the ischaemic penumbra is possible. However, the CBF threshold at which tissue is no longer salvageable differs greatly amongst models. CBF levels below 11ml/100g/min in a baboon model of middle cerebral artery occlusion (MCAO) prevented recovery of cortex-evoked potentials. In a monkey model of transient MCAO, cerebral ischaemia was well tolerated provided CBF levels did not fall below 12ml/100g/min. Moreover, following

reperfusion recovery of the penumbra occurred (Mobley et al., 1988). In permanent MCAO, CBF values above 18ml/100g/min were required to prevent infarction. Jones and colleagues (Jones et al., 1981) found variability of neuronal susceptibility in a feline model of MCAO. 1-2 h of ischaemia was well tolerated by the majority of neurons at CBF levels of 15ml/100g/min. However, recovery of the penumbra was not observed at CBF levels below this. Infarct volume has also been shown to change significantly between 6-72 h following ischaemia but not thereafter (Stoll et al., 1998). Therefore, within 6-72 h of stroke is most likely to be the time limit for therapeutic intervention time.

1.5.3 The Ischaemic Injury Cascade

The ischaemic cascade refers to the series of events that occur as a result of the ischaemia. The cellular injury mechanisms following stroke are made up of both primary and secondary mechanisms. Direct tissue damage occurs following stroke, such that reductions in CBF within the lesion core lead to rapid cell death within minutes (Sims and Anderson, 2002). This is followed by an array of secondary injury processes including excitotoxicity, oxidative stress, inflammation, apoptosis, increased vascular permeability and cerebral oedema (Lo et al., 2001). Heiss and colleagues (Heiss et al., 1999) propose that the majority of the tissue damage occurs as a result of the initial ischaemic insult and that secondary and delayed factors do not greatly contribute to tissue damage. However, the majority of researchers believe that secondary factors significantly contribute to neuronal injury post-stroke.

Cellular Bioenergetic State

Impaired CBF leads to a mismatch between O_2 supply and demand by the tissue, leading to impairment of cellular bioenergetic state. Within just minutes of ischaemia, ATP concentrations fall sharply to values less than 10 percent of normal (Sims and Anderson, 2002). Such a disruption of neuronal ATP levels has widespread and varied effects. Decreased ATP leads to an increase in lactate, causing lactoacidosis. The Na^+/K^+ ATPase, an ATP-dependent ion pump, fails in the absence of sufficient ATP and disrupts ion homeostasis. Massive K^+ efflux and Na^+ influx occurs, accompanied by cellular swelling (Lo et al., 2001; Sims and Anderson, 2002). Finally, no phosphorylation reactions can occur in the absence of ATP, thereby impacting on a wide range of metabolic processes.

Calcium Homeostasis and Excitotoxicity

Ischaemia activates voltage-dependent Ca^{2+} channels, facilitating a massive influx of Ca^{2+} into cells. Indeed, following ischaemia, total neuronal Ca^{2+} content may increase by up to 150 percent of control levels (Kristian et al., 1998; Kristian and Siesjo, 1998). An increase in intracellular Ca^{2+} has many detrimental effects on cells, some of which are outlined below. Excess Ca^{2+} stimulates excitatory amino acid (EAA) release, such as glutamate, which leads to excitotoxicity. This is compounded by the failure of EAA pre-synaptic uptake, further contributing to glutamate accumulation. Glutamate activates N-Methyl-D-Aspartate (NMDA) channels. Magnesium (Mg^{2+}) normally serves as an endogenous blocker of NMDA channels (Altura, 1985; Hallak et al., 1994; Iseri and French, 1984). However, following stroke, Mg^{2+} levels are reduced, thereby allowing NMDA receptor activation. This allows the further influx of Ca^{2+}, leading to a cascade of deleterious

events (Choi, 1994; Kristian and Siesjo, 1998). For example, Ca^{2+} leads to phospholipase C (PLC) and inositol phosphate (IP_3) signalling, as well as activation of proteolytic enzymes that degrade intracellular components such as laminin, spectrin and actin, leading to loss of cellular structure and integrity (Dirnagl et al., 1999). Furthermore, Ca^{2+} may activate phospholipase A_2 (PLA_2) resulting in free radical (FR) generation beyond the capacity of scavenging mechanisms (Phillis and O'Regan, 2003). The increased FR cause lipid peroxidation of the phospholipid bilayer and damage to cell membranes (Lewen et al., 2000). In addition, disruption of the inner mitochondrial membrane causes the mitochondria to become leaky and the mitochondrial permeability transition pore to form, facilitating mitochondrial swelling and cessation of ATP production, which in turn leads to FR production, cytochrome c release and apoptosis. FR induce pro-inflammatory genes, with post-ischaemic inflammation contributing to the neuronal injury seen following stroke.

Despite EAA contributing to Ca^{2+} influx, administration of NMDA receptor antagonists following stroke have produced mixed results. Early studies showed that the NMDA receptor antagonist, CGS-19755, prevented the entry of Ca^{2+} into ischaemic neurons with a resultant improvement in learning ability. However, this was observed without histological protection (Grotta et al., 1990). Conversely, other studies have reported that administration of the NMDA receptor antagonist, MK-801, was found to increase the volume of necrosis and had a limited ability to protect axons, thereby limiting its clinical applications (Yam et al., 2000). As such, it has been proposed that NMDA receptor antagonists may simply delay the evolution of the infarct, rather than conserve cerebral tissue (Valtysson et al., 1994).

1.5.4 Neuronal Death: Necrosis, Apoptosis and Non-Apoptotic Programmed Cell Death following stroke

Neuronal death following stroke is a prolonged process where cells may die by necrosis or apoptosis (Braun et al., 1996). Necrosis usually affects large numbers of cells and is identified by swelling of the cystol, mitochondria and organelles, with subsequent cell rupture and an inflammatory reaction. Conversely, apoptosis usually occurs in discrete cells and is characterised by chromatin condensation, nuclear fragmentation, cell shrinkage, plasma membrane blebbing, formation and phagocytosis of apoptotic bodies and the absence of an inflammatory response. Regardless, any longer than very brief ischaemia leads to neuronal death (Love, 1999; Love, 2003).

Cell death via apoptosis may involve two pathways, the extrinsic pathway triggered by the activation of death receptors, or the intrinsic pathway via the release of cytochrome c. The intrinsic pathway is suppressed by anti-apoptotic Bcl-2 proteins that prevent cytochrome c release (Reed, 2001). There is a large body of evidence confirming that some neuronal death following ischaemia is related to the action of caspases. Caspases-2, 8, 9 and 10 are initiator caspases that transduce a number of pro-apoptotic stimuli into proteolytic activity. Caspases-3, 6 and 7 are effector caspases and are responsible for most of the morphological and biological features of apoptosis (Love, 2003; Reed, 2001). Caspase-3 activation occurs in susceptible cortical and hippocampal neurons following transient focal and transient global ischaemic respectively. Caspase-3 activation also occurs in degenerating CA1 neurons following brief forebrain ischaemia (Robertson et al., 2000). As such, the potential of caspase inhibition for the treatment of stroke has been extensively

investigated (Love, 2003; Mattson, 2000; Onteniente et al., 2003; Reed, 2001; Robertson et al., 2000; Schulz et al., 1999; Unal-Cevik et al., 2004). Administration of caspase inhibitors before of soon after ischaemia consistently reduces infarct volume and the extent of neurological dysfunction (Li et al., 2000a; Reed, 2001). However, some groups speculate that inhibition of caspases may simply delay rather than prevent cell death (Love, 2003; Robertson et al., 2000).

Other forms of cell death have also been described, including a form of non-apoptotic programmed cell death that has been documented in striatal neurons. The receptor ligand-pair, neurokinin 1-SP, was shown to produce such a non-apoptotic form of programmed cell death, and was successfully inhibited with an antagonist for this receptor (Castro-Obregon et al., 2002).

Traditionally, the core of the ischaemic lesion is considered to be necrotic whilst the penumbra is thought to involve apoptotic mechanisms (Onteniente et al., 2003). However, cell and organelle swelling and rupture, the main characteristics of necrosis, are rarely observed in neurons within the core region. Furthermore, caspase activation has been observed within the ischaemic core (Onteniente et al., 2003). Swollen morphology of such neurons is associated with apoptotic features and cytoplasmic vacuolation. Interestingly, several characteristics of apoptosis have been observed in a pro-necrotic setting, such as cell shrinkage and reduction of infarct volume by protease inhibitors or over-expression of bcl-2. These findings led to the controversial theory that cell death proceeds via a number of hybrid pathways, and that under ischaemic conditions apoptosis may be masked by necrosis. In support of this, one group has reported necrotic and apoptotic cell death

mechanisms occurring concomitantly in the same cell after cerebral ischaemia, and suggested that the cell death phenotype of the cell was determined by the relative speed of each process (Unal-Cevik et al., 2004).

1.5.5 Reperfusion Injury

Reperfusion of an ischaemic vascular bed, whether spontaneous or the result of thrombolytic therapy, is essential to salvage ischaemic tissue (Sims and Anderson, 2002; Souza et al., 2002). However, injury is worsened upon reperfusion because reperfusion itself causes stress. Re-delivery of O_2 to the ischaemic tissue results in the formation of reactive oxygen species that cause lipid peroxidation, BBB breakdown and exacerbation of oedema. Indeed, reperfusion injury has been extensively documented in peripheral tissues such as skeletal muscle (Turchanyi et al., 2005), pancreas (Dembinski et al., 2003), intestine (Souza et al., 2002), heart muscle (Przyklenk, 1989; Steenbergen et al., 1990; Wu et al., 2001) and lung (Souza et al., 2002). The release of cytokines can also trigger the migration and adhesion of neutrophils, monocytes and macrophages. Inflammatory cells contribute to cellular damage via further FR production and the release of proteases and vasoconstrictive mediators, thereby amplifying oxidative stress and inflammatory mediated injury (Belayev et al., 1996a; Kastrup et al., 1999; Lo et al., 2001; Yatsu et al., 1987). Reperfusion injury has now been demonstrated in numerous experimental studies of stroke. For example, 3 h of ischaemia followed by 3 h of reperfusion produced much greater injury compared with 6 h of ischaemia (Yang and Betz, 1994). Also, 24 h of pMCAO was found to produce less injury than 2-5 h of reversible occlusion with 24 h of reperfusion (Aronowski et al., 1997). This group also found that 72 percent of brain damage produced by ischaemia

followed by reperfusion was produced by reperfusion injury. Such studies reinforce the contribution of reperfusion injury to overall tissue injury following cerebral ischaemia.

1.6 Ischaemic Brain Oedema

1.6.1 Classification

Cerebral oedema is a common and life-threatening complication of stroke, and is defined as an abnormal accumulation of fluid within the brain (Ayata and Ropper, 2002). Klatzo and colleagues (Klatzo, 1987) were the first group to classify oedema into two categories, based upon the integrity of the BBB: vasogenic and cytotoxic (Petty and Wettstein, 2001).

Cytotoxic Oedema

Cytotoxic oedema is an intracellular type of oedema that occurs secondary to cellular injury and represents a shift of water from the extracellular compartment to the intracellular compartment, accompanied by shrinkage of the extracellular space (De Girolami et al., 1999; Lo et al., 2001). As there is only movement of water between the two compartments, it does not theoretically contribute to a net increase in brain water content and therefore cannot be considered as a major cause of brain swelling. However, this view is highly controversial, and a number of researchers believe that it is in fact the cytotoxic oedema that is the major contributor to brain swelling and not vasogenic oedema. Cytotoxic oedema occurs independently of alterations in BBB and appears to be more prominent in the grey matter (Ayata and Ropper, 2002). Failure of the Na^+/K^+ ATPase is central to the development of

cytotoxic oedema, which is apparent in regions of energy failure (Kuroiwa et al., 1994; Lazovic et al., 2005; Rosenberg and Yang, 2007).

Vasogenic Oedema

Vasogenic oedema occurs in the setting of BBB disruption and injury to blood vessels (Rosenberg and Yang, 2007), and involves the escape of proteins from the vasculature and accumulation in the brain intracellular space (De Girolami et al., 1999; Lo et al., 2001). This causes an osmotic increase at the site of injury and the subsequent movement of water down the osmotic gradient (Gamache, 1987; Yatsu et al., 1987). There is a strong correlation between extravasation of proteins into the extracellular space and the development of vasogenic oedema (Kuroiwa et al., 1985a; Kuroiwa et al., 1985b). Vasogenic oedema is of particular importance in the setting of vascular recanalisation due to the increased risk of haemorrhagic transformation from damaged blood vessels and excess fluid accumulation (Rosenberg, 1999). It is more prominent in white matter (Ayata and Ropper, 2002).

Interestingly, the core and penumbra of the ischaemic lesion have been shown to display different oedema profiles. The core tissue shows a cytotoxic oedema profile whereas the surrounding penumbral tissue shows a vasogenic oedema profile (Ayata and Ropper, 2002). Therefore, identification of the underlying factors associated with vasogenic oedema formation may provide insight into the mechanisms associated with neuronal cell death within the penumbra. Menzies et al (Menzies et al., 1993) found that within the core of the infarct, oedema was maximal at 12 h and remained elevated for 7 d before returning to normal. They discovered the

oedematous changes were associated with increases in Na^+ and decreases in K^+, and these ionic changes accounted for nearly all the observed oedema.

Furthermore, the profile of oedema may also vary according to the perfusion status. For example, in permanent cerebral ischaemia the formation of cerebral oedema is not associated with alterations in BBB permeability, suggesting the oedema is cytotoxic in nature. Conversely, in the setting of transient cerebral ischaemia, oedema is exacerbated by reperfusion and is associated with disruption of the BBB, indicating the oedema is vasogenic in type (Kuroiwa et al., 1988). However, these 2 types of oedema do not necessarily occur in isolation. When the infarct progresses, cytotoxic oedema may occur concurrently with vasogenic oedema (Hakamata et al., 1997). Clinical studies report that oedema is maximal between 1-3 d following stroke (Ayata and Ropper, 2002), whilst experimental studies report its presence as early as 15 mins after the onset of vascular occlusion (Kuroiwa et al., 2007).

1.6.3 Blood-Brain-Barrier Permeability

Breakdown of the BBB is central to the development of vasogenic oedema. The BBB is a complex cellular system comprising cells of cerebral capillaries and post-capillary venules resting on the basal lamina (Lo et al., 2001). It functions to regulate the entry of blood-borne substances that may damage the fragile brain extracellular environment. The BBB also ensures a constant supply of nutrients by specific transport, preserves ion homeostasis within the brain microenvironment, and protects against noxious chemicals, variation in blood composition and breakdown of concentration gradient. It thus facilitates a stable and chemically optimal environment for neuronal function (Fitzgerald and Folan-Curran, 2002).

Passage of substances across the BBB occurs via two different mechanisms, the transcellular and paracellular pathways. The endothelial cell transcellular pathway refers to the passive diffusion of lipophilic compounds with a molecular weight of less than 450 kDa. The second mechanism is the paracellular pathway (between cells) where ions and solutes diffuse between adjacent cells down their concentration gradient (Petty and Lo, 2002).

Tight and adherin junctions form the junctional complexes that make up the BBB. They are made up of a complex network of transmembrane and cystolic proteins (ie., claudin, occludin and adhesion molecules) that allow tight junctions to form a seal and thereby mediate the gate function of the BBB (Lo et al., 2001; Petty and Lo, 2002). Tight junctions are dynamic and responsive, and are regulated by numerous signalling mechanisms to meet the needs and responses of the BBB. They respond to cyclic adenosine monophosphate (cAMP), G proteins, tyrosine kinases, Ca^{2+} and protein kinase C (PKC), amongst others. They also form a seal to prevent paracellular diffusion of solutes from cell to cell. The phosphorylation of transmembrane and accessory proteins is essential in the regulation and establishment of these tight junctions. Adherin junctions act to hold neighbouring cells together and are also important in intracellular signalling (Petty and Lo, 2002).

The Blood-Brain-Barrier in Cerebral Ischaemia

In conditions of ischaemia, there is a loss of microvascular integrity such that alterations in vascular permeability and basal lamina structure occur. Ischaemic injury is initiated by a series of events that occur at the blood-vascular-parenchymal interface. Deprivation of blood flow to downstream microvasculature occurs as a

result of arterial occlusion and activates a cascade of events, resulting in activation of coagulation factors, disruption of the extracellular matrix (ECM) and increased capillary permeability. Endothelial dysfunction occurs and leads to early permeability increases that cause extravasation of plasma components and oedema formation. Loss of basal lamina integrity causes red blood cell leakage and the development of haemorrhagic transformation. Inflammatory cells, cytokines, proteases and free radicals have all been linked to such damage (Lo et al., 2001; Petty and Lo, 2002).

The exact mechanism by which ischaemia disrupts the BBB is debatable, however acute hypertension, hyperosmolar solutions and inflammation have all been implicated. Tight junctions remain intact until very late in infarction and are not primarily responsible for the leakage of oedema fluid. The initial BBB permeability appears to be associated with active pinocytosis by endothelial cells, however the mechanism for the oedema formation is unclear (Ayata and Ropper, 2002). Certainly, FR are known to affect the permeability of blood vessels, while increased glutamate leads to NO release which also opens the BBB (Rosenberg, 1999). The degranulation of mast cells is associated with the genesis of perivascular oedema following focal cerebral ischaemia. Indeed, treatment with a mast cell degranulating inhibitor profoundly reduced BBB damage and leakage, brain swelling and the infiltration of neutrophils (Strbian et al., 2006). In recent years, the role of matrixmetalloproteinases (MMP) has also gained increasing attention. The MMP's are a family of proteases involved in the metabolism of the ECM and are integral to normal functions such as angiogenesis and tissue remodelling (Zhao et al., 2007). Increased levels of MMP-9 have been detected in clinical (Montaner et

al., 2003; Ning et al., 2006) and experimental (Aoki et al., 2002; Romanic et al., 1998; Sumii and Lo, 2002) stroke and are regarded as a negative prognostic indicator (Ning et al., 2006). MMP-9 may promote a breakdown of the BBB through degradation of integral proteins such as laminin (Zhao et al., 2007). Consistent with this, inhibition of MMP-9 is associated with maintenance of BBB integrity.

Temporal Profile of Blood-Brain Barrier Breakdown

The literature is divided as to the exact time course of BBB disruption following stroke, however experimental studies have revealed that there is a biphasic opening of the BBB (Kuroiwa et al., 1985a; Kuroiwa et al, 1985b; Rosenberg and Yang, 2007). Early changes in BBB permeability have been observed in the acute phase following stroke at 2-3 h after the onset of vascular occlusion (Jiang et al., 2005; Rosenberg and Yang, 2007). A second and delayed opening follows, typically observed at 24-48 h, and seems to be associated with more intense blood vessel damage. Findings by Katrup and colleagues (Kastrup et al., 1999) reveal that 3 h of ischaemia followed by 3 h of reperfusion produced a massive, instantaneous breakdown of the BBB, indicating that reperfusion may cause earlier and more severe disruption of the BBB. As such, restoration of CBF following ischaemia may cause hyperperfusion that may exacerbate BBB disruption. Such findings suggest that the integrity of the BBB plays a major role in the pathophysiology of post-ischaemic reperfusion injury and may exacerbate tissue damage. Preston et al (Preston et al., 1993) observed 3 openings of the BBB following forebrain ischaemia immediately following ischaemia, delayed and intensified opening in the striatum and hippocampus after 6 h and a final opening at 6-24 h. Belayev et al

(Belayev et al., 1996b) reported that BBB disruption became apparent at 3-4 h following ischaemia, maximal at 5 h and was still evident as a delayed disruption at 48-50 h. Although little consensus exists, one thing that is agreed upon amongst researchers is that there is more than one period where the barrier is compromised following stroke. Early BBB opening has been attributed to the action of MMP-2, while the late opening has been attributed to the activation of MMP-9 and MMP-3 (Rosenberg and Yang, 2007). Therefore, interference with MMP activation may reduce BBB permeability and subsequent vasogenic oedema formation. Indeed, treatment with broad-spectrum MMP inhibitors significantly reduces BBB permeability following experimental cerebral ischaemia (Sood et al., 2007).

1.6.4 Consequences of Cerebral Oedema

Brain swelling is a direct consequence of cerebral oedema and is a common life threatening complication of stroke. It is a major cause of clinical deterioration within the first 24 h following stroke and the leading cause of death within the first week of stroke. Cerebral oedema may compromise the quality of reperfusion and increase the risk of brain herniation (Ayata and Ropper, 2002; Gartshore et al., 1997; Hanley, 2003). With the mortality of malignant cerebral oedema approaching 80% (Ayata and Ropper, 2002; Broderick and Hacke, 2002) it is essential that it is addressed in a timely fashion. Signs of cerebral oedema formation include a progressive decreased level of consciousness, worsening neurological deficits, headache, nausea, vomiting and increased drowsiness (Ayata and Ropper, 2002). Due to the fixed nature of the intracranial cavity, expansion of one of the components (brain, cerebrospinal fluid or vasculature), is accommodated at the expense of another. When no more accommodation can occur, cerebral oedema

leads to increased intracranial pressure which in turn decreases cerebral perfusion pressure (CPP) and CBF. This compromise in the CBF further aggravates cerebral oedema (Ayata and Ropper, 2002). Regional oedema leads to increases in local tissue pressure, which can cause physical distortion of the adjacent brain. Slow increases in local ICP then occur which may lead to herniation within 24-72 h of stroke (Rosenberg, 1999).

1.6.5 Treatment

Therapeutic intervention for the treatment of cerebral oedema has the capacity to markedly reduce mortality and improve outcome (Hanley, 2003). However, the treatments currently available to patients with cerebral oedema are largely inadequate as they address the symptoms and not the cause of the brain swelling. Such treatments include the use of hyperosmolar agents, barbiturates, corticosteroids, hyperventilation, drainage of CSF and in extreme cases, decompressive craniectomy (Ayata and Ropper, 2002). The aim of decompressive craniectomy is to reduce ICP, prevent fatal brain herniation, increase cerebral perfusion pressure and preserve CBF (Broderick and Hacke, 2002; Robertson et al., 2004). It has been reported to decrease mortality from 80% to 30%, without an increase in the rate of severe disability (Broderick and Hacke, 2002). Nonetheless, it is a highly invasive procedure that is reserved as a last resort. The goal of osmotherapy is to create an osmotic gradient across the BBB to remove water from the brain. However, an intact BBB is required for this mechanism so as to prevent entry of the osmolyte into the brain parenchyma and exacerbation of oedema. In the short-term, osmotherapy may produce decreases in ICP but is only effective for 48-72 h (Broderick and Hacke, 2002). Mild hyperventilation, that is a PCO_2 between

30-35 mmHg, also has a transient effect, lasting up to 12-36 h (Broderick and Hacke, 2002). Following osmotherapy and mild hyperventilation, barbiturates may be used to lower critically increased ICP. However, the mechanism of action is vasoconstriction, so cerebral perfusion may be compromised. In addition, the efficacy is short-lived as once the vasoconstrictive capacity is exhausted no further ICP lowering can be expected (Broderick and Hacke, 2002; Schwab et al., 1997). Corticosteroids have proved ineffective in treating ischaemic brain oedema of the cytotoxic or vasogenic type (Ito et al., 1980). Due to the lack of definitive randomised clinical trials, there is currently insuffucient evidence to support the use of any of the aforementioned agents in the clinical management of cerebral oedema (Broderick and Hacke, 2002; Bereczki et al., 2000; Qizilbash et al., 2002). Hence, numerous clinical and experimental studies are focused on elucidating an effective therapy for the treatment of cerebral oedema. Recently, substance P (SP) has been implicated in the pathogenesis of BBB breakdown and oedema formation following injury to the brain (Donkin et al., 2007; Nimmo et al., 2004; Vink et al., 2003). It offers a potential therapeutic target that would impact on the mechanism of brain oedema formation and is discussed in detail below.

1.7 Substance P

SP is an 11 amino acid peptide that is a member of the tachykinin family, so named for their fast-acting properties (Maggi, 1995), that also includes neurokinin A (NKA), neuropeptide K (NPK), neuropeptide γ (NKγ) and neurokinin B (NKB), amongst others (Harrison and Geppetti, 2001). Tachykinins are highly conserved peptides, indicating an important evolutionary role. SP was first isolated by von Euler and Gaddum in the 1930's as a crude extract from equine brain and gut that

demonstrated potent hypotensive and smooth muscle contractile properties. It was subsequently named 'substance P' for the powder from which it was extracted (Leeman and Ferguson, 2000). SP was identified in high concentrations in the dorsal root ganglia of the spinal cord and this drove speculation that the substance functioned as a neuronal sensory transmitter in the transmission of pain signals (Leeman and Ferguson, 2000). It is now known that SP is indeed released from both central and peripheral endings of primary afferent neurons where it functions as a neurotransmitter (Maggi, 1995; Otsuka and Yoshioka, 1993).

1.7.1 Neuropeptide Synthesis

Tachykinins are produced from the preprotachykinin (PPT) A and B genes. Alternative splicing of the PPTA gene yields four different transcripts. The α- and δ- transcripts may produce SP, NKA, NPK and NKγ, whereas the β- and γ- transcripts give rise only to SP. The PPTB gene only encodes for NKB (Campos and Calixto, 2000; Hokfelt et al., 2000). SP synthesis occurs at the cell body ribosomes where it is then packaged into vesicles and axonally transported to the terminal endings for final enzymatic processing (Hokfelt et al., 2000). In the secretory granules, precursor proteins are stored along with processing enzymes that excise the active peptide from its' pre-peptide and pro-peptide precursors, and subsequently undertake post-translational modifications such as glycosylation, C-terminal amidation and disulphide bond formation (Harrison and Geppetti, 2001; Lundy and Linden, 2004). The biologically active peptide is then stored in large, dense vesicles ready for release. Under normal conditions, substantial amounts of SP are synthesised and stored within neurons (Hokfelt et al., 2000). However,

activation or damage of these neurons results in the rapid release of neuropeptides (Harrison and Geppetti, 2001; Hokfelt et al., 2000).

1.7.2 Neuropeptide Release

Neuropeptides are released in response to Ca^{2+}-dependent depolarisation of neurons, induced by a variety of stimuli including electrical stimulation, pH changes and ligand binding to their receptors, including capsaicin (Lundy and Linden, 2004; Severini et al., 2002). Once released, SP may have direct post-synaptic actions as a neurotransmitter, modulatory function at post-synaptic sites or paracrine functions on non-neuronal targets (Lundy and Linden, 2004). The microenvironment is also likely to dictate the specific actions of SP.

1.7.3 Neuropeptide Localisation

Tachykinins, namely SP and its mRNA, are widely distributed throughout the central nervous system (CNS) and peripheral nervous system (PNS) of mammals (Alves et al., 1999; Harrison and Geppetti, 2001). Specifically, they are found in the brain, gastrointestinal tract, respiratory tract, blood and blood vessels, urinary system, skin and immune system (Severini et al., 2002). However, the distribution of specific transcripts varies according to the tissue with α-PPTA more abundant within the brain, whilst the β-PPTA is more abundant in peripheral tissues (Lundy and Linden, 2004). The main feature of SP immunoreactivity is co-localisation with other classical transmitters such as serotonin and glutamate, and with other neuropeptides such as calcitonin gene-related peptide (CGRP) and NKA (Hokfelt et al., 2000; Ribeiro-da-Silva and Hokfelt, 2000). For example, in the enteric nervous system, up to 6 neuropeptides may be co-localised within one neuron (Lundy and

Linden, 2004). Thus, neurons are capable of releasing a cocktail of agents, thereby producing a spectrum of biological activity.

Localisation within the CNS

SP immunoreactivity has been shown in many brain regions, with the most immunoreactive regions being the amygdala, nucleus caudatus, putamen, globus pallidus, hypothalamus, substantia nigra and locus ceruleus (Severini et al., 2002). Sutoo et al (Sutoo et al., 1999) found moderate SP-like immunoreactivity in the caudate nucleus and putamen and intense SP-like immunoreactivity in the substantia nigra.

Localisation within the PNS

Sensory neuropeptides, including SP, are distributed throughout the PNS. In particular, SP is found in the enteric nervous system that innervates the gut, the respiratory tract in the tracheal and bronchial fibres, the urinary system in the renal pelvis, ureter and bladder, within the immune system in lymphoid organs and within the blood vessels and blood (Severini et al., 2002). Within each tissue, SP has tissue-specific effects. For example in the gut, its release is associated with motility while in the bladder it is associated with micturition.

1.7.4 Metabolism

Once released, SP may be cleared and inactivated by many different proteolytic enzymes. Those enzymes involved in the metabolism of SP include neutral endopeptidase (NEP) (Freed et al., 2001; Matsas et al., 1984), angiotensin-converting enzyme (ACE) (Freed et al., 2001; Skidgel and Erdos, 1987a; Skidgel

and Erdos, 1987b), SP-degrading enzyme (Probert and Hanley, 1987; Freed et al., 2001), post-proline endopeptidase (Blumberg et al., 1980; Freed et al., 2001), cathepsin-D (Azaryan and Gayolan, 1988), SP-hydrolysing enzyme, aminopeptidase P and dipeptidyl aminopeptidase IV (Freed et al., 2001). All of these enzymes degrade SP *in vitro*; however, it is likely that ACE (Koshiya et al., 1984) and NEP are primarily involved in the cleavage of SP *in vivo* due to their cellular location (Harrison and Geppetti, 2001). These enzymes catalyse the degradation of the hydrolytic bonds of SP, rendering it inactive without the carboxyl terminus required to bind NK receptors (Hokfelt et al., 2000). Specifically, NEP has been shown to degrade SP in the brain (Hooper and Turner, 1987), spinal cord (Sakurada et al., 1990) and peripheral tissues (Harrison and Geppetti, 2001), whereas ACE has been shown to degrade SP in plasma, CSF and the brain, and in particular, the substantia nigra (Wang et al., 1991).

Interestingly, many clinical and experimental studies have reported adverse effects of ACE or NEP inhibition, as this exaggerates the effects of tachykinins (Bertrand et al., 1993). In addition to confirming a pivotal role for NEP and ACE in the metabolism of SP, such studies provide further evidence that SP mediates plasma extravasation (Harrison and Geppetti, 2001). These findings are of great importance as ACE inhibitors are a widely used therapeutic agent for the treatment of hypertension, and any elevation in SP levels has the potential to worsen outcome following a cerebral insult such as TBI or stroke. Indeed, ACE inhibitors and NEP have been found to increase plasma extravasation (Cyrino et al., 2002). As such, it has been proposed that the increased activation of tachykinins observed in

pathological conditions may be partially explained by a decrease in the activity of NEP (Maggi et al., 1995).

1.7.5 Receptors

The NK receptors are rhodopsin-like membrane structures that are made up of 7 transmembrane domains connected by intracellular and extracellular loops, coupled to G proteins (Maggi and Schwartz, 1997). There are 3 mammalian tachykinin receptors that have been identified to date, including the neurokinin 1 (NK_1), neurokinin 2 (NK_2), and neurokinin 3 (NK_3) receptors. Each of the tachykinins may act on all these receptors types with varying affinities depending upon receptor availability and neuropeptide concentration (Regoli et al., 1994). However, SP has a high affinity for the NK_1 receptor, NKA for the NK_2 receptor and NKB for the NK_3 receptor (Black, 2002; Carrasco and Van de Kar, 2003).

The NK_1 Receptor

A 403 amino acid residue protein, the NK_1 receptor has been isolated from a number of tissues and from numerous species including the rat brain, salivary glands and lung, and the guinea pig uterus (Maggi, 1995). The NK_1 receptor sequence is highly conserved with only discrete variations between species. For example, the rat/mouse NK_1 receptor sequence contains Leu and Ser at positions 116 and 290 respectively, in comparison to the Val and Ile found in the human NK_1 receptor sequence (Fong et al., 1992; Sachais et al., 1993). An NK_1 autoreceptor has also been characterised purported to be involved in the regulation of SP release (Malcangio and Bowery, 1999; Levesque et al., 2007; Patacchini et al., 2000; Kalsner et al., 2000).

The biological effects of SP are mediated through the NK_1 receptor (Kavelaars et al., 1994). The tachykinins share a structural homology, a carboxyl terminal sequence that reflects their common biological action. Therefore, some cross-reactivity amongst tachykinin receptors exists (Hardwick et al., 1997). NK_1 receptors are found in highest levels in the caudate putamen and superior colliculus, however they are also found in moderate/low levels in the inferior colliculus, olfactory bulb, hypothalamus, cerebral cortex, septum, striatum, mesencephalon and dorsal horn of the spinal cord (Dam and Quirion, 1986). A disparity between SP expression and NK_1 receptor expression does sometimes exist, but this may be explained in terms of the co-localisation of SP with other tachykinins, and hence the presence of other tachykinin receptors within tissues.

NK_1 Receptor Activation and Internalisation

Transduction of the SP signal occurs through the action of G proteins associated with the intracellular domain of the NK_1 receptor. The stimulation of G proteins produces an elevation in cAMP as a secondary messenger, which through a cascade of events leads to the regulation of ion channels, enzyme activity, and changes in gene expression (Bouvier, 2001). Although normally confined to the cell membrane, the NK_1-SP complex is rapidly internalised following SP binding. SP is then removed by endosomal acidification and targeted by the lysosomes for degradation, whilst the NK_1 receptor is then recycled to the cell membrane (Lundy and Linden, 2004).

1.7.6 NK_1 Receptor Agonists and Antagonists

Because of the role of neuropeptides in pain and inflammation, numerous NK_1 receptor angonists with the capacity to activate NK_1 receptors, and NK_1 receptor antagonists with the capacity to block the NK_1 receptor, have been developed. The use of such synthetic agents has greatly advanced the neuropeptide field, enabling researchers to elucidate the role of neuropeptides in normal physiology and in disease. In particular, NK_1 receptor antagonists have been tested in dental pain, osteoarthritis, neuropathic pain and migraine (Hokfelt et al., 2001).

1.7.7 Functions of Substance P

Tachykinins are involved in distinct biological processes such as plasma protein extravasation, vasodilation, smooth muscle contraction and relaxation, airway contraction, transmission of nociceptive responses, salivary secretion and inflammation (Black, 2002; Campos and Calixto, 2000). Indeed, intracerebroventricular injection of SP in rats results in many diverse effects including increased BP and heart rate, increased hindlimb rearing behaviour, scratching, skin biting and grooming. Consideration to some of these roles for SP is given below. SP injection into the lateral septum of rodents was found to induce clear aversive behaviour including freezing and jumping followed by darting behaviour in the elevated plus maze (Gavioli et al., 2002). SP release in the basolateral amygdala of the guinea pig elicits distress vocalisations, which can be inhibited by the NK_1 receptors antagonist L760 735 (Boyce et al., 2001). Post-synaptic dorsal column neurons do not the express NK_1 receptor under control conditions, however in visceral inflammation, de novo expression of the NK_1

receptor occurs, thereby allowing the activation of such neurons by SP (Palecek et al., 2003).

In terms of pathophysiology, SP has been implicated in many conditions including asthma, inflammatory bowel disease, pain, psoriasis, anxiety, migraine, emesis and movement disorders as well as neurological and psychiatric disorders such as psychosis, stroke, migraine, pain and emesis (Rupniak et al., 2000; Snider and Lowe, 1997). Increased SP levels are associated with painful conditions such as peripheral neuropathy and osteoarthritis. Despite these findings, clinical studies of NK_1 receptor antagonists in the majority of these disorders have proved disappointing (Rupniak and Kramer, 1999).

Nociception

Cerebral vessels are innervated by a combination of sympathetic, parasympathetic and trigeminal somatic nerve fibres, all of which are important in cerebrovascular regulation. In particular, the trigeminal system is known to transmit pain sensation from the dura mater and cranial vessels. The perivascular endings of these trigeminal nerves contain neuropeptides including SP, CGRP and NKA. In particular, SP is known to contribute to pain transmission in the spinal cord. Despite this, NK_1 receptor antagonists have failed to produce analgesic effect in a number of different clinical pain models (Wu et al., 2005).

Memory and Learning

SP has been implicated in memory and reinforcement processes. In particular, the amino terminus (NH_2) of SP has been found to be involved in the memory

promoting effects of SP, whilst the carboxyl terminus (CO_2H) is involved in reinforcing properties of SP (Huston and Hasenohrl, 1995). Therefore, the behavioural and neurochemical effects of SP may not only be determined by the site of action within the CNS, but also how SP is metabolised. An NK_1 antagonist (WIN 51,708) was found to block these actions, indicating that the behavioural effects of SP are mediated by the NK_1 receptor (Hasenohrl et al., 2000). Interestingly, administration of SP neutralised age-related learning behaviour deficits, thereby supporting a role for SP in the prevention of memory decline, not just memory formation (Hasenohrl et al., 2000). It has therefore been proposed that neuropeptide levels may change in aged animals and human dementia. Indeed, decreased SP levels have been observed in post-mortem Alzheimer's Disease brains (Bennett et al., 1997).

Anxiety and Depression

SP is released in response to aversive stimuli and is widely expressed in areas of the brain involved in fear producing pathways, including the amygdala, septum, hippocampus, hypothalamus and periaqueductal grey, and as such, injection of SP into regions such as the periaquedutal gray modulates defensive reactions (Rupniak and Kramer, 1999). Furthermore, NK_1 receptors are highly expressed in regions of the brain essential for the regulation of affective and neurochemical behavioural responses to stress. Such findings prompted the investigation of SP antagonists in psychiatric disorders (Kramer et al., 1998). Administration of an NK_1 receptor antagonist attenuated vocalisations in guinea pig pups separated from their mother (Rupniak et al., 2000).

Selective NK_1 antagonists have also been found to have an antidepressant like profile in those with major depression in both rodents and humans. Excessive SP is associated with anxiety, while reduced SP activity is associated with decreased anxiety (Hasenohrl et al., 2000). Consistent with this, SP levels are significantly increased in the serum of depressed patients. Interestingly, antidepressant and anxiolytic drugs have been found to decrease SP levels in several brain regions, suggesting that alteration of neurokinin systems may contribute to the antidepressant and anxiolytic activity of such drugs (Bondy et al., 2003; Kramer et al., 1998). However, the precise mechanism exerting therapeutic effects is unknown. Rupniak and colleagues found an NK_1 receptor antagonist to have antidepressant activity, be well tolerated, cause no increase in adverse side effects compared to placebo, and not result in sexual dysfunction. Moreover, the NK_1 receptor antagonist was found to be as effective as a serotonin re-uptake inhibitor of the Prozac type commonly prescribed for depression (Rupniak et al., 2000). Unfortunately, the high placebo effect observed in modern trials of depression prevented the NK_1 receptor antagonist from having a significant effect in phase III clinical trials.

Recovery Promoting Effects

A number of roles have been proposed for SP beyond that of nociception. SP may enhance neuronal growth and cell survival, while also improving neuronal regeneration and functional recovery following neurotoxic lesions (Hasenohrl et al., 2000). In Alzheimer's disease, SP has the potential to counteract the neurotoxicity of amyloid β. SP also has the potential to exert growth factor-like actions and therefore may provide trophic support within tissues. Furthermore, SP acts as a

dopamine release agonist, which is important in the regulation of the nigro-striatal pathway in Parkinson's disease.

The Inflammatory Response

The role of SP in classical inflammation has been extensively studied. It is well known that SP plays a role in the initiation and potentiation of the inflammatory response and that these effects are mediated by the NK_1 receptor (Guo et al., 2004). SP and its receptor are widely expressed by inflammatory cells, including neutrophils, monocytes, eosinophils, dendritic cells and activated T cells (De Giorgio et al., 1998; Lundy and Linden, 2004; Maggi et al., 1995) suggesting a role in neuroimmunomodulation. Indeed, SP has pleiotropic actions in inflammation, with the capacity to stimulate the degranulation of mast cells, and stimulate cytokine release including that of IL-1β, TNF-α (Lotz et al., 1988) and IL-6 (Brain, 1997; Yamaguchi et al., 2004). Furthermore, SP can modulate the chemotaxis of monocytes (Ruff et al., 1985) and neutrophils (Braun et al., 1996), along with their aggregation, superoxide production (Maggi et al., 1995), adherence to endothelium, lysosomal enzyme release and phagocytic activity by neutrophils (Bar-Shavit et al., 1980; Dianzani et al., 2003). In turn, the cytokines Il-1β, TNF-α and IFN-γ can stimulate the release of SP by brain endothelial cells, further potentiating the inflammatory response and leading to the hypothesis that SP is involved in cytokine-mediated damage to the BBB (Cioni et al., 1998).

1.7.8 Neurogenic Inflammation

The concept of neurogenic inflammation was first documented by Bayliss in 1901, who reported vasodilation of the lower limbs in response to dorsal root ganglia

stimulation. Since then, the definition of neurogenic inflammation has evolved to encompass a painful local inflammatory response characterised by vasodilation, increased vascular permeability and mast cell degranulation (Samsam et al., 2001; Severini et al., 2002). There are also tissue specific responses including smooth muscle contraction/relaxation in the bladder, ionotropic/chronotropic effects on the heart and bronchoconstriction in the airways, amongst others (Black, 2002; Richardson and Vasko, 2002). Neurogenic inflammation has been demonstrated in tissue receiving trigeminal innervation such as oral, nasal, facial and ocular tissue, and may be stimulated by many agents including prostanoids, leukotrienes, histamine and serotonin and also by changes in the extracellular environment such as decreased pH, increased osmolarity, heat, inflammatory conditions and tissue injury (Harrison and Geppetti, 2001; Saria and Lundberg, 1983). The changes in blood vessel size and permeability that occur lead to oedema formation within the tissue (Kuroiwa et al., 1985a; Kuroiwa et al., 1985b). Perhaps the most important factor in neurogenic inflammation is SP, identified to be the most potent initiator of this response (Holzer, 1998; Otsuka and Yoshioka, 1993). However, there is doubt that SP is the sole peptide with the direct/indirect ability to cause vasodilation and plasma extravasation observed in neurogenic inflammation (Severini et al., 2002). Indeed, neurogenic inflammation leads to increases in both PPT mRNA (Harrison and Geppetti, 2001) and NK_1 receptor mRNA (Saria, 1999). Oedema and plasma extravasation in response to SP are due to direct/indirect mechanisms such as the release of CGRP, release of histamine and serotonin from mast cells and the release of prostanoids and NO (Black, 2002; Richardson and Vasko, 2002). Some of the effects of SP are related to the ability of the peptide to activate neutrophils and thereby produce hydrogen peroxide, superoxide anion and NO. The pro-

inflammatory effects of neurokinins are also related to activation of nuclear transcription factors such as NFκB (Campos and Calixto, 2000; Fiebich et al., 2000) that increases cytokine levels (Yamaguchi et al., 2004; Fiebich et al., 2000).

Neuropeptides are localised in capsaicin sensitive neurons and are released from central and peripheral endings of primary afferent neurons in response to various noxious stimuli (Cao et al., 1999; Harrison and Geppetti, 2001). Capsaicin itself causes the release of neuropeptides from these sensory nerve fibres to the point of depletion, hence being referred to as "capsaicin-sensitive" neurons. However, chronic exposure or high concentrations of capsaicin leads to permanent depletion, thereby blocking the genesis of neurogenic inflammation and the resultant oedema. Accordingly, acute and chronic treatment is a useful tool to study the effects of neuropeptides in various physiological and pathological settings.

Neurogenic Inflammation in the PNS

The release of neuropeptides, in particular SP, has long been known to initiate neurogenic inflammation in peripheral tissues such as the skin and trachea (Black, 2002; Severini et al., 2002). For example, arterial administration of SP leads to vasodilation and plasma extravasation. Alves and colleagues found SP, NKA or NKB injection to increase paw oedema, as indicated by increased paw volume (Alves et al., 1999), and other NK_1, NK_2 and NK_3 receptor agonists have also been found to increase paw oedema. Consistent with this, NK_1, NK_2 and NK_3 receptor antagonists inhibited oedema caused by SP, NKA and NKB respectively, in a dose dependent manner. Such findings confirmed previous results that SP, NKA and NKB play a role in the control of plasma extravasation and oedema formation and it

is likely that all three receptor subtypes account for the pro-inflammatory response observed (Alves et al., 1999). Indeed, studies in knockout mice have shown that loss of the NK_1 receptor results in a decrease in plasma extravasation within peripheral tissues. In addition, there was a loss of the chemoattractant influence of SP, blunted noxious chemical signalling, decreased anxiety and blunting of response to danger.

In guinea pig skin, SP causes oedema formation and white blood cell accumulation, both of which are inhibited by co-injection of the NK_1 receptor antagonist RP 67580 (Campos and Calixto, 2000). In mouse ear skin, SP, NKA and NKB all cause oedema formation. While all of these neuropeptides play a role in controlling plasma extravasation and oedema formation, it is SP that is the major contributor. NK_1 receptor antagonists are able to inhibit oedematous responses caused by the various pro-inflammatory agents.

Neurogenic Inflammation in the CNS

In contrast to classical inflammation the concept of neurogenic inflammation in the brain has until very recently remained largely unexplored. Intravenous administration of SP has now been shown to cause a significant increase in plasma extravasation in the dura mater, an effect abolished by pre-treatment with an NK_1 antagonist (Cyrino et al., 2002). Similarly, chemical, electrical or immunological stimulation, or treatment with capsaicin, was found to elicit a neurogenic inflammatory response in the dura mater but not the pia or cerebral cortex (Markowitz et al., 1987). It was previously proposed that the activation of NK_1 receptors on vascular endothelium contributed to cerebral oedema (Stumm et al.,

2001). Subsequent studies in TBI have documented a role of SP and neurogenic inflammation in BBB dysfunction and the genesis of vasogenic oedema (Donkin et al., 2007; Nimmo et al., 2004; Vink et al., 2003). This group was the first to characterise a role for neurogenic inflammation in the brain injury. Taken together, these findings indicate that the release of SP may be central to changes in BBB permeability following cerebral insults. More recently, the transient receptor potential V1 (TRPV1), or the capsaicin receptor, has gained attention as an effector of neuronal injury. Notably, there is a high degree of localisation of the TRPV1 receptor with SP and CGRP (Bae et al., 2004).

1.7.9 Substance P in Hypoxia and Ischaemia

Many groups have hypothesised that antagonists of tachykinin receptors may have several therapeutic applications (Rupniak et al., 2000; Snider and Lowe, 1997; Watling and Guard, 1992). In a model of myocardial ischaemia/reperfusion in the setting of magnesium deficiency, neurogenic inflammation was found to be an early event that initiated inflammatory and pro-oxidative processes that predispose the myocardium to reperfusion injury (Kramer et al., 1997). As such, inhibiting the SP-induced inflammation and pro-oxidative events with the NK_1 receptor antagonist L-703, 606, improved functional recovery and reduced oxidative injury. Furthermore, following hypoxia of the rat carotid body, SP release was found to be increased as a function of the severity of the hypoxic insult (Kim et al., 2001). These findings further suggest that SP release is a tissue response to hypoxia/ischaemia.

In regards to cerebral ischaemia, an increase in SP has previously been reported in the serum of patients with complete stroke and TIA (Bruno et al., 2003). However,

to date only one group has investigated the efficacy of NK_1 receptor antagonists following cerebral ischaemia (Yu et al., 1997). This group reported over-expression of SP in conditions of cerebral ischaemia and hence speculated that SP may play a role in exacerbating ischaemic damage. They administered the NK_1 receptor antagonist SR140333 (i.c.v) and found that it significantly reduced infarct volume and improved neurological function when measured 24 h after focal cerebral ischaemia. Despite these positive findings there has been no further work published in this area. As such, the further investigation of NK_1 receptor antagonists in cerebral ischaemia seems warranted.

1.8 Treatment of Ischaemic Stroke

1.8.1 Thrombolytic Therapy

Thrombotic/embolic strokes with vascular occlusion represent the most common presentation of stroke. As such, restoration of CBF is a key therapeutic strategy for the clinical management of ischaemic stroke. Numerous studies have demonstrated that relief of the obstruction allows for reperfusion of the ischaemic territory and an associated improvement in outcome (Lees, 2000). Although reperfusion of the ischaemic region is necessary to salvage compromised but viable tissue, it may also cause reperfusion injury. As discussed earlier, re-delivery of O_2 to the ischaemic tissue can lead to an exacerbation of the ischaemic injury and extension of the infarct (Przyklenk and Kloner, 1989). Nevertheless, the only therapy available to stroke patients is thrombolysis with tPA. tPA is a naturally occurring serine protease, primarily secreted by vascular endothelial cells, that catalyses the conversion of inactive plasminogen to plasmin, which in turn promotes fibrinolysis, the breakdown of the fibrin/blood clot obstructing blood flow (Longstaff and

Thelwell, 2005). Streptokinase and alteplase are the most widely studied thrombolytic agents.

In 1996, the USA Food and Drug Administration approved the use of the tPA in acute ischaemic stroke within 3 h of symptom onset, on the basis of positive results from a National Institute of Neurological Disorders and Stroke (NINDS) sponsored clinical trial (Kwiatkowski et al., 1999). In this study 624 patients with acute ischaemic stroke were treated with tPA administered intravenously at a dose of 0.9 mg/kg (to a maximum dose of 90 mg), or placebo within 3 h of stroke onset, with approximately half the patients treated within 90 mins. Favourable outcome at 3 months was greater in the tPA group compared to placebo (31-50% compared to 20-37%) with the mortality rates similar in the 2 groups (17% compared to 20%). The major complication of tPA administration that was reported was symptomatic brain haemorrhage that occurred in 6.4% of patients in the tPA group compared to 0.6% of patients in the placebo group.

Despite these positive findings, the use of tPA in clinical stroke has been criticised, mainly due to the short therapeutic window for administration and the risk of haemorrhagic complications (Jaillard et al., 1999; Wang et al., 2004; Barber et al., 2001; Kidwell et al., 2002). Indeed, the majority of patients arrive at hospital many hours and sometimes days after stroke. This means that the vast majority of patients are ineligible for tPA therapy (Hill and Hachinski, 1998). It is therefore not surprising, although somewhat alarming, to discover that as few as 5-15% of patients receive tPA due to time constraints and safety concerns (Marler and Goldstein, 2003). As such, several studies have investigated whether the

therapeutic window for tPA administration may be extended beyond the recommended 3 h. Some have reported than increasing the therapeutic window is associated with a worsened outcome (Hill and Hachinski, 1998). Other groups, however, have shown that those individuals with a perfusion/diffusion mismatch can be identified with modern imaging techniques and the therapeutic window for thrombolysis tailored, and extended up to 9 h after stroke onset without a reduction in treatment effect (Hacke et al., 2005). Nevertheless, careful patient selection is required. Patients with insignificant neurological deficits (NIHSS<4) at 3 h post-stroke are unlikely to derive much benefit from thrombolytic therapy, whereas those with severe stroke (NIHSS>22) may have a greater risk-benefit ratio than in patients with mild stroke (Lees, 2000).

Haemorrhagic transformation of the infarcted tissue may be exacerbated by or precipitated by thrombolysis (Hill and Hachinski, 1998) and reperfusion of damaged tissue may exacerbate BBB dysfunction and cerebral oedema (Przyklenk and Kloner, 1989). As a result, a number of experimental studies have investigated the potential mechanisms of tPA-induced neurotoxicity. Potentiation of NMDA signalling, activation of MMPs with subsequent breakdown of the BBB, haemorrhagic transformation and oedema, and the accumulation of neutrophils and free radicals have all been implicated (Petty and Wettstein, 2001; Van de Werf, 2001; Kahles et al., 2005; Sumii and Lo, 2002; Horwood et al., 2004; Lee et al., 2007; Yang et al., 2007; Zhang et al., 2007; Cao et al., 2006; Lapchak et al., 2002). An increase in MMP-9 levels following tPA administration has been documented in clinical (Montaner et al., 2003; Ning et al., 2006) and experimental stroke (Aoki et al., 2002; Romanic et al., 1998; Sumii and Lo, 2002). This association between tPA

and MMP-9 is purported to be integral to the BBB dysfunction and haemorrhagic transformation associated with thrombolysis. Due to its capacity to metabolise the extracellular matrix, MMP-9 may also attack the neurovascular matrix, compromising vessel integrity and thereby increasing the risk of rupture and haemorrhagic transformation, whilst also increasing the permeability of the BBB. As such, the goal of recent research has been to develop an adjunctive therapy that may be administered with tPA to potentially reduce neurotoxicity and increase the window for thrombolysis. Agents that have been tested to date in pre-clinical studies include FR scavengers, MMP inhibitors and anti-inflammatory agents.

1.8.2 Neuroprotection

Neuroprotection has the potential to be universally offered, alone or in combination with thrombolysis (Lees, 2000), the aim being to salvage ischaemic tissue and improve outcome. The rationale for neuroprotection is to interrupt the biochemical and inflammatory cascades that occur following ischaemia, such as cerebral oedema, free radical formation, impaired Ca^{2+} homeostasis and microvascular changes so as to limit infarct size, increase the time window for reperfusion therapy, minimise the reperfusion injury and improve outcome (Gladstone et al., 2002; Lo et al., 2001). Many potential neuroprotective agents have shown great promise in animal models, but none have proven efficacious despite more than 50 agents being tested in over 114 clinical trials (Gladstone et al., 2002; Heiss et al., 1999). As a result, no neuroprotective agents have been approved for the management of acute ischaemic stroke. Agents that have been tested include NMDA channel antagonists, K^+ channel blockers, Ca^{2+} channel antagonists, cell membrane stabilisers, NOS inhibitors, Na^+ channel blockers, FR scavengers and anti-apoptotic agents, amongst

others (Heiss et al., 1999; Lo et al., 2001). All of these agents showed negative outcomes in phase III trials and were subsequently excluded due to lack of efficacy or an unacceptable side effect profile. In this regard, the stroke field is not alone, with neuroprotective agents also having failed in the treatment of other CNS pathologies such as TBI, despite offering significant protection in animal models (Gladstone et al., 2002). Such findings stress the importance of pre-clinical testing of potential therapeutics in appropriate animal models, as well as demonstrating efficacy when administered in combination with tPA, having a clinically relevant therapeutic window and being applicable to different severities of stroke. It is likely that an effective neuroprotective agent will target multiple aspects of the ischaemic injury cascade which occur at different time-points (Ginsberg, 2003; Heiss et al., 1999; Lee et al., 1999). While some factors may be involved in the ischaemic injury cascade early on following stroke, they may also be involved in reparative and regenerative pathways that occur later on. For example, in the acute phase after stroke MMP's are involved in the breakdown of the BBB and haemorrhagic transformation, but in the chronic phase following stroke, they are involved in remodelling of the tissue.

1.9 Experimental Modelling of Stroke

Many of the biochemical and molecular events of clinical stroke can only be thoroughly investigated with the use of experimental models. Not only do studies in highly reproducible animal models aid in the determination of stroke pathophysiology, but they also may provide information of the efficacy of potential neuroprotective agents (Ginsberg, 2003; Green, 2002). Although no single animal model can reproduce the complex physiological responses of human stroke, they are

an invaluable tool for the study of stroke. Moreover, it is reasonable to assume that a drug that attenuates ischaemia induced damage in appropriate experimental models may also prove effective in humans (Green, 2002).

Rodents are usually the animals of choice for the study of stroke despite the disadvantage of lissencephalic brain structure and small amount of white matter compared to higher species, because the advantages of their use outweigh the disadvantages. Specifically, the advantages include their relatively small size and modest cost, the availability of a large physiological database, and the potential to assess functional outcome with a variety of behavioural tests. The use of higher species is also not always possible due to prohibitive cost and technical limitations.

There are many animal models of ischaemic stroke, broadly classified into global or focal. Models of global ischaemia involve the blockage of major vessels that supply the forebrain (Dobbin et al., 1989; Modo et al., 2000a) and these are not widely used in the study of stroke as they better represent the cerebral consequences of cardiac arrest rather than stroke. Focal ischaemia models involve the occlusion of a specific vessel, usually the middle cerebral artery (MCA). These have particular relevance to the human condition as the majority of clinical strokes result from occlusion within the MCA territory (Yoshimoto et al., 1986). Ischaemia in focal models may be permanent or transient, with removal of the blockage allowing for reperfusion of the ischaemic territory (Sims and Anderson, 2002). Thrombo/embolic models of stroke has been developed to mimic the processes of thrombosis/embolism and thrombolysis (Aoki et al., 2002; Asahi et al., 2000; Kano et al., 2000; Lapchak et al., 2000; Meng et al., 1999; Sumii and Lo, 2002; Toomey

et al., 2002; Vanderschueren et al., 1997). In particular, the intraluminal thread model of MCAO has been extensively used in the study of stroke and is used throughout the current thesis.

1.9.1 Model of ischaemic stroke: middle cerebral artery occlusion

The MCAO model of stroke was first developed in the 1980s (Koizumi et al., 1985; Longa et al., 1989) and since this time has been widely used, with minor modifications, for the study of ischaemic stroke (Belayev et al., 1996a; Irving et al., 2001; Pfefferkorn and Rosenberg, 2003; Schmid-Elsaesser et al., 1998; Turner et al., 2006; Virley et al., 2000). The model produces consistent infarction within the cortex and striatum, with cortical infarction in 80% of animals and striatal infarction in 100% of animals. Such infarction produces a variety of functional deficits that can be readily assessed through the use of behavioural outcome measures. The model has advantages over other models of cerebral ischaemia in than an invasive craniectomy is not required to produce vascular occlusion. A nylene thread with the tip rounded to produce a bulb and coated with either poly-$_{L}$-lysine or silicone is used to produce the vascular occlusion. The thread is then advanced to occlude the origin of the MCA, with access via the carotid arteries. Leaving the thread in place produces permanent occlusion whereas withdrawal of the thread allows for reperfusion of the ischaemic territory. Studies in this model have enabled pre-clinical testing of potential therapeutic agents, in addition to the study of the pathophysiological responses to stroke, in particular, reperfusion injury, BBB dysfunction and cerebral oedema.

1.10 Synopsis

This thesis will examine the potential role of the neuropeptide SP in the pathophysiology of ischaemic stroke. The studies will employ a reproducible thread MCAO model of reversible ischaemic stroke to determine whether SP is upregulated following cerebral ischaemia. This will be investigated in both permanent and transient ischaemia as well as in different grades of ischaemia, thus replicating stroke with different perfusion status and of varying severity. After characterising the SP response to cerebral ischaemia, the thesis will then explore effects of NK_1 receptor antagonist administration with regards to BBB status, cerebral oedema, lesion volume, histological abnormalities and functional outcome. The potential for NK_1 receptor antagonists for the use as an adjunctive therapy to tPA will also be investigated. Complete neuropeptide depletion with capsaicin pre-treatment will then be used to further explore the role of neuropeptides in cerebral ischaemia.

Each chapter will have a brief introduction followed by a summary of the study design and protocol, which will be fully outlined in chapter 2. Although each chapter will report on the findings of that study, such results are likely to have implications not only for the present investigation but also other aspects raised in the thesis, and therefore there will some overlap in discussion between chapters. Finally, a concluding general discussion will integrate the major conclusions drawn from each chapter.

CHAPTER 2:

MATERIALS AND METHODS

2.1 Animal Care

2.1.1 Ethics

The experimental studies presented in this thesis were performed within the guidelines established by the National Health and Medical Research Council (NH&MRC) and were approved by the experimental ethics committees of the Institute of Medical and Veterinary Science (IMVS; 39/04, 101/06) and the University of Adelaide (M-27-2004, M-44-2006).

2.1.2 General

Adult male Sprague-Dawley rats were used in the study. Animals were group housed in a conventional rodent room on a 12 h day-night cycle and provided with a standard diet of rodent pellets and water *ad libitum*. After transport, animals were rested for several days before inclusion in any experiment. At the time of the experiment, they were randomly assigned to treatment and control groups. The exact number of animals used in each experiment is detailed in each relevant chapter.

2.2 Experimental Procedures

2.2.1 Anaesthesia

Halothane or isoflurane

Halothane (Rhone Merieux) and Isoflurane (Abbot Australasia) were obtained as volatile liquids from Lyppards Veterinary Supplies (Adelaide). They were stored at room temperature away from direct heat and sunlight in a drug safe. General anaesthesia was induced by placing the animal in a transparent induction chamber, delivering 3% halothane or isoflurane in O_2 via a calibrated vaporiser at a flow rate

of 1.5L/min. Animals undergoing stroke surgery were then intubated and mechanically ventilated using a rodent ventilator (Harvard) (volume: 2 ml; stroke rate: 95-100 s/min), with anaesthesia maintained at 1.5% halothane or isoflurane delivered in O_2 at a flow rate of 1L/min. Animals undergoing all other procedures received 1.5-2% halothane or isoflurane maintenance anaesthesia through a nose delivered in O_2 at a flow rate of 1.5L/min. While halothane was used in all experiments initially, it was phased out of use in Australia in 2006. Accordingly, later experiments necessitated a switch to isoflurane. The use of appropriate controls ensured that there was no independent effects of anaesthesia within experiments, there was also no difference in any outcome parameter noted using either anaesthetic.

Lignocaine

Lignocaine (Lignocaine hydrochloride, 2% Mavlab, Australia) was supplied as an aqueous solution by Lyppards Veterinary Services (Adelaide) and was stored at room temperature in a drug safe. Lignocaine was used as a local anaesthetic and was administered to all animals following stroke and reperfusion surgery. It was applied directly to the incision site at a dose of 0.5 ml per injection.

Pentobarbital (Lethobarb)

Pentobarbital (pentobarbitone sodium, 60mg/ml; Rhone Merieux) was obtained as an aqueous solution and stored at room temperature in a drug safe. Pentobarbital was used to euthanase animals and was administered via intraperitoneal injection using a 25-G, 12.5 mm needle at a dose of 60 mg/kg. Animals were restrained by

grasping the loose skin of the back of the neck and the needle was inserted into the left caudal area of the abdominal cavity to avoid any vital organs.

2.2.2 Rodent Model of Reversible Middle Cerebral Artery Occlusion

The MCAO model of ischaemic stroke is highly reproducible and has been widely used to study experimental stroke (Koizumi, et al. 1985; Longa et al., 1989; Bederson et al., 1996). The model involves advancement of a nylene thread to occlude the origin of the MCA, with access via the carotid arteries. Withdrawal of the thread allows reperfusion of the whole MCA territory. The thread obstructs blood flow to the basal ganglia, predominantly the middle and posterior portions of the caudoputamen region including the internal capsule and anterior thalamus (Longa et al, 1989). It produces large consistent cortical infarct in 80 percent of animals and striatal infarction in 100 percent of animals (Ginsberg, 2003). The model produces neurological deficits that can be readily assessed (Bederson et al., 1986b; Persson et al., 1989). Figure 2.1 shows the MCA territory anatomy.

Preparation of Nylon Threads

Nylene monofilament thread (4-0 Nylene, #405, Dynek) sutures were straightened by gently heating with an electrocautery unity and then the tip rounded in a flame (BIC cigarette lighter). Threads were subsequently coated in 0.1% poly-L-lysine (Sigma P-1274; mw = 70 000-150 000) and dried at 60°C for 1 h.

Stroke surgery

Animals were fasted overnight and then removed from their home cages, placed in an induction box and anaesthetised using the inhalation anaesthetic, halothane or

NOTE: This figure is included on page 69 of the print copy of the thesis held in the University of Adelaide Library.

Figure 2.1 Middle cerebral artery territory anatomy (Longa et al., 1989). Schematic diagram of the rat brain. Note the passage of the thread from the carotid arteries to the origin of the MCA, indicated in yellow.

isoflurane (3%; 1.5L/min oxygen). After a surgical level of anaesthesia was reached, animals were intubated and mechanically ventilated (volume: 2 ml; stroke rate: 95-100 s/min) using a rodent ventilator (Harvard). Anaesthesia was subsequently maintained at a concentration of 1-1.5% (1L/min O_2 ratio) throughout surgery. When the withdrawal to pain reflex was absent, animals were placed in the supine position on a thermostatically controlled 37°C heat pad. Temperature regulation throughout the surgical procedures was important because not only are animals unable to regulate their own body temperature whilst under anaesthetic, but any deviation from normothermia may potentially improve or worsen outcome (Green, 2002). Right-side MCAO was performed as described in detail elsewhere (Longa et al., 1989). Initially, the neck was shaved using clippers and the area swabbed with alcohol. A midline incision was made and the overlying connective tissue and glands were blunt dissected to reveal the neck muscles, which were subsequently retracted to reveal the carotid arteries. The superior thyroid artery and ophthalmic artery were ligated and the pterogopalatine artery tied off to prevent passage of the thread into this vessel. The common carotid artery (CCA) was clamped, and the nylene thread inserted into the internal carotid artery (ICA) via a puncture in the external carotid artery (ECA). The thread was inserted 17 mm beyond the ECA/ICA bifurcation (or until elastic resistance was felt). The ECA was subsequently tied off, holding the thread in place. The arterial clamp was removed and the sutures and thread trimmed. Lignocaine (as described in Chapter 2.2) was applied to the surgical area and the wound closed with wound clips (9 mm Autoclip wound clips, Becton Dickinson). An additional group of animals were subject to all surgical procedures used to induce MCAO without advancement of the thread

(sham surgery) and this group was used as a surgical/anaesthesia control group throughout the experiments.

Post-Surgery Recovery

Animals were weaned off the ventilator and allowed to recover from anaesthetic on the heating pad. All animals received 15 ml of saline subcutaneously using a 23-G needle, as a measure to prevent dehydration following stroke. Animals were returned to their home cage when they were conscious and mobile.

Reperfusion Surgery

At the pre-determined time-point following the onset of ischaemia, animals were re-anaesthetised, the wound clips removed and the surgical area was cleared to reveal the carotid arteries. Reperfusion of the ischaemic territory was achieved via withdrawal of the thread into the ICA. The wound was treated with lignocaine and then closed with wound clips (as described above). Anaesthesia was discontinued and animals allowed to recover on the heating pad before being returned to their home cage. A subset of animals, the pMCAO group, did not receive the reperfusion surgery.

Exclusion Criteria

Typically, observation of anticlockwise circling by the animals was taken as an indicator of successful occlusion. Hence, animals that did not exhibit anticlockwise circling by 2 h following MCAO were excluded from further study.

Assessments

Following stroke, animals were assessed either for functional outcome by rotarod, bilateral asymmetry test, angleboard test, open field test and neuroscore, oedema formation by wet weight dry weight analysis, BBB integrity by Evan's Blue extravasation, infarct volume using tetrazolium chloride staining, determination of SP levels using ELISA or histological outcome using fluoro jade C (FJC) and H&E staining and SP, APP, GFAP and ED-1 immuohistochemistry.

2.2.3 Perfusion

Perfusion was performed using either 10% formalin (tissue fixation) or saline (Evan's Blue determination) as appropriate. At pre-selected time-points following stroke, animals were anaesthetised (as described in 2.2.1) and placed in the supine position on a wire rack. When a surgical level of anaesthesia was achieved, a bilateral thoracotomy was performed to expose the heart. A blunt 19-G, 37 mm needle was inserted into the apex of the heart (left ventricle) and advanced to rest in the ascending aorta. Heparin (5000 I.U/1ml; David Bull Laboratories) was then slowly injected into the ascending aorta. The right atrium was then incised to allow vascular flushing with either 10% formalin or saline. Perfusion was continued until the fluid from the right atrium ran clear, approximately 10 mins. For histology, animals were left intact for at least 1 h before the brain was removed, to minimise artefactual damage. For Evan's Blue determination brains were rapidly removed following perfusion.

2.3 Drug Treatments

Animals were randomly assigned to the NAT, tPA, NAT/tPA, saline vehicle, capsaicin and capsaicin vehicle treatment groups. Specific details of the study design are provided in each chapter. For intravenous administration, at the pre-determined time-point animals were lightly anaesthetised with 2% halothane or isoflurane (1.5L/min O_2), maintained via a nose cone. The tail was placed in warm water to aid injection into the tail vein. Anaesthesia was discontinued and animals were returned to their home cage when they were conscious and stable, with free access to food and water. For subcutaneous administration, animals were restrained and the injection administered into the subcutaneous space of the neck.

2.3.1 Saline

Following surgery all animals received saline (up to 15ml) subcutaneously as a measure to prevent post-operative dehydration. Vehicle control animals received saline only at a dose of 1ml/kg, with the saline drawn up from a sterile intravenous bag (Baxter Healthcare).

2.3.2 N-acetyl-L-Tryptophan (SP Antagonist)

N-acetyl-L-Tryptophan (NAT) (Sigma, A-6376) was stored at 4°C. A dose of 25 μmoles/kg body weight was prepared by dissolving NAT in saline and adjusting the pH to 7.5 with NaOH. The optimal dose was taken from results from our laboratory's traumatic brain injury studies (Donkin, PhD thesis). Specific details of the treatment regime are outlined in chapters 4-7.

2.3.3 Actilyse (tPA)

Actilyse (Boehringer Ingelheim) was stored at room temperature. The solution was prepared with sterile water, as per the manufacturer instructions. Injections were subsequently prepared and stored at -20ºC. Immediately before use they were removed from the freezer and allowed to thaw at room temperature. A dose of 1mg/kg was administered to a subset of animals at the onset of reperfusion, equating to a volume of 1ml/kg. Further details of the treatment paradigm are outlined in chapter 5.

2.3.4 Capsaicin

A subset of animals were pre-treated subcutaneously with capsaicin or equal volume of vehicle at a dose of 125mg/kg over a 3 d period (50mg/kg day 1, 50mg/kg day 2 and 25 mg/kg day 3) 14 d prior to MCAO surgery. Capsaicin (Sigma, M-2028) was dissolved in a vehicle of 60% alcohol, 20% saline and 20% Tween 80 (Sigma, P-8074). Further details of the treatment paradigm are outlined in chapter 8.

2.4 Neurological Assessment

MCAO produces short-term and long-term motor, sensory and neurological dysfunction (Ding et al., 2002; Modo et al., 2000b). A battery of tests are required to evaluate post-stroke impairments, as MCAO damages various anatomical and functional regions of the brain including the striatum, somatosensory cortex and motor cortex (Modo et al., 2000b; Zausinger et al., 2000). In the present thesis, the rotarod, bilateral asymmetry test, open field, neurscore and angleboard tests were used to assess the functional capacity of animals following stroke. Commencing at

24 h post-surgery, animals were assessed on a daily basis, in a random order, during the early phase of their light cycle to determine their functional outcome. Specific details of the neurological assessment procedure are outlined below.

2.4.1 Rotarod

The motor deficits associated with stroke were assessed using the rotarod (Figure 2.2), which tests the balance and co-ordination aspects of general motor function. Extensively used in mice and rats, the rotarod is an efficient and sensitive measure for assessing the motor deficits associated with brain pathologies such as stroke and TBI (Corbett and Nurse, 1998; DeVries et al., 2001; Rogers et al, 1997; Schallert, 2006) and has been shown to be more effective in revealing injury-induced deficits compared to other motor tasks (Hamm et al., 1994). Briefly, the rotarod comprises a metal frame with a rotating assembly of eighteen 1mm rods. Rats are required to grip the rods in order to walk on the rotarod. Animals were placed on the device and remained stationary for a period of 10 s. The rotations per minute (rpm) of the rods was then increased from 0 rpm to a maximum of 30 rpm, with each speed maintained for 10 s. The time score where the animal completes the 2 min task, falls completely from the apparatus or grips the rungs for 2 consecutive revolutions without walking was recorded. Animals were assessed daily (in the morning) for a 7 d period, commencing at 24 h post-stroke.

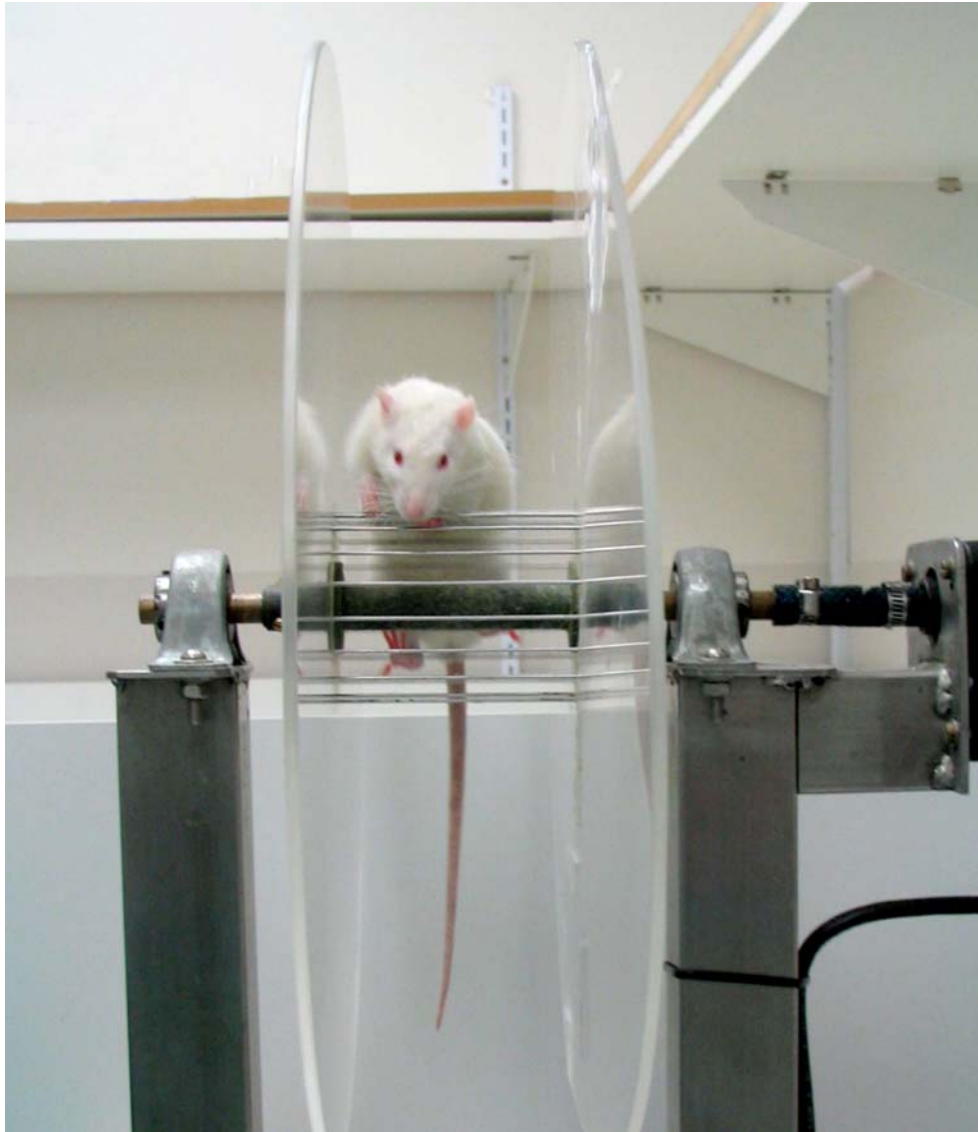

Figure 2.2 Motor function- Rotarod.

Animals are required to walk on the rotarod for the 2 min trial, with the speed increasing by 3 rpm every 10 s, to a maximum of 30 rpm.

2.4.2 Bilateral Asymmetry Test

The bilateral asymmetry test was used as a measure of sensory function following stroke. The "sticky label" test is effective because it is resistant to practice (Modo et al., 2000b), and assesses tactile extinction, while probing sensory neglect (Schallert et al., 1982). It is suitable for the use in stroke studies as animals subject to right-side MCAO frequently experience sensory deficits on the contralateral (LHS) side (Modo et al., 2000b). Briefly, two strips of tape (2cm x 3.5cm; Sigma, L-8519) were applied to the saphaneous part (soft underside) of the forepaws. Time to removal for the left and right forepaws was recorded. Each trial lasted 120 s and animals were given two consecutive trials, separated by a rest period. The mean of the two trials was then taken as their bilateral asymmetry test latency. Animals were assessed for sensory function daily (in the early morning) for a 7 d period following stroke.

2.4.3 Open Field

The open field test (Giulian and Silverman, 1975) was used to assess spontaneous exploratory behaviour, which is considered to reflect stress and anxiety. The open field comprises a white panelled 1 m x 1 m enclosure with 100 equal 10 cm squares marked on the base. Animals are placed in the centre of the enclosure and allowed to explore for 5 mins. The number of squares travelled through by the animals was taken as the spontaneous exploratory behaviour. The amount of freezing behaviour, grooming, rearing and defecation behaviour was also recorded. Spontaneous exploratory behaviour was assessed on days 1, 3 ,5 and 7 (in the morning) post-stroke.

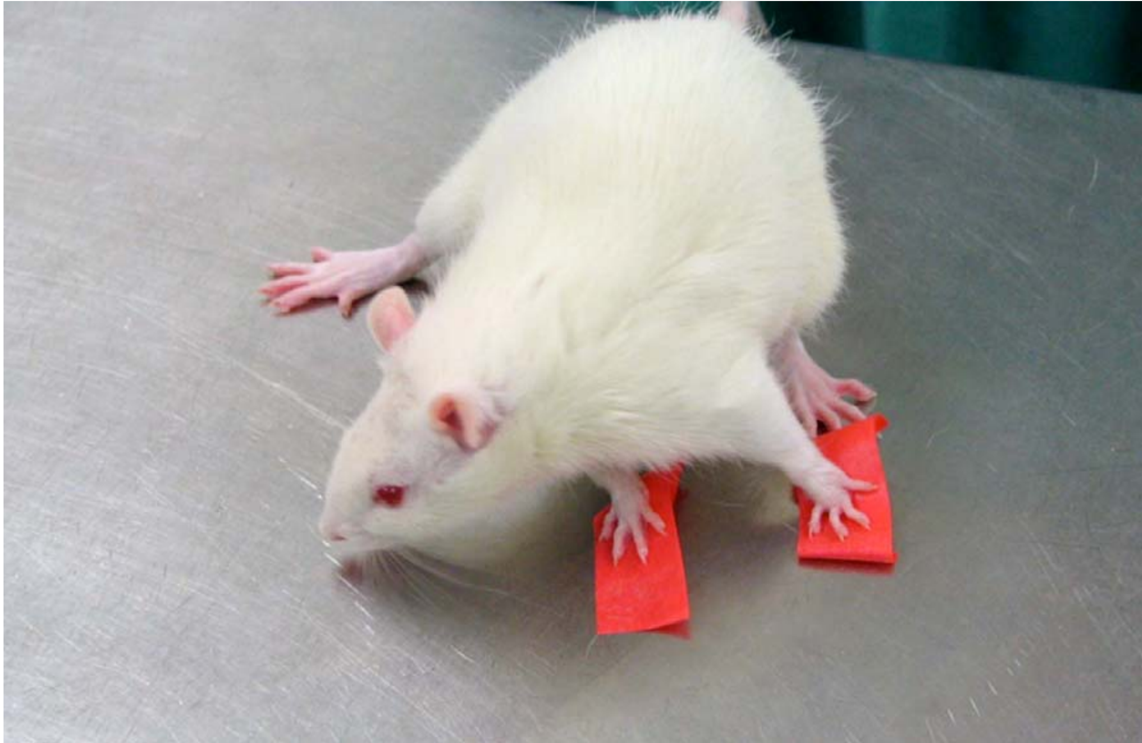

Figure 2.3 Sensory function – bilateral asymmetry test.

Note the position of the tape on the saphenous part of the forepaws. Time to removal on each side was recorded.

Figure 2.4 Spontaneous Exploratory Behaviour – Open Field.
Note the * in the middle of the open field where the animal begins the test. Animals are allowed 5 mins to explore the 1 m by 1 m enclosure. The amount of exploratory behaviour is recorded, including the number of squares travelled through and the amount of rearing behaviour.

2.4.4 Modified Neuroseverity Score (mNSS)

A modified neuroscore (Li et al., 2000b) was used to assess overall neurological function following stroke. Numerous stroke studies have demonstrated that a simple neuroscore is an effective means of assessing the neurological function of animals (Bederson et al., 1986b; Garcia et al., 1995a; Garcia et al., 1995b; Modo et al., 2000b; Rogers et al., 1997; Zausinger et al., 2000). The neuroscore assesses a number of aspects of general function following stroke, such as spontaneous motility, righting reflex, grasping reflex, placing reaction and visual placing (Li et al., 2000b; Modo et al., 2000b), as detailed in Table 2.1 below. The higher the overall score the more severe the observed deficit, as such the rankings were as follows: 1-4 denotes mild injury, 5-9 denotes moderate injury and 10-14 denotes severe injury. The neurological function of animals was assessed daily (in the early morning) following stroke.

2.4.5 Angleboard

The angleboard was used to assess hemiparesis following stroke. Hemiparesis is a common long-term complication of human stroke (Plummer et al., 2007; Rijntjes, 2006). Animals were placed on the angleboard apparatus, which was gently and slowly raised up. The angle at which the animals lost their footing and slid was the angleboard score that was recorded. Animals had 2 trials each on the left and right sides, the mean of which was taken as the angleboard score for each side. Angleboard function was determined daily (in the early morning) following stroke.

Motor Tests
Muscle status-hemiplegia - raising rat by the tail: - Flexion of forelimb - Flexion of hindlimb - Head moved more than 10º to the vertical axis within 30 s
Placing rat on the floor: - Inability to walk straight - Circling toward paretic side - Fall down to paretic side
Abnormal movements: - Immobility and staring - Tremor (wet-dog shakes) - Myodystony, irritability, seizures, myoclonus
Sensory tests: - Placing test (visual and tactile test) - Proprioceptive test (deep sensation, pushing the paw against the table to stimulate limb muscles)
Reflexes: - Pinna reflex (a head shake when touching the auditory meatus) - Corneal reflex (an eye blink when lightly touching the cornea) - Startle reflex (motor response to a brief noise)

Table 2. 1 Modified neuroseverity score.

One point is awarded for the inability to perform the task or for the lack of a tested reflex.

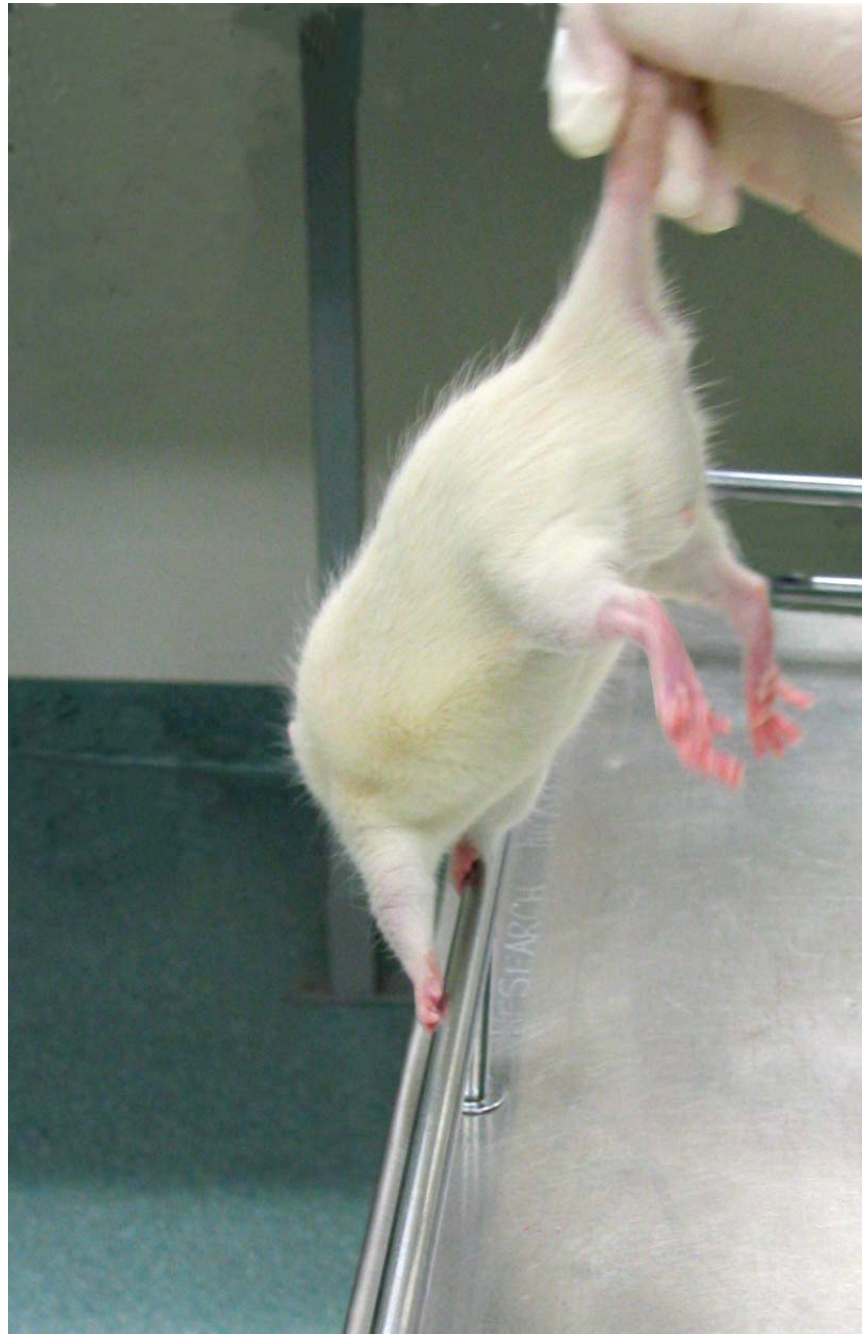

Figure 2.5 Neurological function – modified neuroseverity score.
An example of one of the neuroscore tests. Here the animal is being assessed for the placing test.

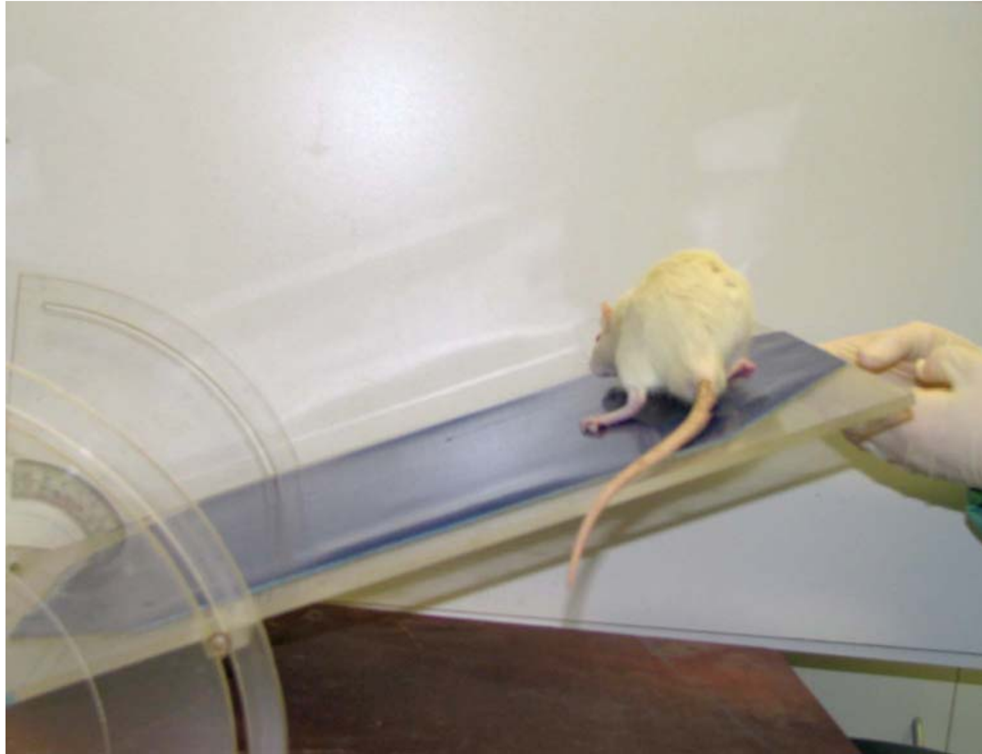

Figure 2.6 Hemiparesis – Angleboard.

The angleboard is slowly lifted and the angle at which the animal loses its footing is recorded. Here, the animal is being tested for hemiparesis on the LHS of the body.

2.5 Histological Analysis

2.5.1 Perfusion Fixation and Brain Sampling

Following perfusion (Section 2.2.3) the body was left intact for at least 1 h before decapitation and brain removal to minimise artefactual damage. Brains were stored in 10% formalin for at least 7 d before being processed for histology. Brains were then blocked in a rodent brain blocker (Kopf, PA002) and sectioned into consecutive 2 mm slices. Slices were then processed overnight. The tissue processing was as follows: 20 mins each of graded ethanol baths (50%, 70%, 80%, 95%, 100%, 100%), followed by 2 xylene baths of 1.5 h each and finally followed by paraffin baths of increasing time (30, 60, 60, 90 mins). Finally, brain slices were embedded in paraffin wax and serial 5 µm sections were cut using a microtone (Micron). Sections were then stained as detailed in 2.4.4-2.4.9.

2.5.2 Haematoxylin & Eosin Staining (H&E)

Haematoxylin & eosin (H&E) staining of the brain sections was carried out to allow observation of neuronal changes within the infarcted brains. The staining was carried out as follows: slides were initially de-waxed by moving the slides through 2 changes of xylene (2 mins) and then into 2 changes of ethanol (2 mins). Slides were then placed in haematoxylin for 5 mins, washed under running water and then placed in acid alcohol. Next, slides were washed in water and then placed in lithium carbonate for 1 min, washed again and then placed in eosin for 1 min. Slides were then placed straight into 2 changes of ethanol (2 mins) and then into 2 changes of histolene (2 mins). Slides were cleared and mounted in DPX before being assessed using light microscopy (Olympus).

2.5.3 Immunohistochemistry for Substance P (SP)

Sections were incubated with a monoclonal antibody specific for SP to enable the visualisation of those neurons, glia and vessels expressing SP. Following EDTA retrieval, sections were incubated in SP primary antibody (Santa Cruz, sc-9758; 1/2000) at room temperature overnight. Sections were then washed in phosphate buffered saline (PBS) (2 x 3mins) before being incubated in an anti-goat IgG HRP conjugated secondary antibody (BA-9500; 1/250) for at least 30 mins at room temperature. Sections were once again washed in PBS (2 x 3 mins) and then incubated in the tertiary streptavin peroxidase conjugate (SPC) (Pierce) (1/1000), for at lest 1 h at room temperature. The immunocomplex was subsequently visualised, following wash in PBS (2 x 3 min), with 3,3' diaminobenzidine tetrahydrochloride (DAB; Sigma, D8001) as a chromogen in the peroxidase reaction. Sections were then assessed using light microscopy (Olympus).

2.5.4 Immunohistochemistry for Amyloid Precursor Protein (APP)

Sections were stained with monoclonal antibody specific for APP to enable the visualisation of those neurons, glia and vessels expressing APP. APP is an extremely sensitive marker of axonal injury (Blumbergs et al., 1995), used successfully in our laboratory in trauma studies (Van Den Heuvel et al., 1999; Van Den Heuvel et al., 1998). Following citrate retrieval, sections were incubated in APP primary antibody (Boéhringer 22C11; 1/1000) overnight at room temperature. Sections were then washed in PBS (2 x 3mins) before being incubated in an anti-mouse IgG HRP conjugated secondary antibody (BA-2000; 1/250) for at least 30 mins at room temperature. Sections were once again washed in PBS (2 x 3 mins) and then incubated with the tertiary antibody, SPC (1/1000), for at lest 1 h at room

temperature. The immunocomplex subsequently was visualised, following wash in PBS (2 x 3 min), with DAB (Sigma, D8001) as a chromogen in the peroxidase reaction. Sections were then assessed using light microscopy (Olympus).

2.5.5 Immunohistochemistry for Glial Fibrillary Assocaited Protein (GFAP)

Sections were stained for GFAP to identify the astrocytic response to ischaemia throughout the infarcted hemisphere. Following citrate retrieval, sections were incubated in GFAP primary antibody (Dako, Z0334; 1/ 30 000) overnight at room temperature. Sections were then washed in PBS (2 x 3mins) before being incubated in an anti-rabbit IgG HRP conjugated secondary antibody (BA-1000; 1/250) for at least 30 mins at room temperature. Sections were again washed in PBS (2 x 3 mins) and then incubated in the tertiary antibody, SPC (1/1000), for at lest 1 h at room temperature. The immunocomplex subsequently was visualised, following wash in PBS (2 x 3 min), with DAB (Sigma, D8001) as a chromogen in the peroxidase reaction. Sections were then assessed using light microscopy (Olympus).

2.5.6 Immunohistochemistry for ED-1

Sections were incubated with the ED-1 antibody, raised against the CD68 antigen, to detect the presence of macrophages/activated microglia throughout the infarcted hemisphere. Following citrate retrieval, sections were incubated in ED-1 primary antibody (AbD Serotec, MCA341R; 1/400) overnight at room temperature. Sections were then washed in PBS (2 x 3mins) before being incubated in an anti-mouse IgG HRP conjugated secondary antibody (BA-2000; 1/250) for at least 30 mins at room temperature. Sections were again washed in PBS (2 x 3 mins) and then incubated in the tertiary antibody, SPC (1/1000), for at least 1 h at room

temperature. The immunocomplex subsequently was visualised, following wash in PBS (2 x 3 min), with DAB (Sigma, D8001) as a chromogen in the peroxidase reaction. Sections were then assessed using light microscopy (Olympus).

2.5.7 Fluoro Jade C (FJC)

The Fluoro Jade C antibody was used to detect degenerating neurons following ischaemia. Sections were de-waxed and then taken through 70% alcohol (2 mins) and 2 changes of dH_20 (2 mins). Sections were then incubated in 0.06% potassium permangenate (10 mins) and then washed in 2 changes of dH_2O (2 mins) before being incubated in FJC solution (in 0.1% glacial acetic acid). After washing 3 times in dH_20, sections were then dried at 45°C for 10-15 mins. Slides were then taken though histolene (2 x 2 mins) and mounted in DPX. All sections were assessed using fluorescence microscopy (Olympus).

2.6 Oedema Study

2.5.1 Wet Weight – Dry Weight

The amount of brain water was calculated using the wet weight–dry weight method. At 24 h post-reperfusion, animals were re-anaesthetised with 3% halothane or isoflurane (1.5L/min O_2) until a surgical level of anaesthesia was reached. They were then decapitated with a guillotine and the brain rapidly removed. The olfactory bulbs and cerebellum were discarded and the left and right hemispheres separated and placed in separate pre-weighed vials, with quick-fit lids to prevent evaporation. Vials were immediately weighed to obtain wet water content. Lids were removed and the vials placed in a 100°C oven for 72 h, before being re-

weighed after 72 h to obtain the dry weight. The percentage of brain water was subsequently calculated as follows:

$$\% \text{ Water} = \frac{(\text{Wet Weight} - \text{Dry Weight})}{\text{Wet Weight}} \times 100$$

2.7 Assessment of Blood Brain Barrier Permeability

2.6.1 Evan's Blue Extravasation

Evan's Blue (EB; MW 69 000; Sigma, E-2129) extravasation was used to assess permeability status of the BBB following cerebral ischaemia. EB binds to serum albumin that cannot normally penetrate the BBB. Following disruption of the BBB the EB-albumin complex gains access to the brain through the breached BBB. The amount of EB within the brain tissue gives information on the degree of BBB permeability. At the pre-determined time-point animals were anaesthetised with halothane or isoflurane. The chest cavity was rapidly opened and the animal transcardially perfused with saline until the perfusate from the right atrium ran clear. Following the saline flush, the brain was rapidly removed and the olfactory bulbs and cerebellum discarded and left and right hemispheres separated and placed in separated vials. The hemispheres were then weighed and homogenised in 2.5 ml PBS. 2.5 ml of trichloroacetic acid (Sigma, T-0699) was then added to the homogenate and the samples vortexed for 2 mins. The samples were then stored at 4°C overnight before being centrifuged at 1000G for 30 mins. The absorbance of the supernatant was measured at 610 nm using a UV/Vis spectrophotometer. The level of extravasated EB within the tissue was then determined using a previously obtained EB standard curve, and was expressed as μg/mg brain tissue.

2.7 Assessment of Infarct Volume

2.7.1 TTC

Infarct volume was determined using 2,3,5-triphenyltetrazolium chloride (TTC) staining. At 24 h post-stroke animals were decapitated under halothane or isoflurane anaesthesia. Their brains were then rapidly removed and TTC (Sigma, T-8877) staining was used to determine infarct volume as described elsewhere (Bederson et al., 1986a; Li et al., 1997; Vivaldi et al., 1985). TTC reacts with a reaction product of the mitochondrial respiratory chain and therefore stains viable mitochondria. Non-infarcted tissue stains a red colour and infarcted tissue remains a pale cream/white colour. Using a brain matrix (Kopf, PA002) the brain was cut into 2 mm slices and placed into tris saline (Sigma, F-1503). The slices were then incubated in 3% TTC at 37ºC under dark room conditions for 30 mins, turning once. Anterior and posterior sides of all brain slices were digitally scanned (Canon). The degree of cortical, striatal and total infarction was determined by an blinded observer, experienced in the evaluation of infarct determination, using Adobe Photoshop.

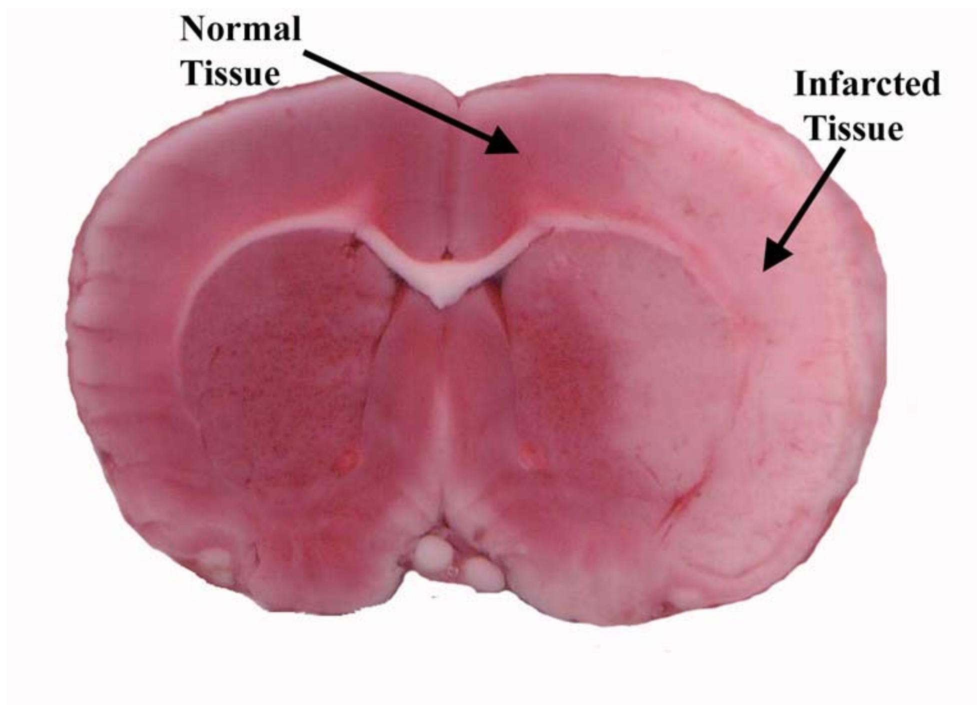

Figure 2.7 Degree of Infarction – TTC staining.

This figure shows a representative brain slice stained with TTC. The dark pink/red area represents normal tissue whereas the pale area within the right hemisphere represents infarcted tissue.

2.8 ELISA for SP

At 24 h post-reperfusion, animals were re-anaesthetised with 3% halothane or isoflurane (1.5L/min O_2) until a surgical level of anaesthesia was reached, and then the brain was rapidly removed. The olfactory bulbs and the cerebellum were discarded and the left and right hemispheres separated. Brains were stored at -80°C until use. Samples were rapidly thawed in a 60 degree water bath and prepared to a dilution of 400 ng protein per 100 μl in tris buffered saline (TBS). To prepare the ELISA, 100 μl of sample was loaded per well into a 96 well plate (Nunc, F96 Maxisorp). Blank wells with no loaded protein were included as a control. Protein was allowed to coat the wells overnight, and stored at 4°C in a humid container. The protein was then tipped off and the wells blocked with 0.5% gelatin in TBS, at room temperature with gentle agitation for 1 h. The tray was then washed in TBS (3 x) and incubated in primary antibody for SP (Chemicon, AB1566; 1/1000) for at least 1 h at 37° C in humid container. Following wash in TBS (3 x) the tray was then incubated in an anti-rabbit secondary antibody (Molecular and Life Sciences Biobar; 1/500) for at least 1 h at 37° C in a humid container. The tray was then washed in TBS (4 x) and the liquid substrate system, 3,3'-5,5'-tetramethylbenzidine (TMB; Sigma, T-8665) was used to develop reaction, with 100μl added per well. The reaction was then stopped with 50μl of 0.5M H_2SO_4/ well. The absorbance at 414 nm was then recorded.

2.9 Statistical Analysis

All parametric data was analysed using analysis of variance followed by Bonferroni post-tests. The non-parametric data was analysed using Kruskal Wallis ANOVA

followed by individual Dunn's multiple comparisons test. All parametric data are expressed as mean +/- SEM.

CHAPTER 3:
CHARACTERISATION OF THE SUBSTANCE P RESPONSE FOLLOWING PERMANENT VERSUS TRANSIENT MIDDLE CEREBRAL ARTERY OCCLUSION

3.1 Introduction

Stroke affects more than 15 million people globally each year. Of these, approximately 5 million die and 5 million are left permanently disabled. Hence, stroke is a significant health problem with billions of dollars spent each year on the hospitalisation and rehabilitation of stroke patients. Identification of factors involved in the injury cascades following ischaemia that lead to cell death, cerebral oedema and the resultant functional deficits will prove useful in the development of effective therapeutic agents.

Substance P (SP) is a neuropeptide that is the major initiator of neurogenic inflammation, a process characterised by vasodilation, increased vascular permeability and tissue swelling. The role of neurogenic inflammation in tissue swelling has been well documented in peripheral tissues such as the skin, however its potential role in brain pathologies has only recently been studied. Reports from our laboratory have detailed a role for neurogenic inflammation following TBI, where increased SP immunoreactivity in perivascular tissue was associated with breakdown of the BBB with subsequent vasogenic oedema formation and greater functional outcome deficits (Nimmo et al., 2004; Vink et al., 2003). To date, few studies have investigated the role of SP following cerebral ischaemia (Stumm et al., 2001; Yu et al., 1997). Although an over-expression of SP has previously been reported, there have been no follow up studies. Given that trauma and stroke share a number of secondary injury factors, SP release may also be involved in the genesis of cerebral oedema following stroke.

Although reperfusion of the ischaemic territory through restoration of blood flow is desirable to salvage ischaemic tissue and limit infarction, the contribution of reperfusion injury to tissue damage following ischaemia is well documented. The reintroduction of blood to the ischaemic tissue may further exacerbate tissue damage via a number of mechanisms including the production of free radicals (Belayev et al., 1996b; Kastrup et al., 1999; Lo et al., 2001; Yatsu et al., 1987). Indeed, SP release has been implicated in free radical formation (Weglicki, 1998). However, the role of neurogenic inflammation in transient and permanent ischaemia has not been documented. The aim of the present study was therefore to investigate the role of neurogenic inflammation in transient and permanent cerebral ischaemia, with particular reference to the SP response.

3.2 Study Design

The present study was divided into 2 components: immunohistochemistry and ELISA. Animals (n=47) were subject to MCAO (as described in 2.1.2). At the pre-determined time-points, animals were then either perfusion-fixed with 10% formalin for immunohistochemistry or the brain rapidly removed for ELISA determination of SP levels. These procedures are outlined briefly below.

3.2.1 Immunohistochemistry

Animals in the tMCAO group received 2 h of occlusion followed by either 5 h, 24 h or 7 d of reperfusion. Animals in the pMCAO group received 7 h or 26 h of occlusion, which was the equivalent time-point to the tMCAO group. Sham MCAO animals were included as controls, however the contralateral (non-infarcted) hemisphere of injured animals also served as an intrinsic control. At the specified

time-point, animals were transcardially perfused with formalin and their brains blocked, processed, sectioned and stained (as detailed in Chapter 2.4.2; 2.4.4-2.4.8). SP, APP, ED-1, GFAP, FJC and H&E staining was then performed on the tissue and assessed using light and flurorescence microscopy, as appropriate.

3.2.2 Enzyme Linked Immunosorbent Assay (ELISA)

A subset of animals were used for the determination of SP levels within the brains of sham and tMCAO (24h) animals. As described in Chapter 2, brains were removed fresh and processed for protein extraction. SP levels of the right hemisphere were then determined using ELISA.

3.2.3 Statistical Analysis

The ELISA data was analysed using ANOVA followed by individual Student Newman-Keuls post-hoc tests. The level of significance was taken at $p<0.05$.

3.3 Results

Previous studies have shown that openings of the BBB occurs at various time-points following stroke (Kuriowa et al, 1985a, Kuriowa et al, 1985b, Kastrup et al, 1999, Belayev et al, 1996). In particular, Preston and colleagues (Preston et al., 1993) reported openings of the BBB immediately after stroke and at 6-24 h after ischaemia. Hence, the 5 h and 24 h post-reperfusion time-points were chosen to coincide with such openings.

Haemorrhagic transformation was a common complication of MCAO surgery, especially when associated with reperfusion. Figure 3.1 shows as example of

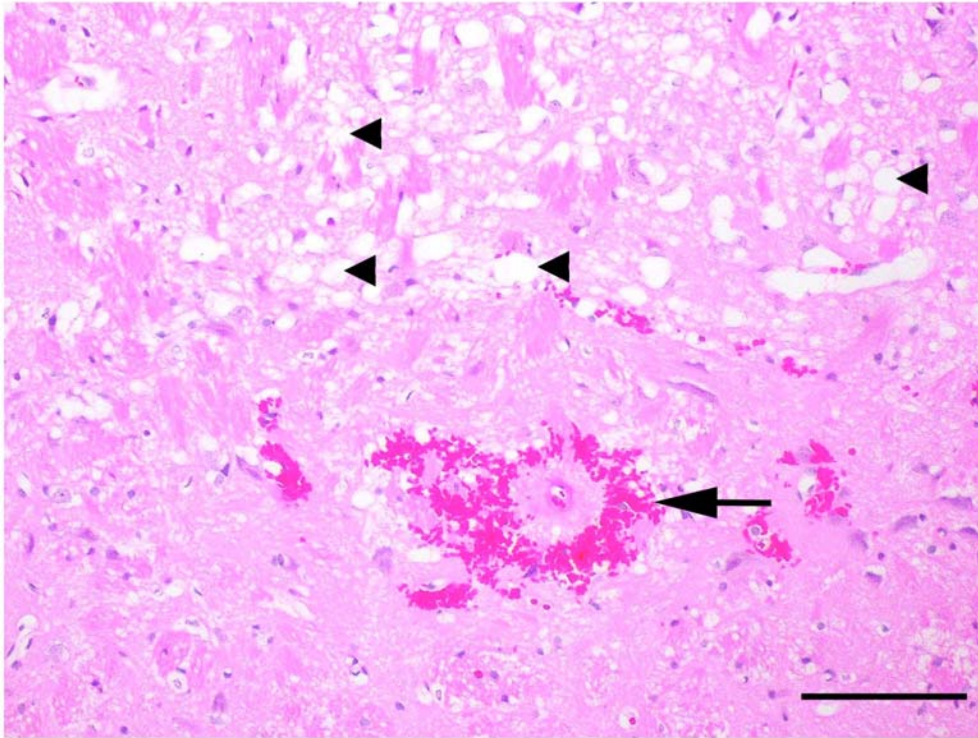

Figure 3.1 Permanent versus transient stroke. Haemorrhagic Transformation – H&E stained sections (Bar = 100 μm).

Haemorrhagic transformation was observed within the parenchyma and around blood vessels, seen here in the white matter (arrow). Note also the severe vacuolation (arrowheads) of the tissue.

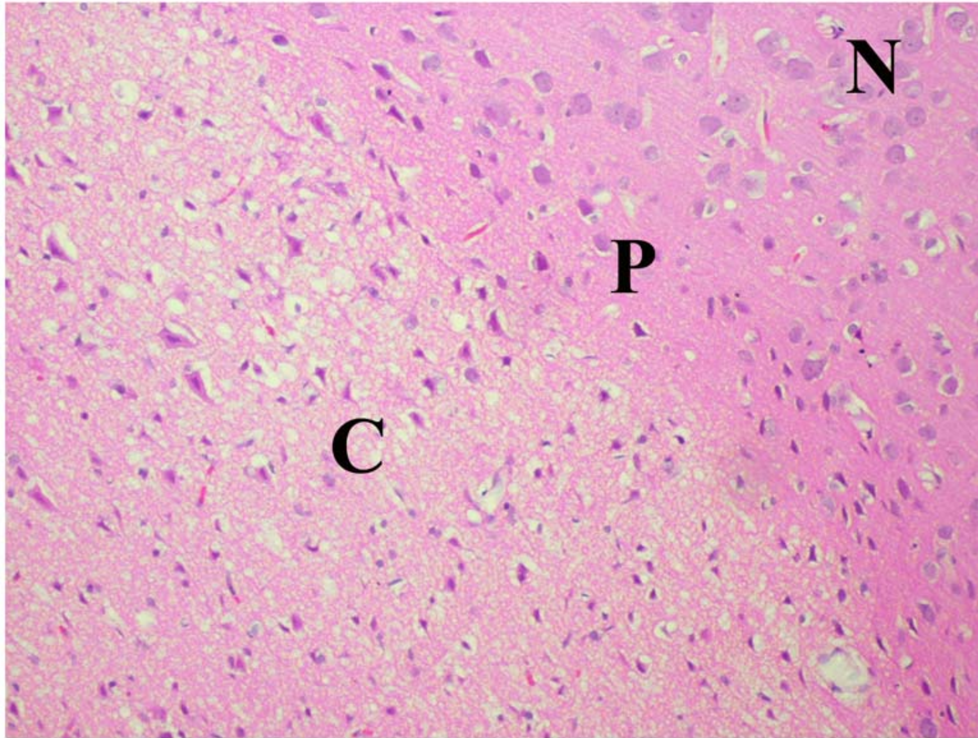

Figure 3.2 Permanent versus transient stroke. Infarction – H&E stained sections (10x).
Clear demarcation between the core (C), penumbra (P) and normal (N) tissue is evident. Normal neurons appear round and healthy. Neurons within the penumbra show DCC. Whilst neurons within the infarct core showed a mixture of DCC and RCC. Vacuolation of the parenchyma and tissue pallor is also evident.

haemorrhagic transformation observed around blood vessels and within the parenchyma. Figure 3.2 shows the clear demarcation between the core, penumbral and normal tissue seen with H&E stain. Cells within the ischaemic core show red cell change (RCC). Cells within the ischaemic penumbra display dark cell change (DCC), indicative of cell damage. Although the interpretation of DCC is somewhat controversial, it is generally accepted that it represents a cell-stress response where the cell may recover should the injurious agent be removed. However, this is discussed in greater detail later in the chapter.

3.3.1 pMCAO

General Pathology: H&E

H&E staining was used to assess general pathology and morphology of the tissue following 7 h and 26 h of permanent ischaemia. No abnormalities were seen on H&E in sham animals in either the cortex (Figure 3.3) or white matter (Figure 3.4). After 7 h of MCAO the right hemisphere had developed marked infarction with substantial vacuolation and retraction of the parenchyma, and the clear development of cell injury as indicated by DCC. By 26 h the infarct had progressed to occupy much of the right hemisphere (Figure 3.3-3.4), with the ischaemic damage much more advanced than that seen at 7 h. Both RCC and DCC were apparent. Significant loss of normal cortical and white matter architecture was observed. Hemispheric enlargement and midline shift were commonly observed in the 26 h group, indicative of cerebral oedema (Figure 3.5).

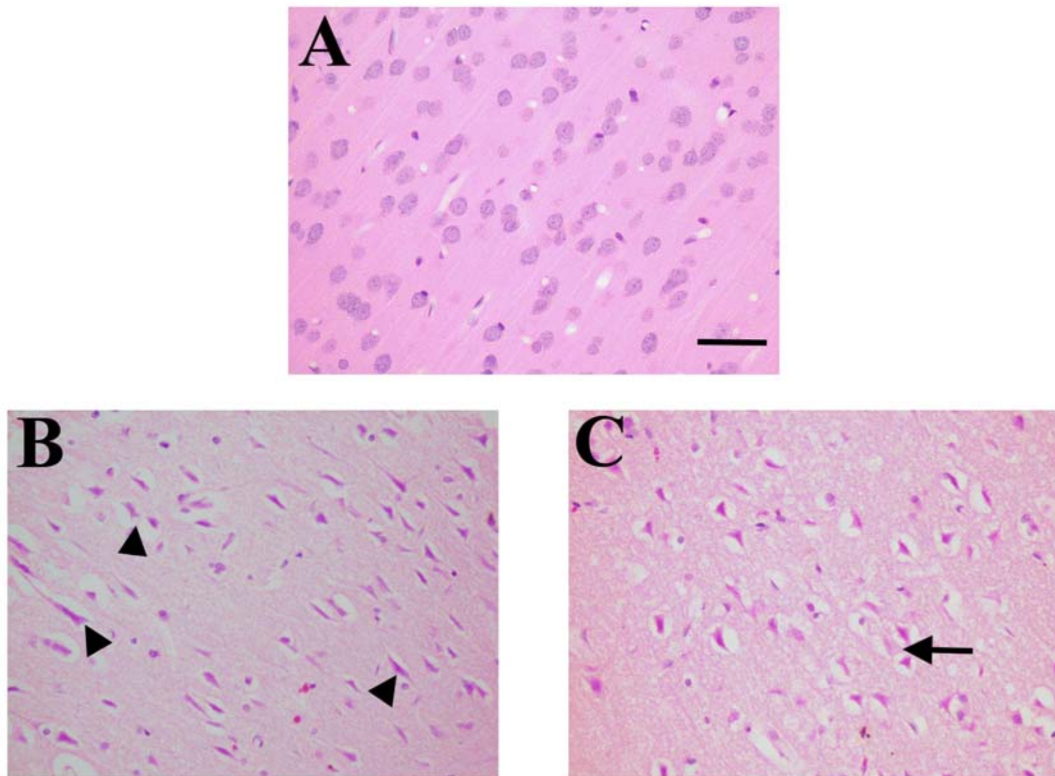

Figure 3.3 Permanent versus transient stroke. pMCAO Cortex – H&E stained sections (Bar = 100 μm).

No abnormalities were detected in sham tissue with neurons demonstrating normal appearance (A). After 7 h of ischaemia significant infarction as indicated by vacuolation, DCC (Arrowheads) and tissue pallor is noted (B). Infarction was more advanced after 26 h of ischaemia (C) with further vacuolation, retraction of parenchyma, tissue pallor and cell injury/loss evident. At 26 h post-ischaemic onset, both DCC and RCC (arrow) were noted (C).

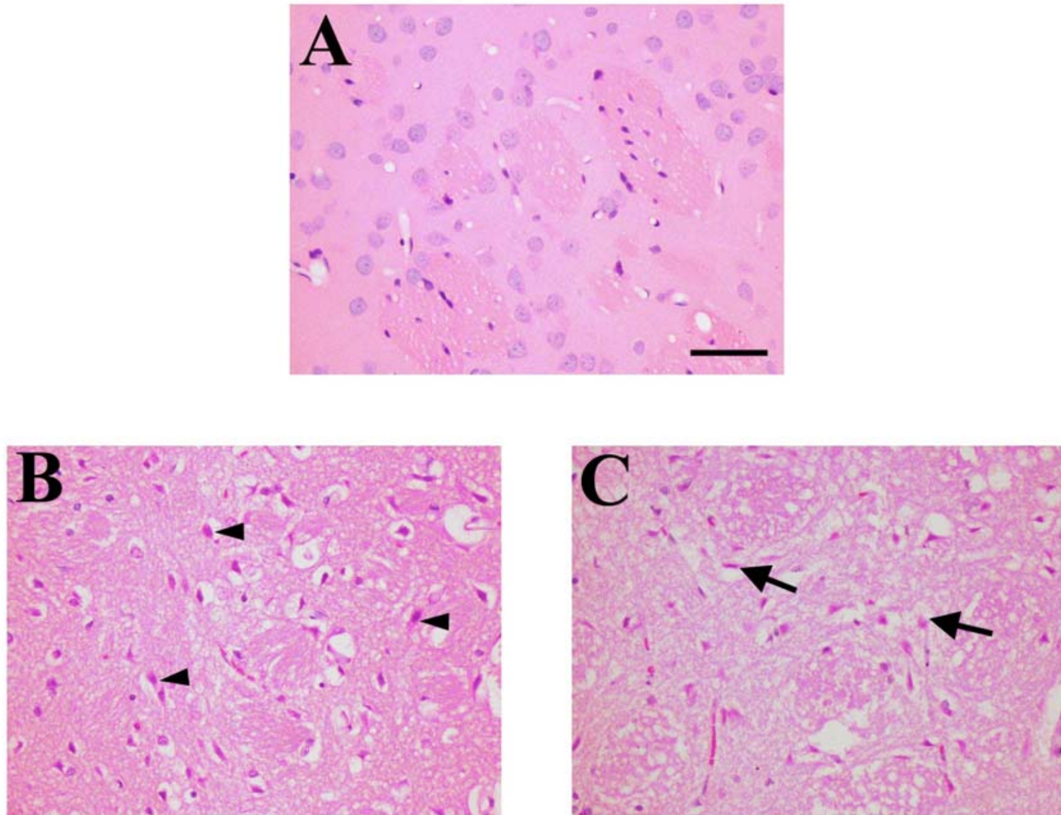

Figure 3.4 Permanent versus transient stroke. pMCAO White Matter – H&E stained sections (Bar = 100 μm).

No abnormalities were apparent in the white matter of sham tissue, with healthy neurons observed (A). After 7 h of ischaemia vacuolation of the white matter was clearly apparent in addition to DCC (B; arrowheads). Infarction was more advanced after 26 h of ischaemia (C) with further destruction of the white matter evident, with RCC (arrows) and a clear loss in normal tissue architecture and evidence of cell loss.

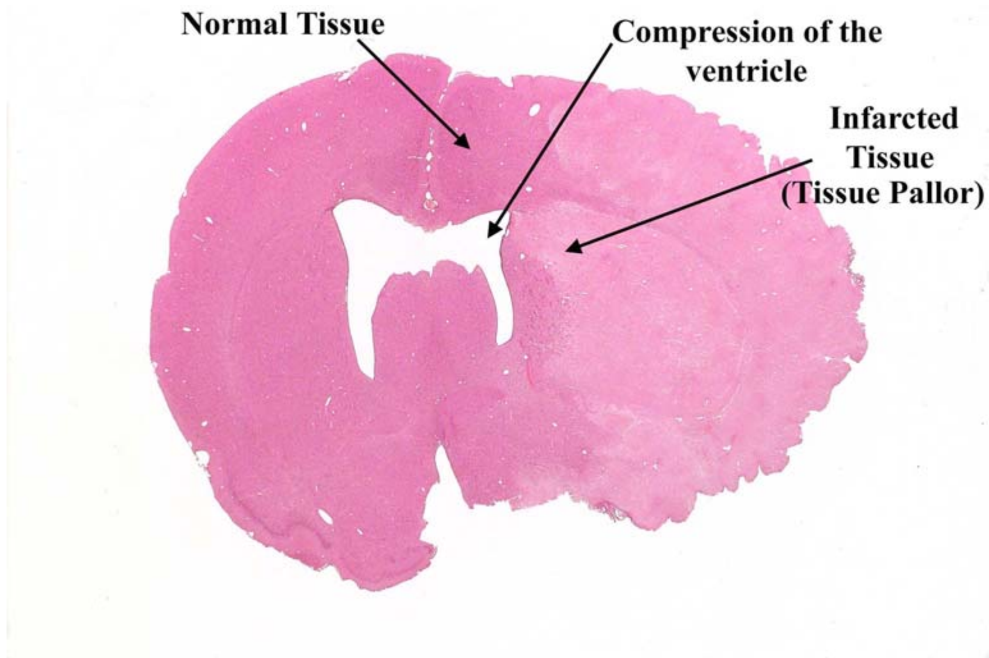

Figure 3.5 Permanent versus transient stroke. pMCAO – H&E stained sections (Bar = 100 μm).
Note the hemispheric enlargement on the right hemisphere, indicative of cerebral oedema. Note also the tissue pallor that delineates the infarct. Tissue was extremely friable.

SP Response: SP Immunohistochemistry

In sham animals light staining of neurons within the cortex and perivascular tissue was seen (Figure 3.6-3.7). After pMCAO, there was little change in the SP immunoreactivity at either the 7 h or 26 h time-points. Apart from increased parenchymal staining, there was no change around blood vessels (Figure 3.6). The increased parenchymal immunoreactivity was more evident in cortical sections (Figure 3.7). The increased parenchymal staining was consistent with earlier reports of increased glial SP immunoreactivity after stroke.

Axonal Injury: APP Immunohistochemistry

APP immunohistochemistry was used to determine the degree of axonal injury and neuronal injury following 7 h and 26 h of permanent ischaemia. Extensive axonal injury, as indicated by APP immunoreactive retractions balls, was observed following pMCAO. Axonal injury was apparent as early as 7 h following ischaemic onset and was more intense and widespread by 26 h post-ischaemic onset (Figure 3.8). Axonal injury was observed throughout the white matter and was especially prominent within the white matter tracts and bundles. An increase in neuronal APP immunoreactivity was also seen following pMCAO. In particular, at 7 h following ischaemia onset shrunken, injured neurons within the cortex showed an increase in APP expression (Figure 3.9). By 26 h post-ischaemic onset the APP immunoreactivity was more intense, seen as very dark brown-orange staining within injured neurons. Previous studies have suggested that such APP immunoreactivity is indicative of a neuronal stress response (Van Den Heuvel et al., 2000b; Van Den Heuvel et al., 2000a).

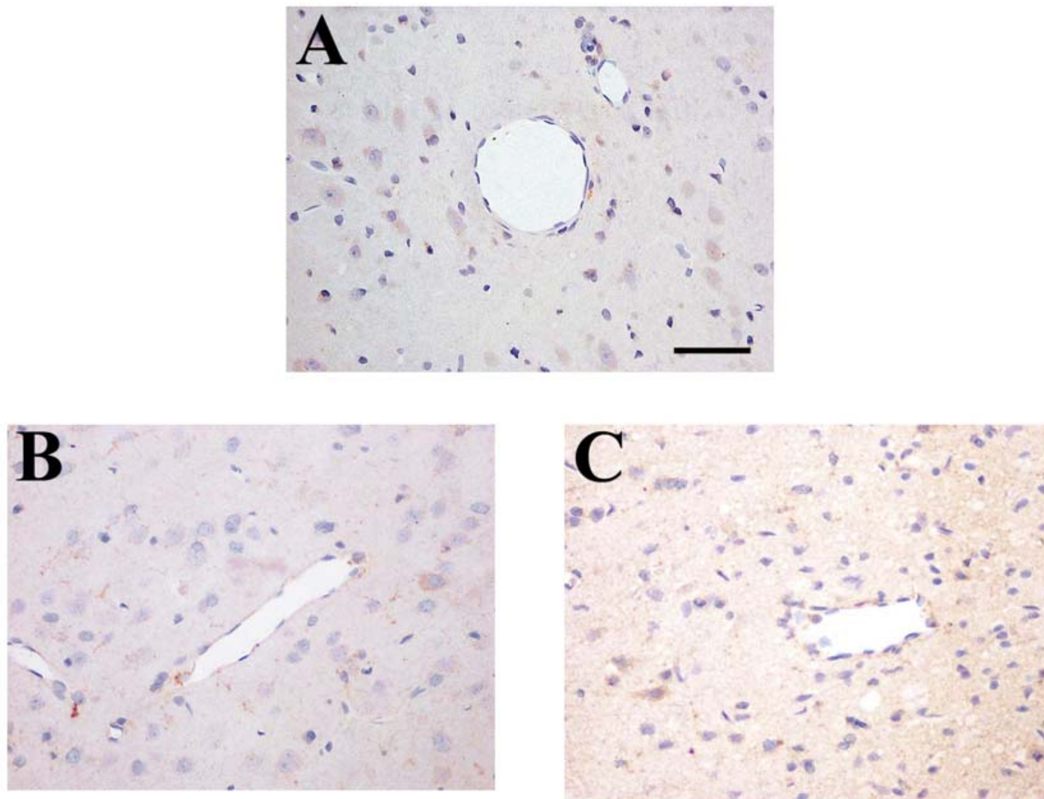

Figure 3.6 Permanent versus transient stroke. pMCAO Perivascular Tissue – SP stained sections (Bar = 100 μm).

Light SP immunoreactivity was observed around blood vessels and surrounding neurons in sham tissue (A). After both 7 h (B) and 26 h (C) of ischaemia the level of perivascular and neuronal SP immunoreactivity appeared comparable to shams, however an increase in parenchymal staining was observed.

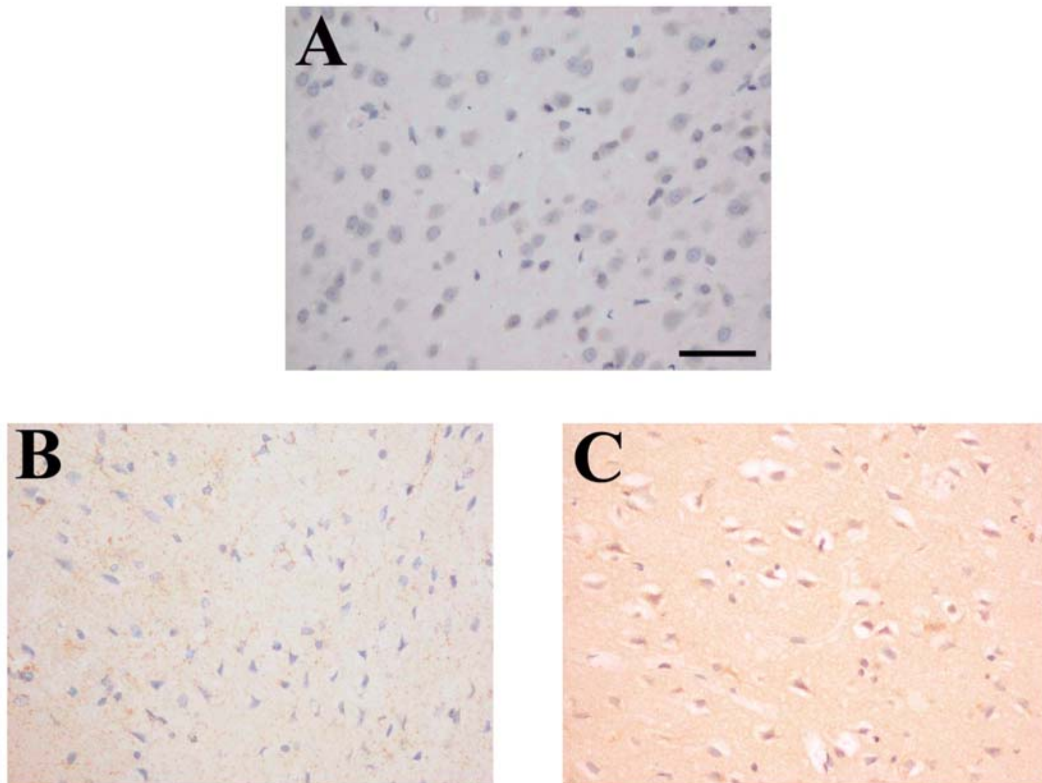

Figure 3.7 Permanent versus transient stroke. pMCAO Cortex – SP stained sections (Bar = 100 μm).

Faint SP immunoreactivity is observed in cortical neurons of sham animals (A). Following 7 h of ischaemia there was an increase in parenchymal staining for SP but a decrease in neuronal SP staining (B). After 26 h of ischaemia more neuronal SP immunoreactivity was evident than at 7 h but this was also associated with increased background staining (C).

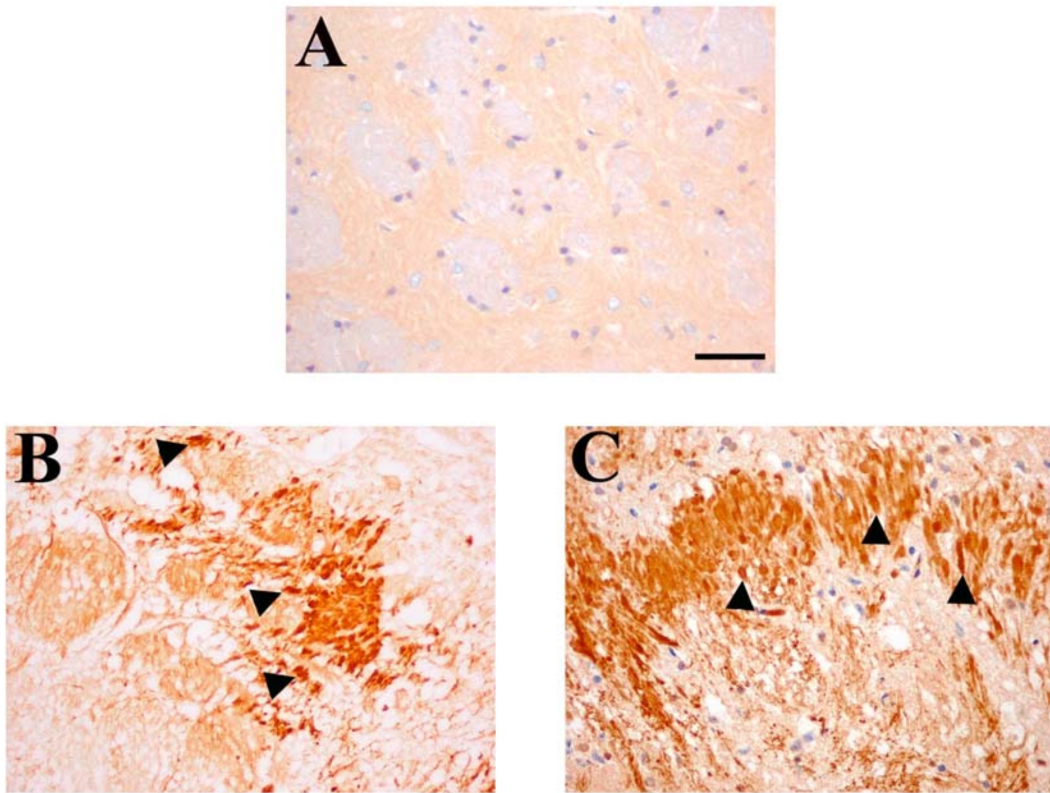

Figure 3.8 Permanent versus transient stroke. pMCAO White Matter – APP stained sections (Bar = 100 μm).

No APP immunoreactivity of the white matter bundles was observed in sham animals (A). Following 7 h of ischaemia there was a significant increase in APP staining and florid axonal injury (arrowheads) within the white matter, seen as an intense orange-brown staining (B). After 26 h of ischaemia, the axonal injury within the white matter was further advanced.

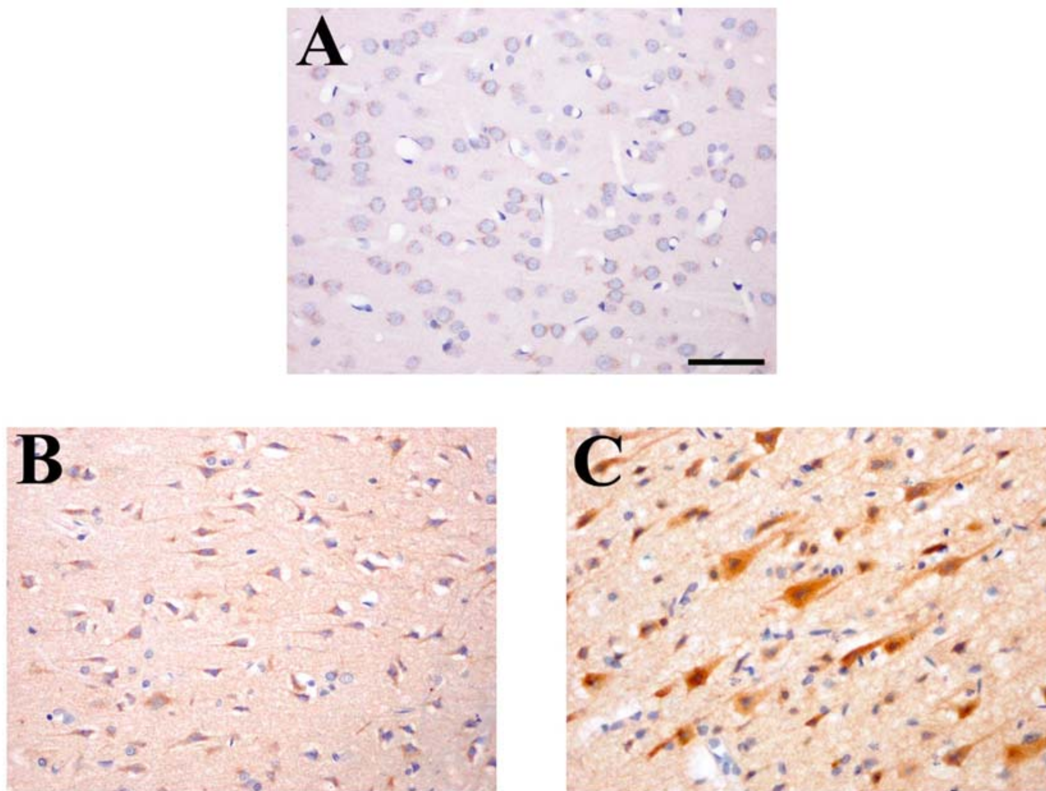

Figure 3.9 Permanent versus transient stroke. pMCAO Cortex – APP stained sections (Bar = 100 μm).

In sham tissue, normal, healthy neurons showed faint APP immunoreactivity (A). Following 7 h of ischaemia there was an increase in APP immunoreactivity of injured and shrunken, cortical neurons (B). Following 26 h of ischaemia, a more profound increase in cortical APP immunoreactivity was observed (C).

Degenerating Neurons: FJC

FJC was used to identify degenerating neurons following 7 h and 26 h of pMCAO. The cortex (Figure 3.10) and white matter (Figure 3.11) of sham animals showed no signs of degeneration by FJC staining. After 7 h of ischaemia there were FJC positive cells throughout the infarcted hemisphere. Such cells were shrunken and with pronounced vacuolisation, indicative of significant cell stress/injury. By 26 h, more extensive vacuolation of the parenchyma was evident along with marked cell loss within both the cortex and white matter.

3.3.2 tMCAO

General Pathology: H&E

General pathology and tissue morphology was assessed by H&E staining at 5 h, 24 h and 7 d following reperfusion. No abnormalities were seen on H&E stain within the cortex of sham animals (Figure 3.12). At 5 h post-reperfusion, the infarction was beginning to develop with injured cells displaying DCC. By 24 h post-reperfusion little of the hemisphere was spared with the infarct extending to include a large proportion of the striatum and regions of the cortex. The infarct core was characterised by shrunken neurons surrounded by a retracted parenchyma, with numerous cells showing RCC. In the surrounding penumbral regions, cells displaying DCC were evident. Early inflammatory cells were seen to infiltrate the infarct, in particular, neutrophils. By 7 d post-reperfusion the infarct was associated with a profound gliosis reaction, characterised by the proliferation of glial cells and the influx of blood-borne inflammatory cells such as macrophages. The gliosis occupied the majority of the infarcted hemisphere but was confined to regions of tissue destruction, and as such it was not observed in areas of tissue preservation.

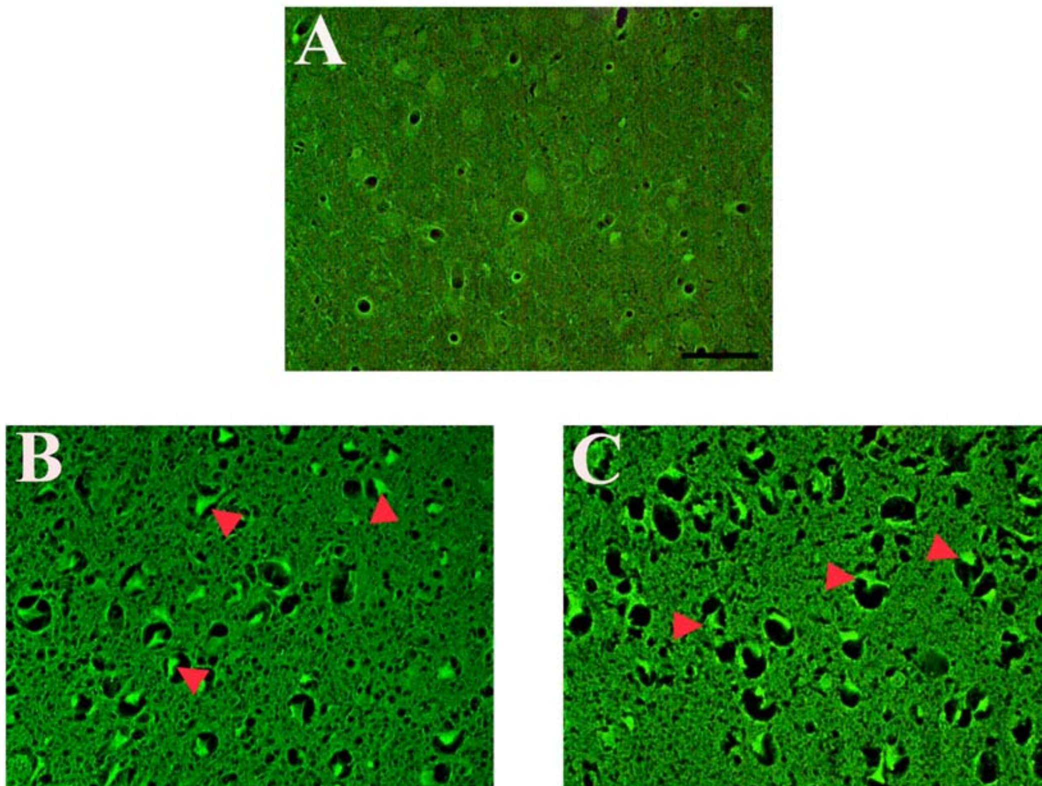

Figure 3.10 Permanent versus transient stroke. pMCAO Cortex-Degenerating Neurons – FJC stained sections (Bar = 100 µm).

The cortex of sham animals showed no signs of degeneration (A). Following 7 h of ischaemia the cortex was dominated by FJC positive neurons (arrowheads) that were shrunken and retracted with vacuolation of the surrounding parenchyma also evident (B). These changes were further exacerbated following 26 h of ischaemia (C).

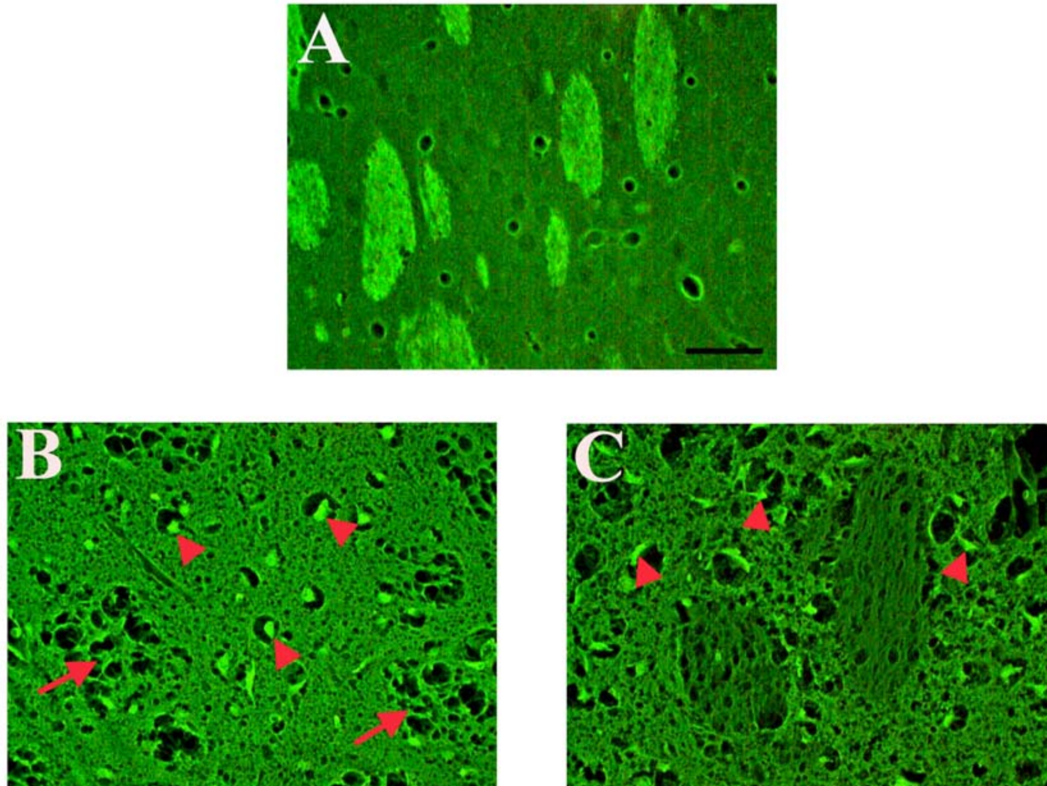

Figure 3.11 Permanent versus transient stroke. pMCAO White Matter-Degenerating Neurons – FJC stained sections (Bar = 100 μm).

The white matter of sham animals showed no signs of degeneration (A). After 7 h of ischaemia there is a clear loss of white matter architecture with vacuolation of the white matter bundles and the presence of degenerating neurons (B; arrowheads). Destruction of the white matter was further advanced following 26 h of ischaemia (C).

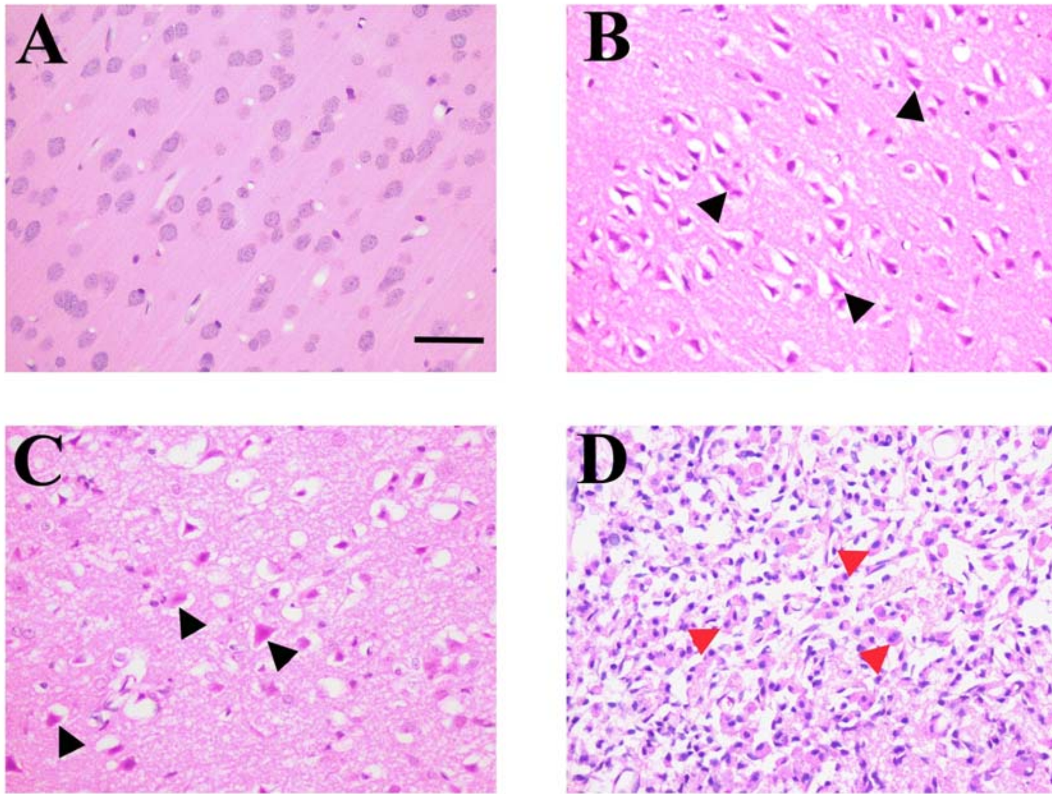

Figure 3.12 Permanent versus transient stroke. tMCAO Cortex – H&E stained sections (Bar = 100 µm).

No abnormalities were seen in sham tissue (A). Following 5 h of reperfusion there was a marked change in the cortex. Neurons were retracted and displaying signs of cell stress (DCC) and there was significant vacuolation (black arrowheads) and pallor of the parenchyma (B). These indicators of infarction were further advanced following 24 h of reperfusion. In addition, the presence of lethally injured cells displaying RCC was evident as well as evidence of cell loss (C). After 7 d of reperfusion (D) the ischaemic lesion was dominated by an extensive gliosis reaction, including the influx of inflammatory cells (macrophages; red arrowheads).

Gliosis is the key component of the cellular response to CNS injury (Stoll et al., 1998; Stoll and Schroeter, 2000).

No abnormalities were seen in the white matter of sham animals (Figure 3.13). At 5 h of reperfusion there were dark cells present throughout the white matter, which was associated with vacuolation of the parenchyma. By 24 h of reperfusion, cells were profoundly shrunken and parenchymal vacuolation was more advanced. There was evidence of cell loss in association with a loss of normal architecture within the white matter. Swelling of the infarcted hemisphere was also apparent with midline shift observed in some animals, indicative of cerebral oedema, as seen in the representative H&E and TTC images in Figure 3.14.

SP Response: SP Immunohistochemistry

The SP immunoreactivity of penumbral blood vessels and cortical neurons was assessed at 5 h, 24 h and 7 d following reperfusion. In sham animals very light SP immunoreactivity was observed in perivascular tissue. At 5 h post-reperfusion SP immunoreactivity was increased compared to shams, and was evident in neuronal, glial and perivascular tissue of the ischaemic penumbra (Figure 3.15-3.16). Specifically, the SP immunoreactivity around penumbral blood vessels was especially prominent. Vessels within the core of the infarct displayed less SP immunoreactivity than those in the penumbra, suggesting that the reperfusion was associated with increased SP immunoreactivity. By 24 h post-reperfusion, the perivascular SP immunoreactivity was markedly increased compared to shams.

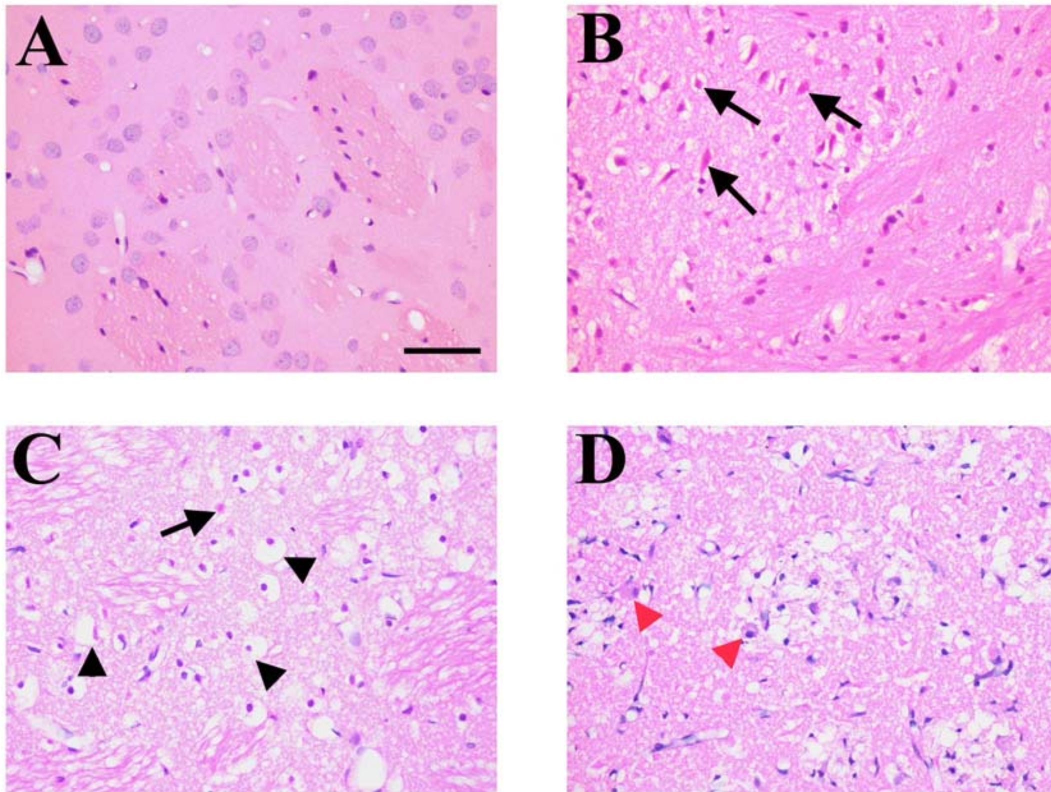

Figure 3.13 Permanent versus transient stroke. tMCAO White Matter – H&E stained sections (Bar = 100 μm).

No abnormalities were observed in the white matter of sham animals (A). At 5 h post-reperfusion, cells showing DCC were apparent along with some vacuolation (black arrowheads) of the parenchyma (B). By 24 h post-reperfusion, extensive vacuolation and cell injury (arrows) was apparent (C). By 7 d post-stroke, there was complete loss of normal white matter architecture with extensive cell loss and vacuolation. The influx of macrophage was also seen (D; red arrowheads).

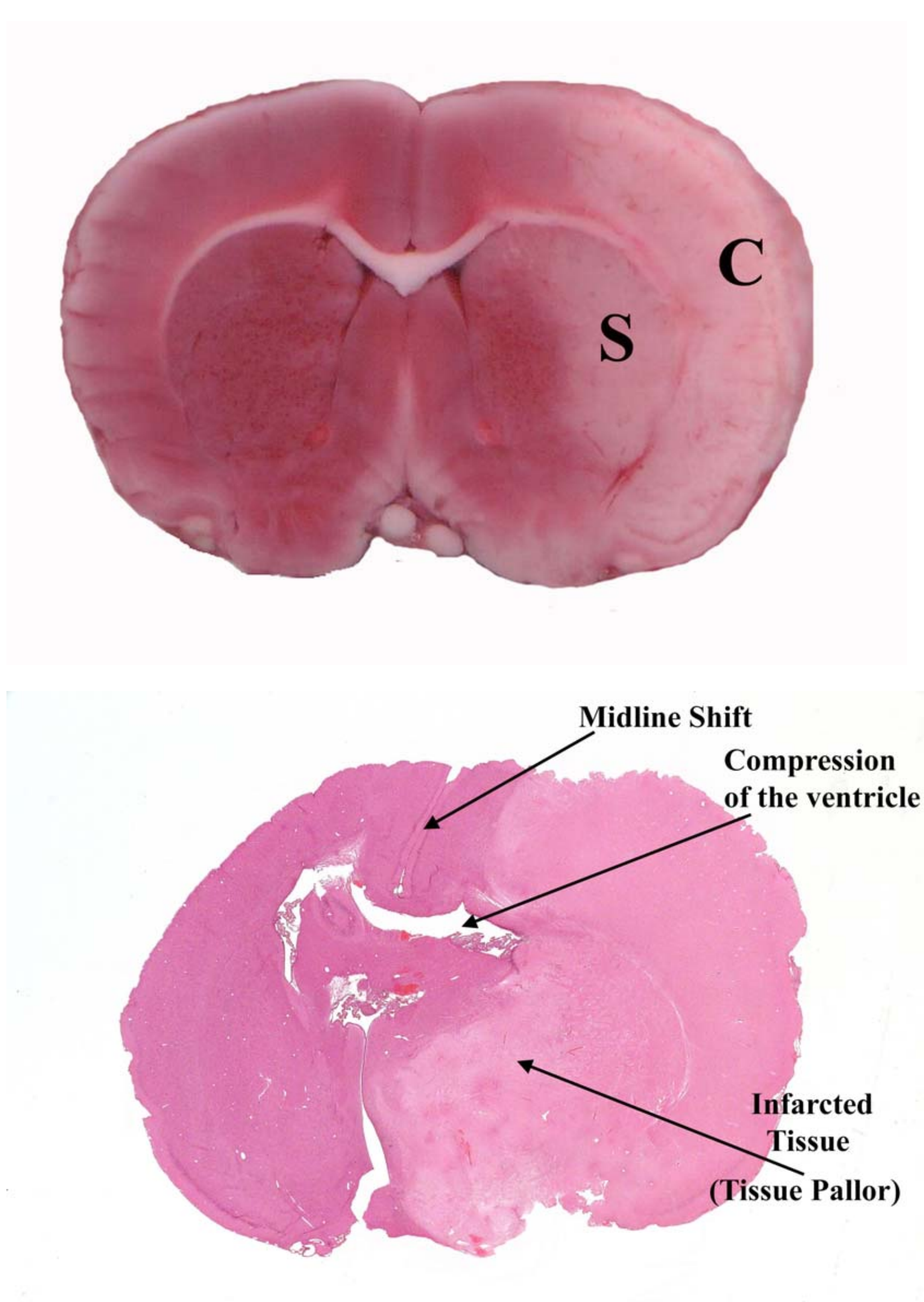

Figure 3.14 Permanent versus transient stroke - tMCAO.

The top panel shows a representative TTC stained brain slice. The C and S denote the regions of the cortex and striatum respectively that was infarcted. The bottom panel shows a H&E stained brain slice, showing the profound hemispheric swelling, midline shift, indicative of cerebral oedema, and the pallor of the infarcted tissue. Tissue was extremely friable.

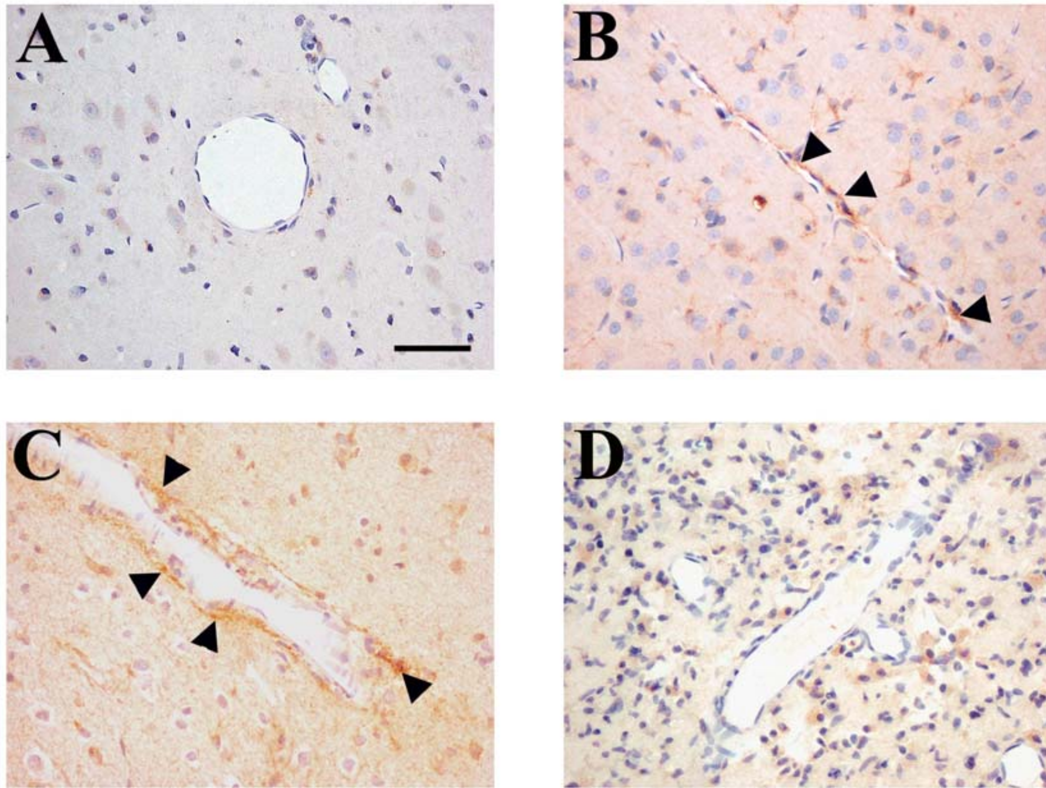

Figure 3.15 Permanent versus transient stroke. tMCAO Perivascular Tissue – SP stained sections (Bar = 100 μm).

Sham tissue showed negligible perivascular SP immunoreactivity (A). Following 5 h of reperfusion there was an increase in perivascular SP immunoreactivity (arrowheads) that was associated with an increase in parenchymal staining (B). After 24 h of reperfusion, the perivascular SP immunoreactivity was further increased (C). At 7 d post-reperfusion no SP immunoreactivity was seen around blood vessels. Extensive gliosis occupied the hemisphere that was associated with the influx of macrophages (D).

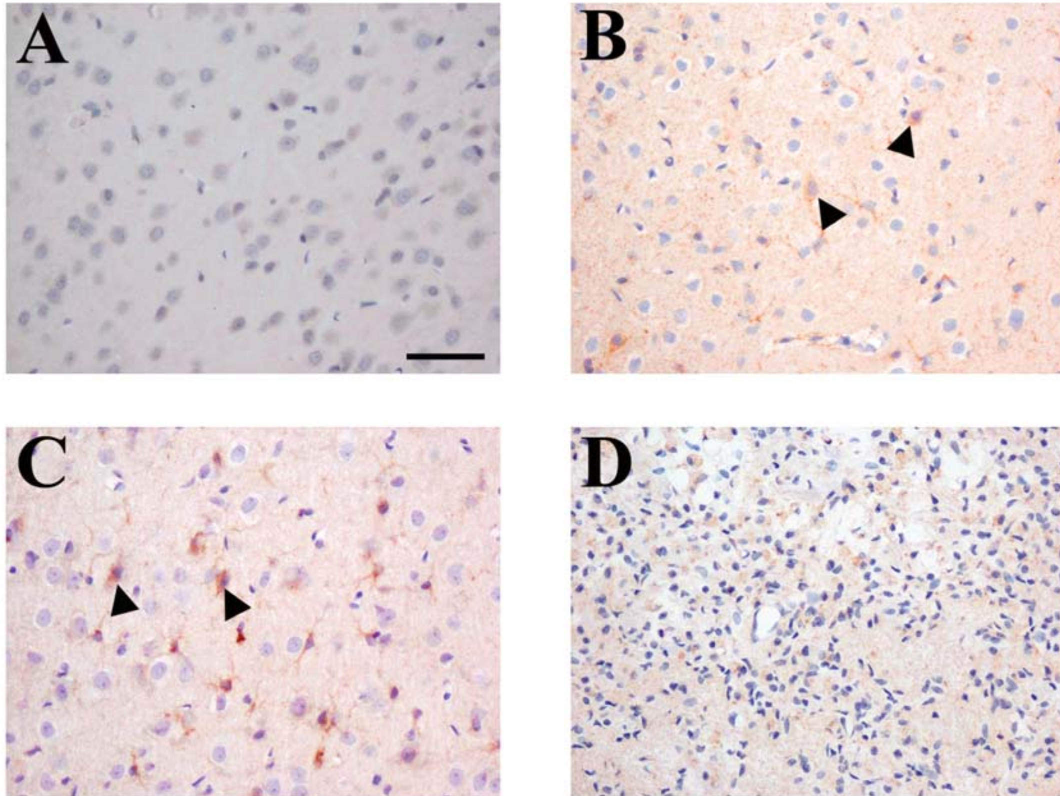

Figure 3.16 Permanent versus transient stroke. tMCAO Cortex – SP stained sections (Bar = 100 µm).

Sham tissue showed only faint neuronal SP immunoreactivity (A). After 5 h of reperfusion scattered neurons showing increased SP immunoreactivity (arrowheads) were seen within the ischaemic penumbra (B). By 24 h post-reperfusion such neurons displayed more intense SP immunoreactivity (C). Neuronal SP immunoreactivity was not seen at 7 d post-reperfusion due to the extensive gliosis that occupied the cortical tissue (D).

However, by 7 d post-reperfusion, SP immunoreactivity was not easily identified due to the extensive gliosis that occupied the majority of the ischaemic hemisphere. Sham animals showed very low levels of SP immunoreactivity within cortical neurons (Figure 3.16). At 5 h of reperfusion a subset of neurons displayed increased SP immunoreactivity. By 24 h post-reperfusion the neuronal SP immunoreactivity was quite extensive and was mainly confined to cells displaying an injured morphology (shrunken and retracted from the parenchyma). After 7 d of reperfusion, as was the case with the perivascular SP immunoreactivity, neuronal SP staining was not readily identified due to the gliosis reaction that consumed the ischaemic hemisphere.

Axonal Injury: APP Immunohistochemistry

APP immunoreactivity was assessed at 5 h, 24 h and 7 d following reperfusion of the ischaemic territory. Florid axonal injury, as demonstrated by swollen APP immunoreactive axons, was seen as early as 5 h post-reperfusion (Figure 3.17). By 24 h post-reperfusion the axonal injury had advanced to include moderately sized retraction balls. Significant APP immunoreactivity was still evident at 7 d post-reperfusion with larger retraction balls observed, as compared to the more acute time-points.

Light APP immunoreactivity was observed within cortical neurons of sham animals. After 5 h of reperfusion in cortical tissue, the expression of APP was increased as compared to shams (Figure 3.18). Following 24 h of reperfusion neurons were more shrunken and contracted and still expressing more APP than shams. Such increased neuronal expression of APP was still apparent by 7 d post-reperfusion.

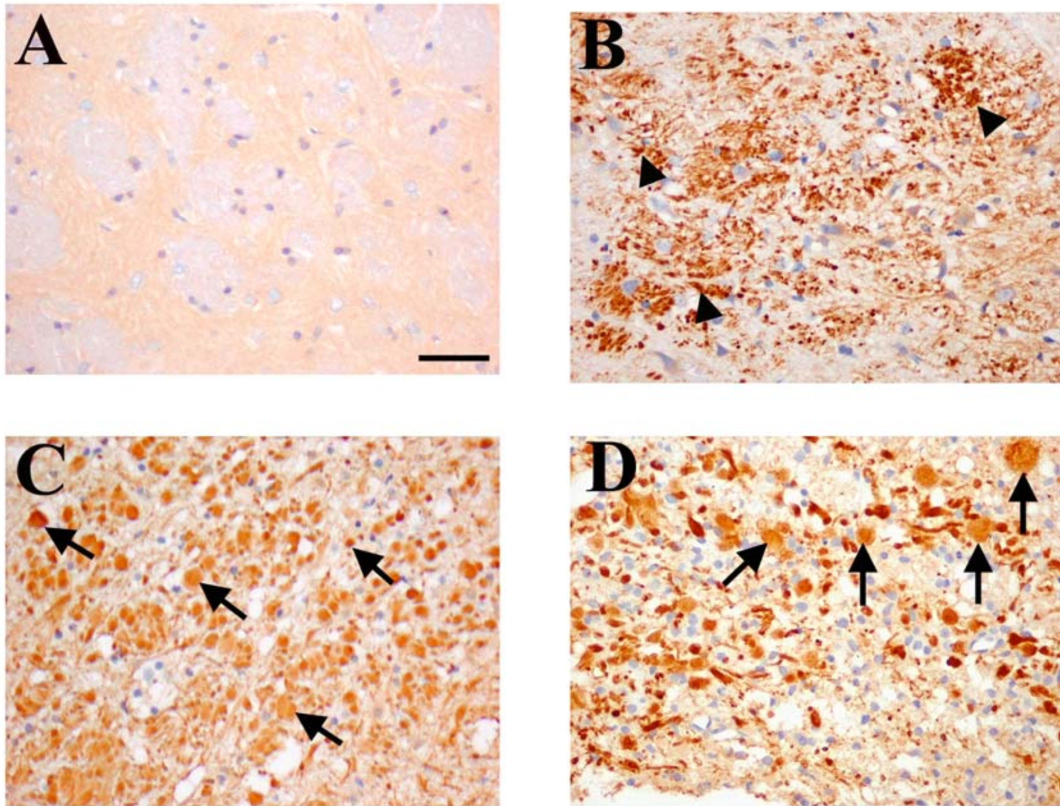

Figure 3.17 Permanent versus transient stroke. tMCAO White Matter – APP stained sections (Bar = 100 μm).

No APP immunoreactivity within the white matter bundles was seen in sham tissue (A). After 5 h of reperfusion, florid axonal injury was seen throughout the white matter (B). By 24 h post-reperfusion, the axonal injury (arrowheads) had advanced and retraction balls (arrows) dominated the white matter (C). By 7 d post-reperfusion, large retraction balls were apparent throughout the white matter (D).

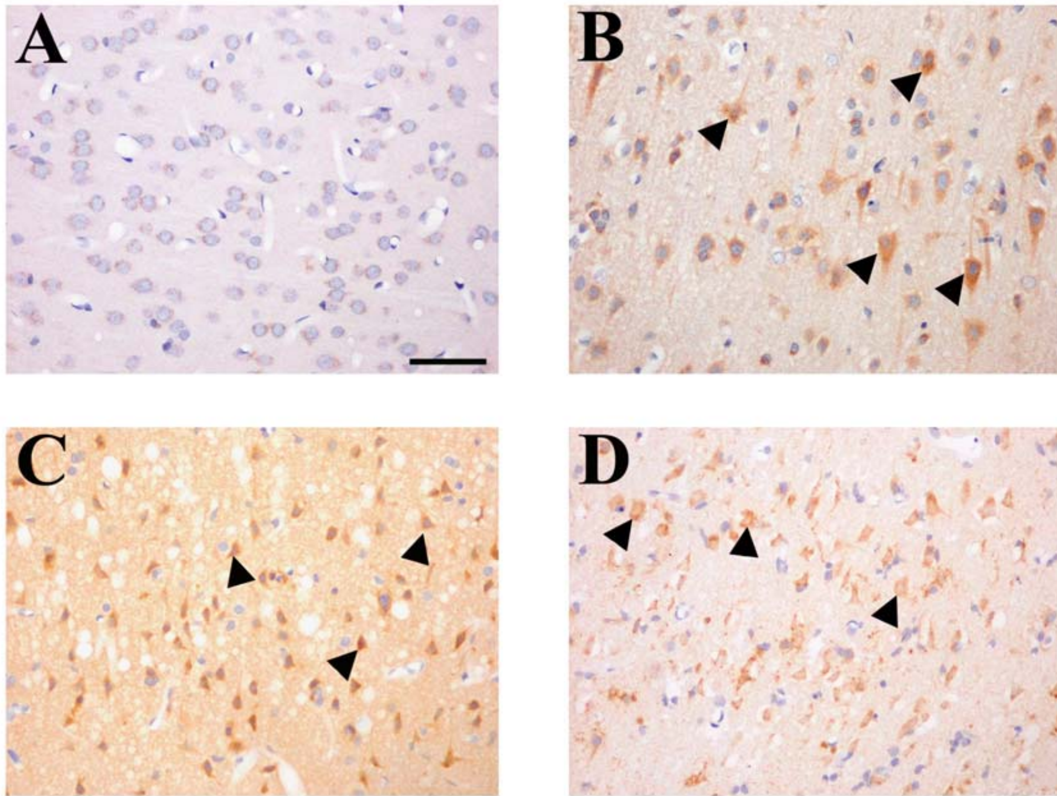

Figure 3.18 Permanent versus transient stroke. tMCAO Cortex – APP stained sections (Bar = 100 μm).

Faint neuronal APP immunoreactivity was seen in sham tissue (A). After 5 h (B) and 24 h (C) of reperfusion an increase in neuronal APP immunoreactivity (arrowheads) was observed. Substantial APP immunoreactivity of neurons was still evident at 7 d post-reperfusion (D).

Degenerating Neurons: FJC

Fluoro Jade C was used to identify degenerating neurons at 5 h, 24 h and 7 d post-reperfusion. In sham tissue, there were no signs of tissue abnormalities with no FJC positive neurons observed (Figure 3.19-3.20). Following tMCAO there was a profound increase in the appearance of FJC positive neurons within the cortex and white matter. This response was seen at both 5 h and 24 h following reperfusion, with the number of degenerating neurons far greater in the 24 h group. Clearly, the infarct had advanced substantially by this stage as evidenced by the large number of FJC positive cells present. By 7 d of reperfusion FJC positive neurons were not visible due to the extensive gliosis that occupied the ischaemic infarct. This was consistent with the H&E stain (Figures 3.10-3.11).

As the gliosis reaction was so extensive in the 7 d group it was difficult to assess the degree of immunoreactivity of the various stains and the amount of tissue preservation. Accordingly, we used ED-1 and GFAP immunohistochemistry to further characterise the tissue response to ischaemia.

Astrocyte Response: GFAP Immunohistochemistry

GFAP immunoreactivity was assessed at 7 d post reperfusion to determine the extent of the astrocytic response to infarction. At 7 d post-reperfusion there was a profound increase in the number of GFAP immunoreactive cells in the boundary zone of the infarct (Figure 3.21), such that they completely delineated the infarct. Such cells were only found in regions of viable tissue and were not observed within the core of the infarct and were seen to have an activated morphology, in addition to hyperplasia and hypertrophy of the astrocytes. Loss of GFAP immunoreactivity

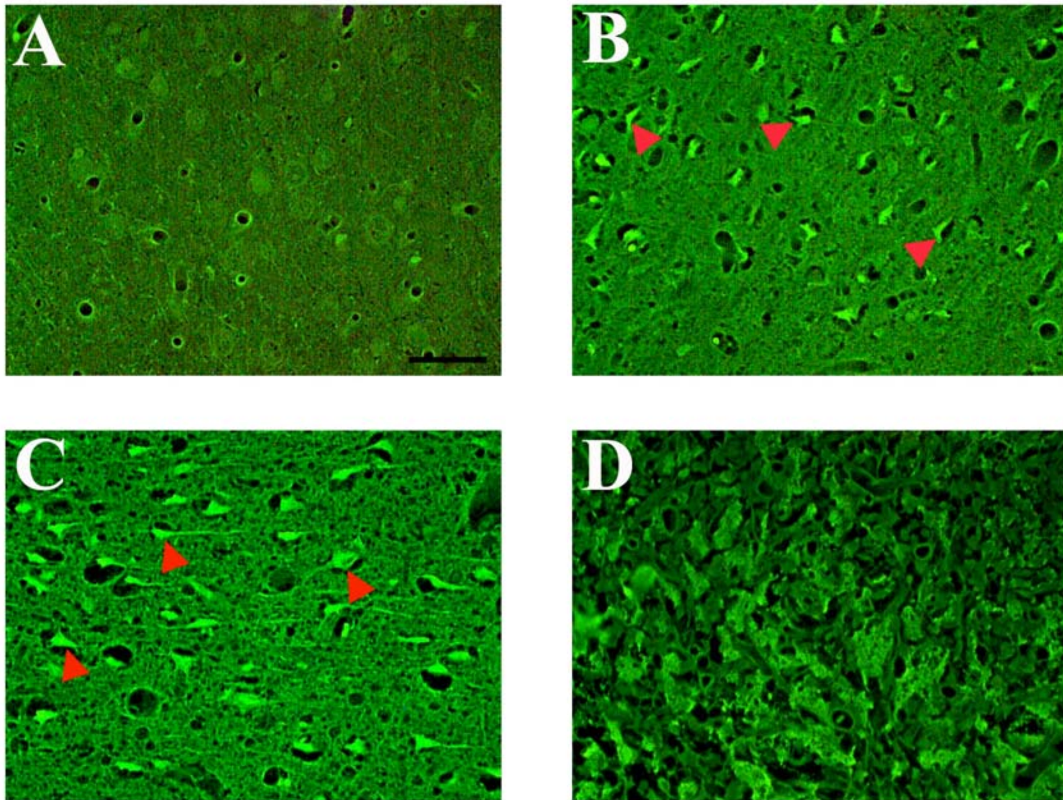

Figure 3.19 Permanent versus transient stroke. tMCAO Cortex-Degenerating Neurons – FJC stained neurons (Bar = 100 μm).

The cortex of sham animals showed no signs of degeneration (A). After 5 h of reperfusion shrunken, degenerating neurons (arrowheads) surrounded by a retracted parenchyma were seen throughout the cortex (B). At 24 h post-reperfusion there was a further increase in the number of degenerating neurons, as well as increased cell loss and disruption of normal cortical architecture (C). By 7 d post-reperfusion few degenerating neurons were seen as the gliosis reaction dominated the infarct zone (D).

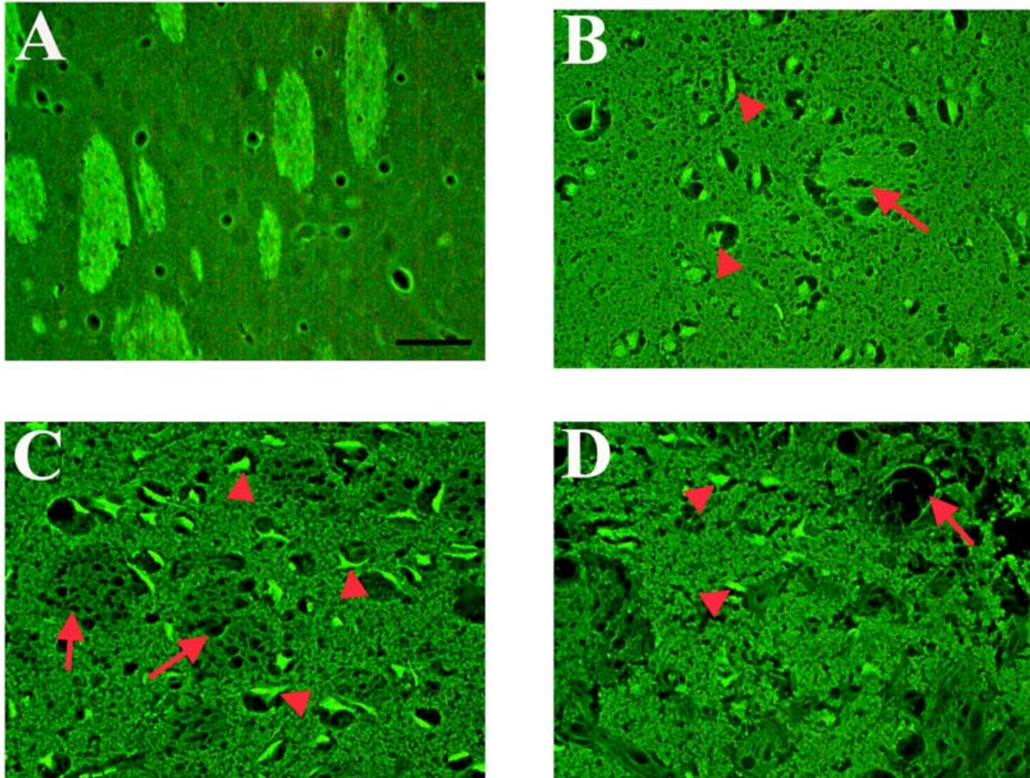

Figure 3.20 Permanent versus transient stroke. tMCAO White Matter-Degenerating Neurons – FJC stained neurons (Bar = 100 μm).

The white matter of sham animals showed no signs of degeneration (A). After 5 h of reperfusion vacuolation of the white matter was apparent along with shrunken, injured cells (B) (arrowheads). Following 24 h of reperfusion, vacuolation and cell injury were more advanced (C). Few degenerating neurons were observed at 7 d seen as the gliosis occupied much of the white matter (D).

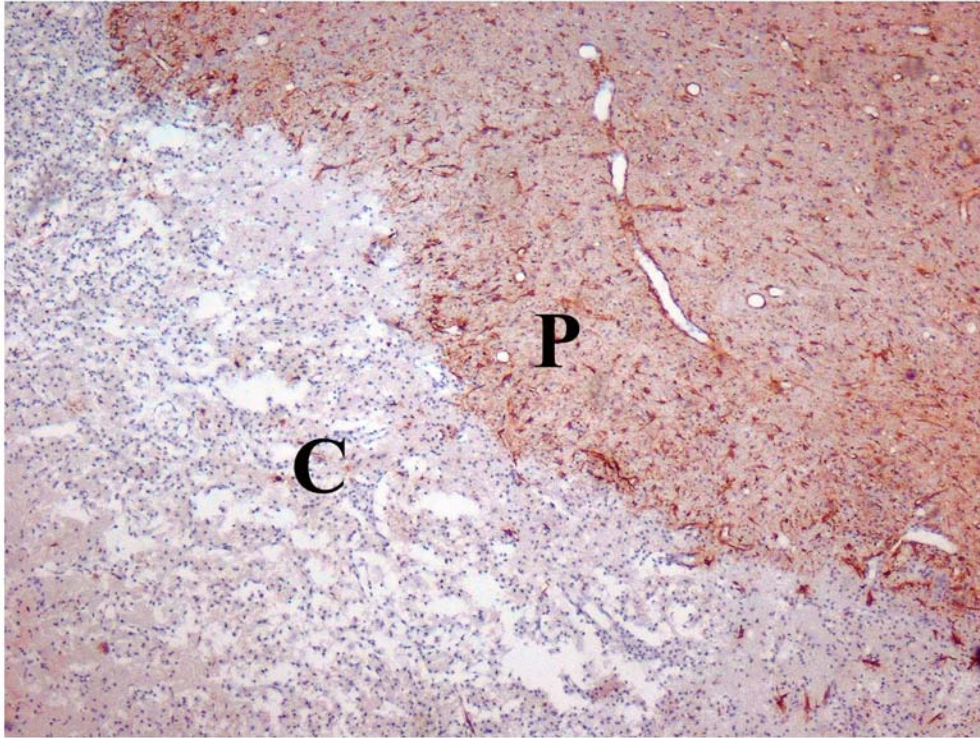

Figure 3.21 Permanent versus transient stroke. tMCAO - GFAP stained sections (4x).

GFAP staining clearly delineated the infarcted tissue. GFAP positive cells were only seen in areas of tissue preservation (P), not in the gliosis-ridden core (C). Tissue was extremely friable.

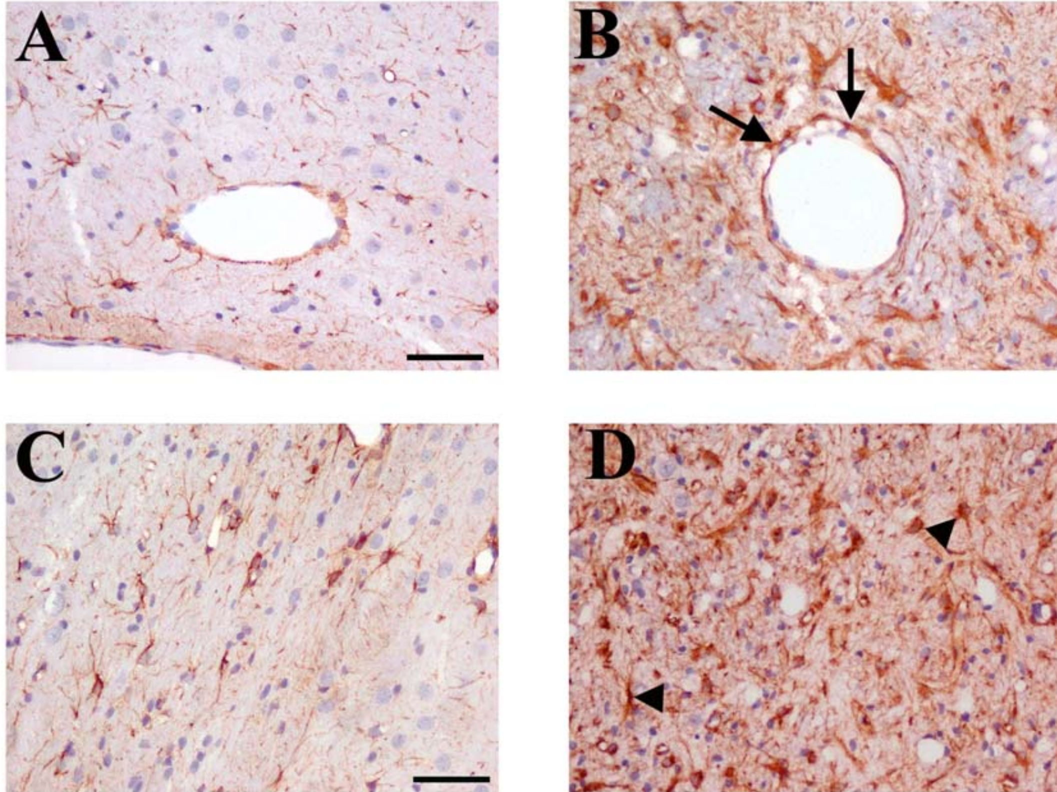

Figure 3.22 Permanent versus transient stroke. tMCAO GFAP stained sections (Bar = 100 μm).

Perivascular Tissue:
In sham tissue, GFAP positive cells were seen in close association with blood vessels (A). After 7 d of reperfusion more intense GFAP immunoreactivity (arrows) was seen around vessels in penumbral tissue (B).

Penumbral Tissue:
In sham tissue the white matter showed a scattering of GFAP positive cells (C). Following 7 d of reperfusion more cells in the white matter were GFAP positive (arrowheads) and such cells displayed an activated morphology (D).

within the infarct core is characteristic of MCAO (Chen et al., 1993; Garcia et al., 1993; Li et al., 1995; Liu et al., 1999). In sham animals, GFAP immunoreactive cells were found throughout the hemisphere, present as resting cells with fine processes (Figure 3.22).

Macrophage/Activated Microglia Response: ED-1 Immunohistochemistry

ED-1 immunohistochemistry was used to assess the macrophage/activated microglia response at 7 d post-reperfusion. Both blood-derived and microglial derived macrophages can be detected with the ED-1 stain, and there is no morphological way to differentiate between these two lineages. No ED-1 immunoreactivity was observed within the cortex of sham animals (Figure 3.21). However, following tMCAO there was a profound increase in the number of ED-1 positive cells. By 7 d post-reperfusion the ED-1 positive cells were present in vast numbers within the core of the infarct, where gliosis was widespread. Such confluent ED-1 immunoreactivity is indicative of a massive macrophage/activated microglia response to infarction and tissue injury. Macrophages/activated microglia are recruited to the tissue to clear tissue debris and assist in the formation of a glial scar.

In sham animals, macrophages/activated microglia were not observed to occupy the perivascular space, however at 7 d post reperfusion, macrophage/activated microglia were commonly seen in close proximity to blood vessels (Figure 3.22).

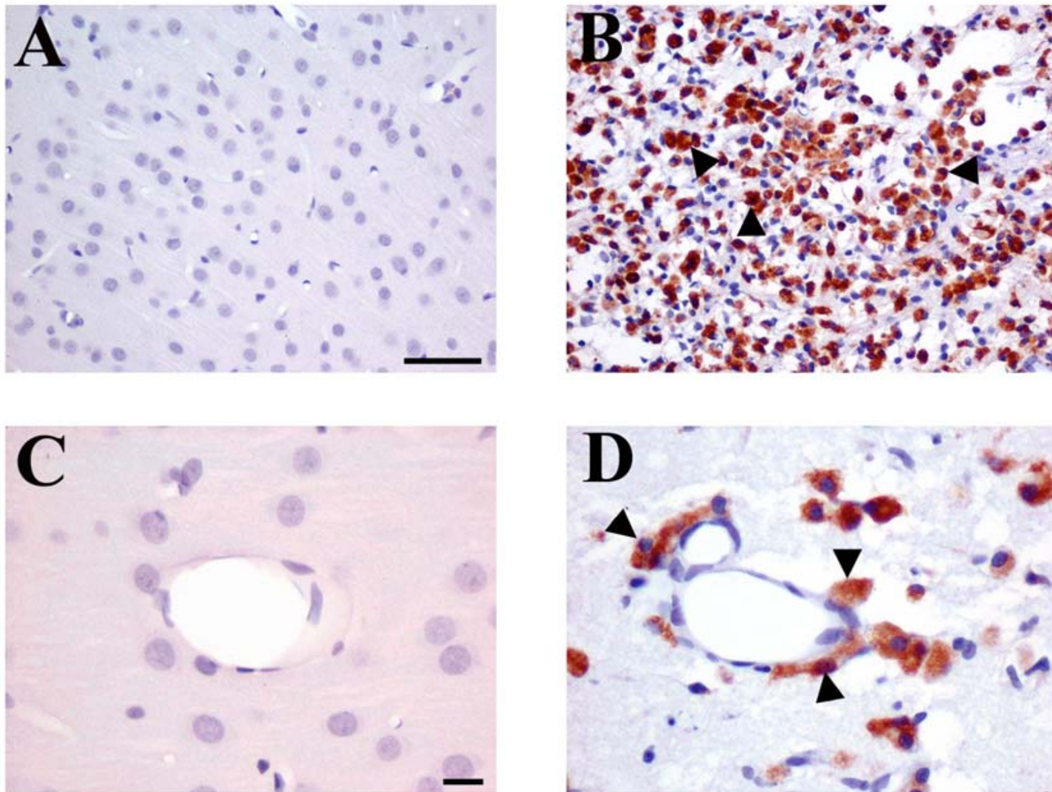

Figure 3.23 Permanent versus transient stroke. tMCAO – Macrophage/Activated Microglia response - ED-1 Stained sections (Bar = 100 μm).

Cortical Tissue:
No ED-1 positive cells were seen in sham tissue (A). At 7 d post-reperfusion, ED-1 positive cells (arrowheads) dominated the infarct and were seen in areas of prominent gliosis (B).

Perivascular Tissue:
No ED-1 positive cells were seen in perivascular tissue of sham animals (A). Following 7 d of reperfusion, ED-1 positive cells (arrowheads) were commonly seen in close association with blood vessels (B).

3.3.3 pMCAO versus tMCAO

Examining the immunohistochemistry sections it was clear that there are a number of immunoreactivity differences between the tissue response to permanent ischaemia, and that of ischaemia with reperfusion. The following section briefly summarises some of these differences.

General Pathology: H&E

More advanced infarction was observed in the tMCAO group compared to the pMCAO group (Figures 3.3-3.4; 3.12-3.13). The influx of early inflammatory cells such as neutrophils was not observed in the pMCAO animals. This may be due to the fact that blood flow is required to bring such inflammatory cells to the site of injury.

SP Response: SP Immunohistochemistry

The SP response to ischaemia appears to differ with the ischaemic conditions (Figure 3.24). It is not only the duration of ischaemia but also the presence or absence of reperfusion that affects the magnitude of the SP response. More SP immunoreactivity, especially of perivascular tissue, was observed in the tMCAO animals compared with the pMCAO animals. Although the perivascular SP response was virtually absent in the pMCAO group, neuronal SP immunoreactivity was still observed, although to a lesser degree than the tMCAO group.

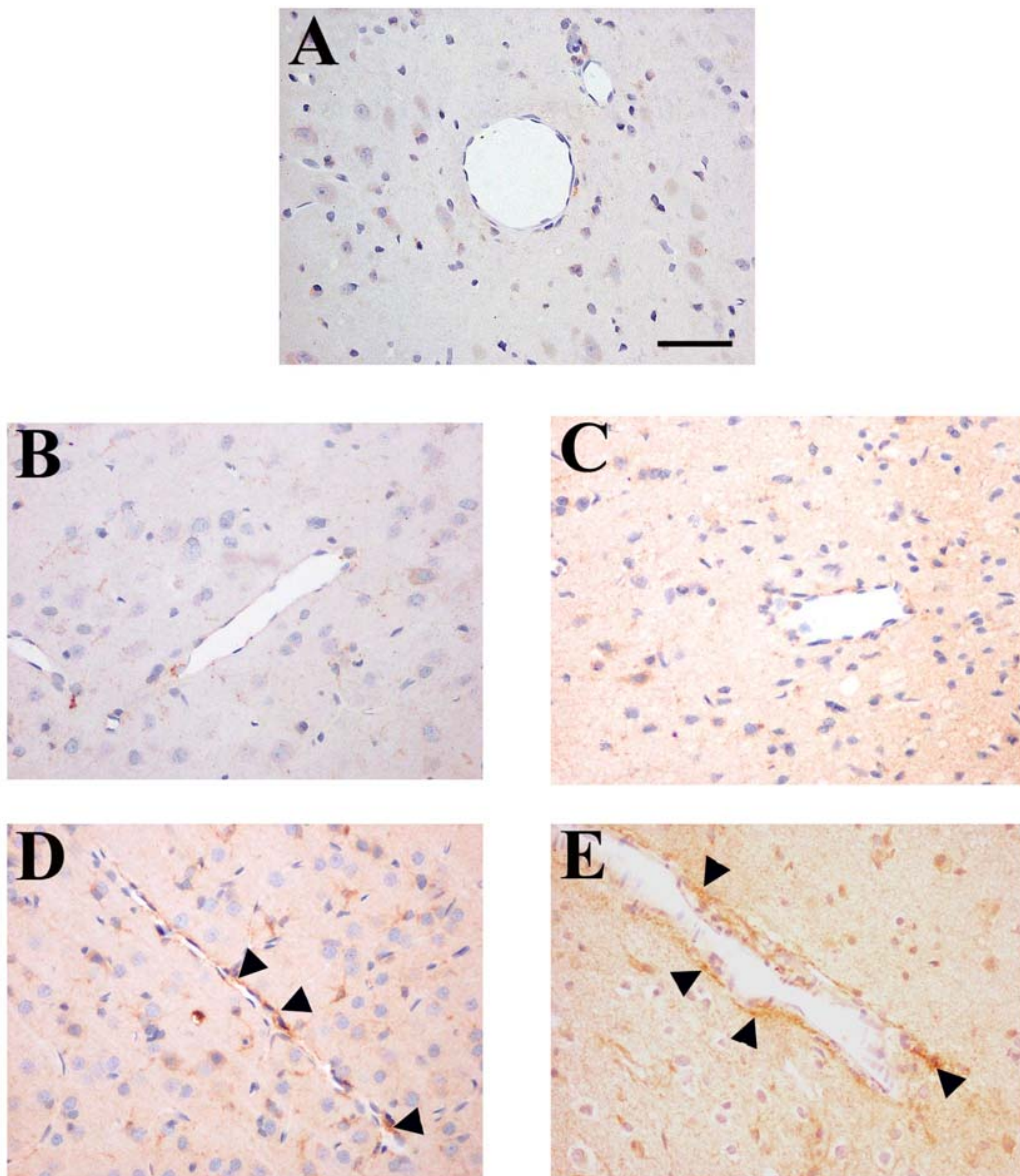

Figure 3.24 Permanent versus transient stroke. pMCAO versus tMCAO – SP stained sections (Bar = 100 μm).

Sham tissue showed negligible SP immunoreactivity around vessels (A). Following 7 h (B) or 26 h (C) of ischaemia there was no apparent difference in perivascular SP immunoreactivity (arrowheads). However, following 5 h (D) and 24 h (E) of reperfusion there was a profound increase in perivascular SP immunoreactivity.

Axonal Injury: APP Immunohistochemistry

A significant increase in the number of APP immunoreactivity axons was observed following both transient and permanent ischaemia with the APP response to ischaemia being comparable for both ischaemic conditions. However, retraction balls of increased size and number, indicative of axonal injury, were observed in tMCAO tissue (Figures 3.7, 3.15).

Degenerating Neurons: FJC

The number of FJC positive neurons was comparable between the pMCAO and tMCAO groups. Both ischaemic conditions produced widespread neuronal injury with marked infarction of the right hemisphere (Figures 3.10-11, 3.19-20).

3.3.4 ELISA for Substance P

In figures 3.15-3.16 we observed an increase in SP immunoreactivity within the infarcted hemisphere at 24 h following tMCAO. As such, ELISA was used to quantitate this increase in SP. ELISA reveals that there was a significant ($p<0.05$) increase in SP protein at 24 h post-stroke in vehicle animals compared to shams (Figure 3.25). This was consistent with the qualitative results of the SP immunohistochemistry study.

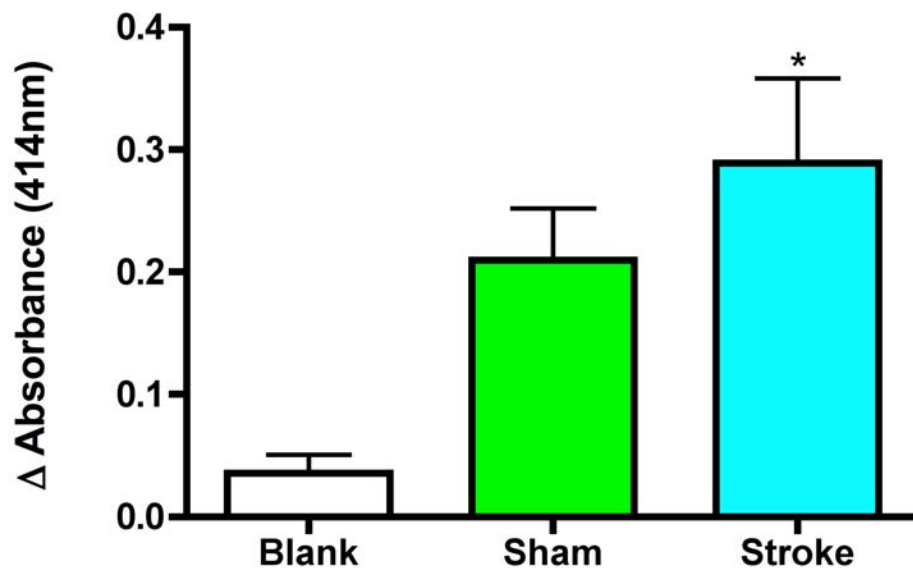

Figure 3.25 Permanent versus transient stroke. Level of SP within the infarcted hemisphere measured at 24 h post-reperfusion, as assessed by ELISA.

Following 24 h post-reperfusion there was a significant ($p<0.05$) increase in the amount of SP protein within the infarcted hemisphere, as compared to shams (* denotes $p<0.05$ versus sham; Blank n=2; Sham n=9; Vehicle n=8; NAT n=11).

3.4 Discussion

In this study we have shown that SP is profoundly increased following cerebral ischaemia with reperfusion, but not following permanent ischaemia. Previous studies from our laboratory have shown that SP release is a ubiquitous feature of TBI, regardless of whether the insult is mild, moderate or severe (Donkin et al., 2007; Chen et al., 1993; Garcia et al., 1993; Li et al., 1995). However, it appears that in the setting of cerebral ischaemia, that SP may not be a consistent feature of stroke and is limited to ischaemia with a reperfusion component. The SP immunoreactivity observed was particularly evident in perivascular tissue of the ischaemic penumbra, as well as neuronal and glial tissue within this region.

The levels of SP observed within the tissue gradually increased with increasing time following transient stroke, with the protein detection methods revealing a significant increase in SP levels within the infarcted hemisphere at 24 h post-reperfusion. Indeed, SP immunoreactivity was maximal at this time-point, but was markedly decreased by 7 d. BBB openings have been reported at various time-points following stroke, and the 24 h time-point when SP was markedly increased in our studies is in keeping with such reports (Preston et al., 1993). The present findings therefore suggest that the function of the increased SP release may be altered BBB permeability and the subsequent genesis of vasogenic oedema. Indeed, it has previously been reported that stimulation of NK_1 receptors in cerebral endothelium may contribute to ischaemia-induced oedema formation and recruitment of inflammatory cells (Stumm et al., 2001). This concept will be further explored in chapter 4.

The immunohistochemistry experiments showed that the increased SP immunoreactivity was localised to perivascular, glial and neuronal tissue within the penumbra of the ischaemic lesion. Yu et al (1997) previously reported an over-expression of SP following cerebral ischaemia (Yu et al., 1997). The findings of the present study confirm such reports. Interestingly, the SP responses differed between the pMCAO and the tMCAO group. Although neuronal SP immunoreactivity was increased compared to shams in the pMCAO group, the tMCAO group demonstrated greater SP immunoreactivity within the infarcted hemisphere, within both neuronal and perivascular tissue. Such findings suggest that the injury mechanisms leading to tissue damage and cell death may differ in permanent and transient ischaemia. Indeed, reperfusion injury is known to exacerbate ischaemic damage through the generation of free radicals, amongst other injury factors (Belayev et al., 1996b; Kastrup et al., 1999; Lo et al., 2001; Yatsu et al., 1987). These findings also suggest that reperfusion/some residual blood flow is required for the perivascular SP response to occur. However, the fact that perivascular SP was only observed in the reperfusion groups suggests that blood flow is important for this response, and cellular components of the blood and/or blood vessels may contribute to this response. This was an interesting observation and confirms that reperfusion may be deleterious by initiating harmful injury cascades. As such, SP release may be included as a newly identified injury factor following ischaemia and reperfusion. That fact that neuronal SP was still observed in the pMCAO suggests that reperfusion/blood flow is not entirely required for this cellular response. This may be related to neuronal cell death, independent of the vascular effects (Bederson et al., 1986a).

As outlined above, cells showing DCC were seen throughout the infarcts of both pMCAO and tMCAO animals. As briefly mentioned earlier, the interpretation of DCC remains controversial. A number of researchers believe that it is due to artefactual damage as a result of the fixation process (Brierly and Brown, 1981; Cammermeyer, 1961), whilst others acknowledge it is a reversible cellular response that occurs following hypoglycaemia and is indicative of reversible cell injury (Agardh et al., 1980; Auer et al., 1984; Auer et al., 1985). Our laboratory has previously shown that DCC represents a reversible form of cellular injury whereby cells may return to their normal state should the injurious agent be removed, or progress to an irreversibly injured state should the injurious agent persist (Turner ert al., 2004a; Turner et al., 2004b). In the present experiments, there were marked differences in the DCC observed in sham and experimental tissue. If DCC did actually represent artefactual change it would have been observed in sham tissue, which was not the case.

Inflammatory mechanisms modulate the proliferation, activation and activity of astrocytes. Indeed, astrocyte hypertrophy and hyperplasia with alterations in GFAP immunoreactivity are the most characteristic features of focal cerebral ischaemia (Clark et al., 1993), and a loss of GFAP immunoreactivity within the infarct core is a consistent feature of MCAO. As GFAP synthesis is not possible in the necrotic core, GFAP positive cells are localised to the boundary zone of the infarct. This pattern of staining is likely to be a reflection of the viability of the cells, where GFAP staining is found in areas where cells are destined to survive. The hypertrophy of astrocytes is most likely due to the cells reacting to changes in the extracellular environment such as alterations in amino acid and ion concentration.

While the hyperplasia within the infarct boundary zone is most likely a response to the injured neurons within the necrotic core (Chen et al., 1993; Garcia et al., 1993; Li et al., 1995).

Macrophages, as indicated by the increased ED-1 immunoreactivity, were recruited to the infarcted tissue by 7 d post-reperfusion and were seen to completely occupy the infarcted area. This is consistent with previous reports that macrophages invade the necrotic infarct from 7 d post-stroke and persist for as long as 21 d post-stroke (Lehrmann et al, 1997). Macrophages/activated microglia rapidly respond to injury, secreting chemokines and cytokines and become phagocytic when neurons are damaged (Orr et al., 2002). Phagocytic transformation is associated with ED-1 expression. It is unclear what the function of the macrophage response is, whether it be to enhance the ischaemic damage by secreting toxic substances or to protect neurons by indirect mechanisms (Stoll et al., 1998; Stoll and Schroeter, 2000). However, macrophages/activated microglia have been reported to contribute to the gradual expansion of cerebral infarction through the production of the cytokine IL-1β (Mabuchi et al., 2000). Therefore, it is likely that the macrophages/activated microglia were recruited to the infarcted area to assist in the clearance of damaged/necrotic tissue and facilitate the formation of a glial scar (Schroeter et al., 1994). Interestingly, macrophages have been shown to produce and secrete SP (Weinstock et al., 1990). Also, SP is a chemotactic factor for monocytes, the inactive precursor of macrophages (Ruff et al., 1985). Thus the increased SP observed following tMCAO may have recruited inflammatory cells to the infarcted area.

Axonal injury is a consequence of traumatic, ischaemic or chemical injury to an axon and leads to the accumulation of APP due to disruption/impairment of the fast axoplasmic transport along the axon (Lewis et al., 1996). This leads to the formation the distinctive axonal swellings/retraction balls. Therefore, APP is a sensitive marker of axonal injury (Blumbergs et al., 1994; Blumbergs et al., 1995) and is widely used to determine the degree of axonal damage following a number of brain pathologies such as TBI and ischaemia. APP immunoreactivity was seen as early as 5 h post-reperfusion in these studies. It does take some time for retraction balls to develop and axonal injury to become apparent, however, axonal injury may be observed as early as 1.5 h following trauma (Van Den Heuvel et al., 1999). In regards to the alterations in neuronal APP immunoreactivity, what the increased APP means for neurons is unclear. However in light of the many neurotrophic roles that APP plays in neurite outgrowth (Breen et al., 1991; Small et al., 1994), cell-cell interactions (Beyreuther et al., 1996) and synaptic formation and function (Moya et al., 1994) the increased APP may be of benefit to neurons following stroke. Indeed studies in TBI have shown that administration of soluble APP-α, the main derivative of APP, was effective in maintaining motor function whilst also markedly reducing axonal injury (Thornton et al., 2006). Alternatively, the increase in APP may represent a neurodegenerative response to injury, as over-expression of APP has been reported to be neurotoxic and induce cell death (Bertrand et al., 2001).

3.5 Conclusions

The findings of the present chapter have characterised an increase in SP within the infarcted hemisphere following transient ischaemia. We also show that that the injury mechanisms that lead to tissue damage differ in tMCAO versus pMCAO, as

reperfusion adds another component. As reperfusion of the ischaemic territory was associated with a significant increase in SP levels, the tMCAO model of ischaemia was chosen for the remainder of the studies in this thesis. This ischaemic model is considered comparable to human stroke whereby reperfusion of the ischaemic area is facilitated with thrombolysis. The next step was to determine what effect inhibiting SP action had on BBB status, cerebral oedema, histological outcome, infarct volume and functional outcome following tMCAO, and this is the subject of Chapter 4.