Characterising the role of the neuropeptide substance P in experimental subarachnoid haemorrhage

Christine Barry

Discipline of Anatomy and Pathology, School of Medical Sciences

The University of Adelaide

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DECLARATION

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PUBLICATIONS

The following articles have been published or accepted for publication during the period of my PhD candidature, and sections of these articles are included in the present thesis.

Published papers

Barry C, Helps S, van den Heuvel C and Vink R (2011) Characterizing the role of the neuropeptide substance P in experimental subarachnoid haemorrhage. *Brain Research* doi:10.1016/j.brainres.2011.02.082

Barry C, Koblar S and O'Carroll D (2007) Frontier technologies for brain repair. *Australasian Science* 28:16-18.

Published abstracts (reviewed)

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ABBREVIATIONS

°C	degrees Celsius
µL	micro litres
µm	micrometres
5-HT	5-Hydroxytryptamine (serotonin)
ACE	angiotensin converting enzyme
aCSF	artificial cerebrospinal fluid
ANOVA	analysis of variance
ATP	adenosine triphosphate
BBB	blood-brain barrier
BP	blood pressure
CBF	cerebral blood flow
CGRP	calcitonin gene-related peptide
cm	centimetres
CNS	central nervous system
CPP	cerebral perfusion pressure
CSD	cortical spreading depolarisation
CSF	cerebrospinal fliud
d	day
DAB	diamimobenzidene
eNOS	endothelial nitric oxide synthase
G	gauge
h	hours

H and E	haematoxylin and eosin
H^+	hydrogen ion
ICA	internal carotid artery
ICH	intracerebral haemorrhage
ICP	intracranial pressure
icv	intracerebroventricular
iv	intravenous
IU	international units
K^+	Potassium ion
L/min	litres per minute
MABP	mean arterial blood pressure
MCA	middle cerebral artery
mg/ml	milligrams per millilitre
mins	minutes
ml	millilitres
mm	millimetres
mmHg	millimetres of mercury
mRNA	messenger ribonucleic acid
n	number
NAT	n-acetyl-L-tryptophan
NEP	neutral endopeptidase
NK1	tachykinin receptor to which SP binds selectively
NK2	tachykinin receptor to which NKA binds selectively
NK3	tachykinin receptor to which NKB binds selectively

NKA	neurokinin A
NKB	neurokinin B
NMDA	N-methyl-D-aspartate
NO	nitric oxide
pCO_2	carbon dioxide partial pressure
PNS	peripheral nervous system
pO_2	oxygen partial pressure
PPT	preprotachykinin
rpm	revolutions per minute
Rx	treatment
s	seconds
SAH	subarachnoid haemorrhage
SEM	standard error of the mean
SD	standard deviation
SP	substance P
TRPV1	transient receptor potential vanilloid 1

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ABSTRACT

Background

Raised intracranial pressure (ICP) following SAH predicts poor outcome and is due to hemorrhage volume and possibly brain oedema, hydrocephalus and increased volume of circulating intracranial blood. Interventions that reduce oedema may therefore reduce ICP and improve outcome. The neuropeptide substance P (SP) mediates vasogenic oedema formation in animal models of ischemic stroke, intracerebral hemorrhage and brain trauma, and may contribute to the development of increased ICP. Blockade of the SP NK1 tachykinin receptor using n-acetyl-l-tryptophan (NAT) reduces brain oedema and improves outcome in these models. This intervention had not previously been tested in models of SAH. This study therefore assessed whether SP mediates oedema formation in experimental SAH, and whether NAT treatment impacted on ICP and functional outcome.

Methods

SAH was induced in adult male Sprague-Dawley rats by either injection of autologous blood into the prechiasmatic cistern (injection SAH) or by endovascular arterial puncture of the Circle of Willis (filament SAH). NAT was injected (i.v.) at 30 minutes after induction of SAH. Subgroups were assessed for brain water content, immunoreactivity to SP, albumin immunoreactivity and functional outcome at 5, 24 and 48 hours, or ICP and cerebral perfusion pressure during SAH and over the following 5 hours.

Results

In both models a primary ICP increase occurred during SAH and a secondary ICP increase occurred within 2 hours. Injection SAH was followed by a non-significant increase in brain water content and caused no functional deficits. In contrast, brain oedema followed filament SAH ($p < 0.001$) and correlated with functional deficits ($r = 0.8$, $p < 0.01$). Increased albumin immunoreactivity ($p < 0.001$) indicated vasogenic brain oedema. Cerebral perfusion pressure was diminished after filament SAH and some animals demonstrated plateau waves of ICP. NAT treatment did not improve ICP, oedema or outcome.

Conclusion

SAH produced secondary ICP elevation, vasogenic brain oedema and functional deficits, but it is unclear if oedema contributed to ICP. Blockade of SP did not improve any outcome parameters, suggesting that SP-mediated neurogenic inflammation may be less critical to outcome than other factors in these models.

Chapter 1

Introduction

1 Introduction

1.1 Definition of subarachnoid haemorrhage

A subarachnoid haemorrhage (SAH) is a bleed into the subarachnoid space surrounding the brain and spinal cord. It may occur spontaneously or due to trauma. In this thesis, the term will refer to haemorrhage into the cerebral subarachnoid space that occurs in the absence of trauma.

1.2 Epidemiology and outcome

Spontaneous SAH affects up to 700 000 people worldwide each year (Ingall *et al* 2000), including some 1 600 Australians (Anderson C 2000). Over 40% die within 28 days, 30% of survivors have long-term dependency and the mean age of those effected is 49 years (Ingall *et al* 2000; van Gijn and Rinkel 2001). Even for those deemed to have a good outcome at the time of discharge from hospital, ongoing disability is extremely common, including cognitive deficits, severe depression and endocrine disorders (Dimopoulou *et al* 2004; Hutter and Gilsbach 1993). Neurological deficits that present as minor on formal testing can significantly impact on the ability of SAH survivors to return to their previous employment and social roles (Ropper and Zervas 1984).

SAH is also present in over 40% of cases of moderate or severe head injury (Servadei *et al* 2002), where its presence is associated with very poor prognosis, that is related to the amount of subarachnoid blood (Chieregato *et al* 2005; Servadei *et al* 2002). Traumatic SAH is usually caused by a deceleration injury, most commonly a motor vehicle accident or a fall.

1.3 Aetiology

Cerebral aneurysm rupture accounts for most cases of spontaneous SAH (85%). Arteriovenous malformations (10%) and arterial wall weakness due to infection, cancer or certain genetic syndromes (5%) are less common causes (Johnson *et al* 2007; Rinkel *et al* 1993; van Gijn and van Dongen 1980; van Gijn *et al* 2007). Family history is associated with around 10% of SAH cases (Ronkainen *et al* 1993). Cerebral aneurysms develop at the branching sites of major cerebral vessels (Figure 1.1). These regions appear to be subject to particularly strong forces related to blood flow turbulence (Kim *et al* 1992) rather than being areas of weakness per se. Aneurysms develop over time and SAH due to aneurysm rupture is rare below the age of 20 years. SAH in the young is more commonly due to congenital arteriovenous malformations (Heiskanen 1989).

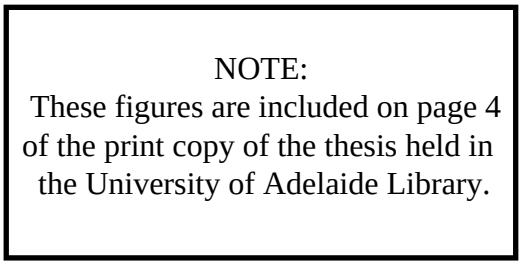


Figure 1.1 A: View of the base of the brain with common sites of aneurysm formation circled (adapted from (van Gijn *et al* 2007). B: Dissected Circle of Willis showing 3 aneurysms (Brisman *et al* 2010).

Intracerebral aneurysms are found in one to five per cent of all adults (King 1997) and most aneurysms are small with a low risk of rupture (approximately 0.7 per cent per year) (Rinkel *et al* 1998). Risk of rupture increases with aneurysm size (Nahed *et al* 2005), onset of symptoms such as headache (Rinkel *et al* 1998) or moderate to extreme physical exertion (Anderson *et al* 2003). Three modifiable risk factors account for 60% of ruptured aneurysms; hypertension, smoking and excess alcohol intake (more than two standard drinks per day) (Johnston *et al* 1998; Longstreth *et al* 1992; Nahed *et al* 2005; Rosen *et al* 2005; Ruigrok *et al* 2001).

Over 90% of patients survive the initial bleed long enough to receive medical attention (Huang and van Gelder 2002; Ingall *et al* 2000), however 42% die within 28 days. Most deaths occur early after the ictus: 37% within 24 hours; 60% within 48 hours; and 75% within one week (Ingall *et al* 2000). So whilst a therapeutic window may exist, new interventions are needed to target very early mechanisms of secondary injury following SAH.

1.4 Pathophysiology of subarachnoid haemorrhage

SAH causes reduced cerebral blood flow, reduced cerebral autoregulation and cerebral ischaemia. These are related to raised ICP and decreased CPP (Bederson *et al* 1995; Nornes 1978), loss of blood-brain barrier integrity (Scholler *et al* 2007; Sehba *et al* 2007), acute and delayed vasoconstriction (Bederson *et al* 1998; Rabinstein *et al* 2003; Rabinstein *et al* 2004) and microvascular platelet aggregation (Sehba *et al* 2005). Both primary injury mechanisms (occurring at the ictus) and secondary mechanisms (biochemical and physiological responses) evolving over time after the SAH bleed may contribute to mortality and morbidity.

Following SAH, various mechanisms contribute to secondary brain injury resulting in damage that is not limited to the distribution of the ruptured vessel, but often affects the brain more globally (Rabinstein *et al* 2005; Voldby *et al* 1985b). Contributing factors include raised intracranial pressure (ICP), cerebral oedema, hydrocephalus, cerebral vasospasm, cerebral microvascular dysfunction, ischaemic depolarisations and re-bleeding. Improved understanding of these mechanisms will aid the development of new therapies to reduce secondary injury following SAH and improve outcome.

1.4.1 Raised intracranial pressure following SAH

Definition of ICP and overview

Intracranial pressure (ICP) is the pressure inside the skull that is exerted by its contents (i.e. brain, blood and CSF). ICP varies with body position, particularly the position of the head in relation to the heart. In a supine adult ICP is 7 to 15 mmHg and in standing it falls to around -10 mmHg (Steiner and Andrews 2006). There is a small variation with breathing and the cardiac cycle (Figure 1.2), whilst abrupt changes in intra-thoracic/intra-abdominal pressure (such as coughing) cause an abrupt change in ICP.

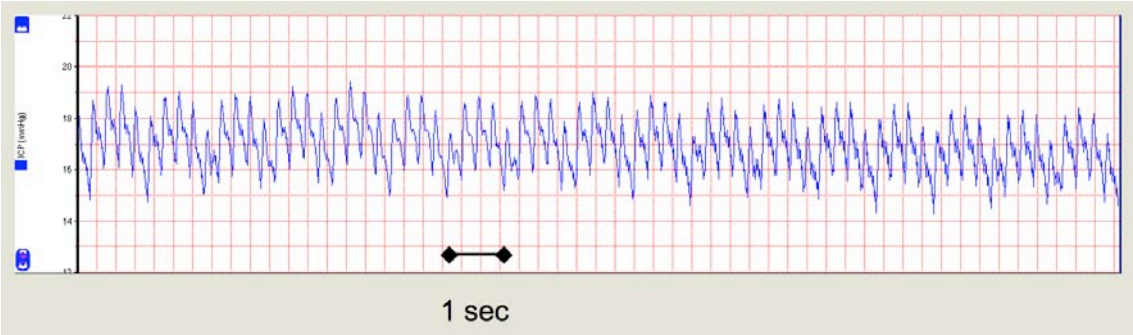


Figure 1.2 ICP trace (rat) showing small variation (~ 1 mmHg) with the respiratory and cardiac cycles.

Intracranial volume – pressure relations

The Monro-Kellie hypothesis describes the pressure-volume relationship between ICP and skull contents. It proposes that intracranial volume is relatively fixed due to the rigidity of the adult skull, and therefore for ICP to remain constant, the combined volume of intracranial contents (brain, blood and CSF) must also remain constant. An increase in one component must be offset by a decrease in another, or ICP will rise (Mokri 2001). For example, increased brain volume (due to tumour or haemorrhage) may be compensated for by displacement of CSF along the craniospinal axis or venous blood into the external

jugular vein (Dunn 2002). Alterations in CSF production and absorption may also occur (Mokri 2001).

However, compensatory mechanisms are limited. Under normal conditions, a small increase in the volume of intracranial contents will have minimal impact on ICP, but as the volume of intracranial contents increases, compliance is reduced. Further small increases in intracranial volume may then cause ICP to increase exponentially (Figure 1.3). Following SAH, the volume of extravasated blood may substantially reduce intracranial compliance (the ability to absorb subsequent increases) and any additional intracranial volume (e.g. due to brain oedema) could potentially have a relatively large impact on ICP.

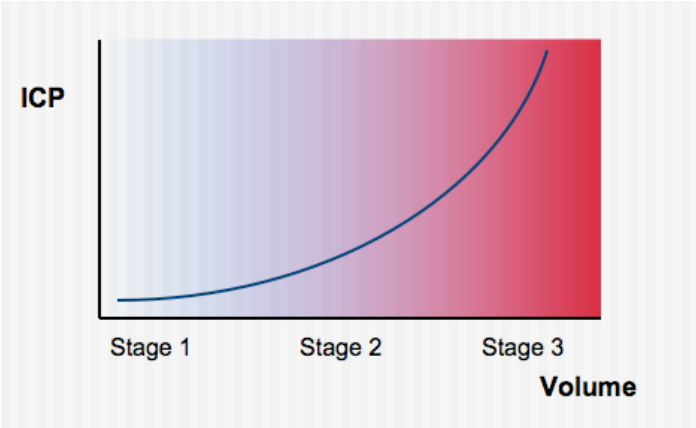


Figure 1.3 Compliance curve for intracranial pressure. Stage 1: When ICP is within normal range, small increases in intracranial volume are managed by compensatory mechanisms. Stage 2: As ICP begins to increase intracranial compliance is reduced and ICP begins to rise more rapidly with additional intracranial volume. Stage 3: As compensatory mechanisms are exhausted, a small additional intracranial volume results in a dramatic ICP increase.

Raised ICP is an independent predictor of poor outcome following SAH. High intracranial pressure can damage brain tissue via several mechanisms. It can restrict cerebral blood flow by reducing cerebral perfusion pressure and disrupting cerebral autoregulation (see 1.4.1), obstruct CSF outflow (see 1.4.3), cause brain herniation and trigger reflex bradycardia (see 1.4.1).

Mechanisms of ICP increase following SAH

Several mechanisms may contribute to raised ICP following SAH. The initial bleed resulting in a volume of extravasated blood in the subarachnoid space may be considered the primary mechanism of ICP increase. Secondary ICP increases may be caused by re-bleeding or by physiological responses to subarachnoid blood, as well as brain oedema, cerebral vasodilation and hydrocephalus. Each of these is discussed below.

Impact of intracranial hypertension on cerebral blood flow

A constant cerebral blood flow is vital as the brain is reliant on aerobic metabolism and has little capacity for anaerobic metabolism. Cerebral autoregulation provides a constant brain blood flow over a range of cerebral perfusion pressures and is mainly achieved by dilation and constriction of small arteries and arterioles in response to changing arterial pressure and local metabolic demands. Small vessels constrict in response to increasing blood pressure and dilate in response to CO_2 accumulation or lowered pH, so as to deliver more blood to areas with high metabolic demands. These changes in vessel tone ensure that a relatively constant cerebral blood flow and adequate O_2 delivery are maintained. However, autoregulation only functions over a defined range of cerebral perfusion pressure (CPP).

CPP is the pressure driving cerebral blood flow. It is determined by the difference between mean arterial blood pressure and ICP (or cerebral venous pressure in cases where cerebral venous pressure exceeds ICP) (Rosner *et al* 1995). An increase in ICP will lower CPP, unless arterial blood pressure increases sufficiently to compensate. If CPP is too low, changes in vessel diameter may no longer adequately control cerebral blood flow. CO_2 accumulation in the cerebral circulation may lead to vasodilation, increasing the volume of intracranial blood and therefore increasing ICP and reducing CPP in a vicious cycle. If CPP falls to around 40 mmHg, maximal vasodilation may result, with complete failure of autoregulation, and severely compromised brain tissue oxygenation (Rosner and Becker 1984).

Intracranial hypertension and brain herniation

Raised ICP from a localised lesion (such as a haematoma) may cause a shift of brain tissue within the skull. Midline shift and brain herniation, including subfalcine, transtentorial and tonsillar herniation, have been observed following SAH (Baraff *et al* 2010; Koenig *et al* 2008). Subfalcine herniation is displacement of the cingulate gyrus under the falx cerebri, transtentorial herniation is displacement of the medial aspect of the temporal lobe against or below the tentorium cerebelli and tonsillar herniation is displacement of the cerebral tonsils through the foramen magnum.

Midline shift may result in further complications such as ventricular compression, obstruction of CSF flow and hydrocephalus. Brain herniation is potentially fatal due to the crushing injury and ischaemia of vital brain regions, including vessels. For example tonsillar herniation may compress vital respiratory and cardiac centres within the medulla.

However, brain herniations have also been identified in the CT scans of SAH patients who were alert and had normal clinical neurological signs (Baraff *et al* 2010).

Cushing reflex

High ICP following SAH may trigger the Cushing reflex (Brinker *et al* 1992; Wan *et al* 2008), which is characterised by bradycardia (following an initial period of tachycardia), widening pulse pressure (rising systolic, declining diastolic) and irregular breathing. The physiological relevance of this response and the underlying mechanisms are controversial. One traditional view is that the Cushing response indicates pre-terminal brain stem damage and impending circulatory failure, caused by sympathetic nervous system (SNS) over-activity in response to mechanical distortion of the medulla. More recently it has been hypothesized that this response to brain ischaemia may restore CPP when ICP is extremely elevated, and that elevated systolic BP is the key feature of the response (Wan *et al* 2008).

The Cushing response is triggered by chemoreceptors in the hypothalamus in response to increased H^+ and increased pCO_2 concentrations (Barbiro-Michaely and Mayevsky 2003). These metabolites accumulate rapidly during brain ischaemia. A sympathetic response is initiated with activation of arterial $\propto$-1 adrenergic receptors. This results in vasoconstriction, increased total peripheral resistance and increased blood pressure. Cardiac contractility and cardiac output are also increased. The increased blood pressure stimulates carotid baroreceptors, triggering a parasympathetic nervous system response including bradycardia mediated by the vagus nerve.

Experimentally, the Cushing response tends to be triggered by sustained moderate increases in ICP rather than dramatic increases (Barbiro-Michaely and Mayevsky 2003; Marshman 1997). A sudden, large ICP increase is more likely to induce an arterial hypotension response (Marshman 1997). This supports the view that the (hypertensive) Cushing response is manifest by a functioning autoregulatory system to maintain or regain CPP. Indeed, following human SAH, the Cushing response has been observed to sustain CPP in the presence of high ICP, and aid in patient recovery (Wan *et al* 2008). However, the Cushing response after SAH may also result in further ICP increases (Brinker *et al* 1992), presumably by increasing brain blood volume. This can trigger a vicious cycle of increasing BP and ICP, as well as reduced cerebral blood flow (Brinker *et al* 1992; Ebel *et al* 1996) and poor outcome.

1.4.2 Brain oedema

Brain oedema is an excess of water content within brain tissue. Two main types of oedema have been described, vasogenic and cytotoxic oedema (Klatzo 1967), with both forms potentially occurring following SAH (Doczi 1985). As brain oedema may contribute to brain volume and therefore increased ICP after SAH, interventions that reduce oedema may reduce ICP and improve outcome.

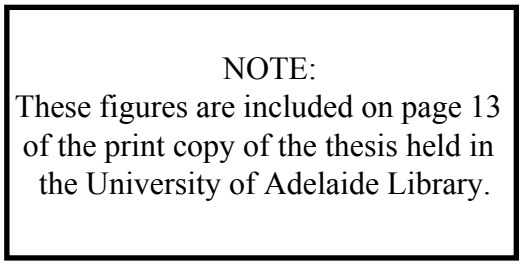
Cytotoxic oedema

Cytotoxic or cellular oedema is an increase in intracellular fluid volume caused by movement of ions and water from the brain's extracellular space to the intracellular space (Kimelberg 1995; Klatzo 1967). When brain blood flow is normal, oxygen and glucose supplied to the brain enables normal functioning of energy-dependent ion transporters that are integral to cell homeostasis. If oxygen and glucose availability are reduced, adenosine

triphosphate (ATP) production in neurons and neuroglia is reduced and ATP-dependent ion pumps may fail. This failure leads to influx of sodium ions into cells, with chloride ions and water following along osmotic gradients, resulting in cellular swelling. Due to failure of ATP-dependent glutamate transporters, extracellular glutamate accumulation and over activation of NMDA receptors occurs with consequent accumulation of intracellular calcium. The unregulated calcium influx triggers biochemical cascades that may lead to cell death by necrosis and apoptosis (Kimelberg 1995; Sureda 2000).

Vasogenic oedema

Vasogenic oedema is an increase in brain fluid volume cause by movement of blood plasma contents from the lumen of brain microvessels (capillaries and post-capillary venules) to the brain's extracellular space. Under normal conditions, the blood-brain barrier prevents the movement of ions or proteins from blood vessels to brain tissue, with the osmotic gradient associated with the blood contents (ions and proteins) also ensuring that water is retained in the blood (Abbott *et al* 2006; Segal 2007). The integrity of the blood-brain barrier is maintained by specialised features including tight junctions between endothelial cells, pericytes (perivascular macrophages), a basal lamina (basement membrane) containing type IV collagen, laminen and fibronectin around vessels and astrocytic end feet abutting microvessels. Brain nutrients cross the blood-brain barrier via specific transporters so a precisely regulated microenvironment is maintained for reliable cell signalling within the brain (Figure 1.4) (Olsen 2010; Rosenberg *et al* 1998; Segal 2007).



(Olsen 2010) (Segal 2007)

Figure 1.4 Components of the blood-brain barrier that maintain a constant extracellular environment within brain tissue. A: Tight junctions seal gaps between endothelial cells. A basement membrane completely encapsulates pericytes and endothelial cells. Astrocytic end feet envelope ~ 99% of cerebral vasculature. B: Even small ions cannot cross tight junctions (1) or permeate endothelial cell membranes (2). Specific carriers and ions channels (3-5) control their transport. Enzymes (6) provide additional protection and efflux pumps (7) can return fat-soluble molecules to the circulation.

Breakdown of the blood-brain barrier enables large proteins that are normally retained within blood, such as albumin, to cross the walls of brain microvessels and enter the brain parenchyma. Water then follows along an osmotic gradient, so that brain water content increases. Two obvious sequelae are that the biochemical homeostasis of the extracellular

environment within the brain may be lost, and that the volume of the brain may be increased, with an associated increase in local pressure.

It has already been shown both clinically and in experimental animals that SAH leads to breakdown of the blood-brain barrier and vasogenic brain oedema formation (Doczi 1985; Gao *et al* 2008; Johshita *et al* 1990; Ostrowski *et al* 2005; Park *et al* 2004; Scholler *et al* 2007; Yatsushige *et al* 2006; Yatsushige *et al* 2007). Furthermore, the extent of brain oedema has recently been shown to correlate with the degree of functional deficits following experimental SAH (Thal *et al* 2009a). Accordingly, interventions targeting oedema following SAH might show promise as a therapeutic intervention.

Various mechanisms are implicated in blood-brain barrier breakdown and oedema formation following SAH. Evidence points to opening of tight junctions between cerebral endothelial cells (Doczi 1985), abnormal vesicular transport through endothelial cells (Doczi 1985; Nakagomi *et al* 1989), apoptosis of endothelial cells (Park *et al* 2004) and degradation of the basal-lamina (Scholler *et al* 2007; Yatsushige *et al* 2007). Blood-brain barrier disruption commences within minutes of SAH and progresses over days (Scholler *et al* 2007; Sehba *et al* 2007). Thus a therapeutic window may exist in which an appropriate mediator may be targeted.

A range of inflammatory mediators have also been shown to be involved in blood-brain barrier opening, including bradykinin (acting on B2 receptors), serotonin (acting on 5-HT2 receptors), histamine (acting on H2 receptors) and arachidonic acid (Abbott 2000). The neuropeptide substance P (SP) also increases blood-brain barrier permeability (Hu *et al*

2005) and appears to be an important mediator of blood-brain barrier breakdown and brain oedema formation in a number of brain injury models. This is discussed in greater detail later in this chapter (1.6).

1.4.3 Hydrocephalus

Acute hydrocephalus (CSF accumulation and ventricular expansion within 72 hours) occurs in 20 to 30% of aneurysmal SAH patients and is more common with larger haemorrhages (Demirgil *et al* 2003; Hasan *et al* 1989c; van Gijn *et al* 1985). CSF production (which occurs predominantly via the choroid plexus in the lateral ventricles) does not appear to be increased following SAH and may in fact be decreased (Kosteljanetz 1987; Kosteljanetz 1988; Marmarou *et al* 1987), possibly as a compensatory mechanism for raised ICP. Rather, CSF out flow may be obstructed by intraventricular blood or by ventricular compression, leading to accumulation within the ventricles and periventricular brain damage (Marmarou *et al* 1987; Rinkel *et al* 1992; Shimoda *et al* 1999). CSF re-absorption may also be reduced through blockage of arachnoid villi by blood products or fibrin deposition (Heinsoo *et al* 1998), the latter being a cause of later onset hydrocephalus (Julow 1979).

1.4.4 Cerebral vasospasm

Cerebral vasospasm is sustained constriction of cerebral arteries causing reduced cerebral blood flow and, frequently, diminished perfusion in the distal territory of the artery (Weidauer *et al* 2007). Acute and delayed (late) vasospasm are both common following SAH. Acute vasospasm is maximal 10 minutes following the initial bleed and may persist for hours. One study identified acute vasospasm in 13% of SAH patients on admission (Qureshi *et al* 1999). It appears to be caused by vasoconstrictors such as endothlin-1 and

serotonin contained in extravasated blood, making direct contact with the external aspect of cerebral arteries (Ansar *et al* 2007b; Seifert *et al* 1995).

Late vasospasm occurs in 30% to 70% of survivors. It typically begins 3 to 5 days after the ictus, is maximal at day 5 to 14 and resolves gradually over 2 to 4 weeks (Fisher *et al* 1980; Heros *et al* 1983). Late vasospasm may be diffuse and affect multiple vessels (Rabinstein *et al* 2005). It causes the appearance of delayed neurological deficits in around 50% of cases (Mayberg *et al* 1994), with new deficits becoming permanent in about half of these cases (Heros *et al* 1983). Late vasospasm is considered a major cause of potentially preventable secondary injury following SAH and as such has been a major focus of SAH research (Binaghi *et al* 2007; Fisher *et al* 1980; Voldby *et al* 1985a).

A number of studies have investigated the roles of many potential mediators of vasospasm including endothelin-1 (Fassbender *et al* 2000; Seifert *et al* 1995), oxyhaemoglobin and reactive oxygen species (Takenaka *et al* 1993; Toda *et al* 1991), prostaglandins and vasoactive amines (Chehrazi *et al* 1989; D'Avella *et al* 1990; O'Neill *et al* 1992), and substance P (Delgado-Zygmunt *et al* 1990). The potential benefit of many interventions have also been characterised, including calcium channel blockers (Barth *et al* 2007b; Brandt *et al* 1991; Haley *et al* 1993; Shibuya *et al* 1992), antioxidant therapy (Asano and Matsui 1999), magnesium (Schmid-Elsaesser *et al* 2006; van den Bergh *et al* 2005), methylprednisolone (Chyatte *et al* 1987), tissue plasminogen activator (Findlay *et al* 1995), cyclosporin A (Manno *et al* 1997), statins (Tseng *et al* 2005; Tseng *et al* 2007b) and endothelin receptor antagonists (Barth *et al* 2007a).

Evidence indicates that multiple factors contribute to the development of delayed vasospasm, including vessel damage at the time of the ictus (due to very high ICP, ischaemia and high arterial blood pressure) and subsequent damage due to factors including sustained exposure to deleterious blood breakdown products. Cerebral endothelial cell damage may reduce their capacity to produce nitrous oxide, which mediates vasodilation (Kavanagh and Kam 2001). There are also changes in the content of perivascular nerves, including depletion of vasodilatory neuropeptides (Edvinsson *et al* 1994; Nozaki *et al* 1990). Cerebrovascular smooth muscle cells demonstrate increased expression of receptors for vasoconstrictors (Ansar *et al* 2007a; Vikman *et al* 2007) and diminished vasodilation responses (Mayberg *et al* 1990; Pasqualin *et al* 1992; Sobey and Quan 1999; Wickman *et al* 2003).

Whilst vasospasm has been considered the major, treatable cause of secondary injury following SAH for many years, clinical trails that prevented vasospasm with a selective endothelin A receptor antagonist (Clazosentan) did not improve patient outcome (Macdonald *et al* 2007). Accordingly, the research focus has shifted to characterising pathophysiological features of SAH that effect the brain more globally, such as global cerebral ischaemia, microcirculation dysfunction and cortical spreading depression (Macdonald *et al* 2007). It also seems worthy to note that whilst late vasospasm is considered a potentially treatable cause of delayed brain injury, secondary injury mechanisms following SAH probably contribute significantly to mortality and morbidity before the delayed vasospasm period, which begins 3 to 5 days or more after SAH.

1.4.5 Cerebral microvascular dysfunction

Damage to the cerebral microcirculation may cause widespread secondary injury following SAH. Contributing factors include microvessel plugging by activated platelets (Akopov *et al* 1996; Sehba *et al* 2005) and endothelial cell damage (Ostrowski *et al* 2005; Park *et al* 2004). Indeed, widespread platelet aggregation has been noted within minutes of experimental SAH, effecting microvessels of the hippocampus, striatum, frontal cortex and other brain regions (Sehba *et al* 2005). High ICP at the ictus may also cause a crush injury to cerebral endothelial cells and trigger apoptosis (Ostrowski *et al* 2005; Park *et al* 2004). Both of these factors may contribute to diffuse brain injury following SAH. Consistent with this CT scans of SAH patients revealed that infarctions involving the territory of a single artery occur in only 52% of cases. Lesions were often both superficial and deep and unrelated to aneurysm site (Rabinstein *et al* 2005), with new infarctions following SAH often located contralateral to the haemorrhage (Brouwers *et al* 1992).

1.4.6 Ischaemic depolarisations

Cerebral infarctions following SAH may result in necrotic brain regions surrounded by an ischaemic penumbra, an area of damaged but potentially viable tissue. Unstable neurons and astrocytes in the penumbra when subject to reduced perfusion may trigger depolarisations that may inturn destabilise adjacent regions, causing progressive ischaemia (Strong *et al* 2007). Ischaemic depolarisations range from localised events to self-propagating waves of mass depolarisation, and may be a major secondary injury mechanism following SAH (Dreier *et al* 2006; Macdonald *et al* 2007; van den Bergh *et al* 2002). A clinical study identified mass depolarisations (cortical spreading depression) in

72% of patients with high grade SAH (Dreier *et al* 2006). New infarctions in these patients were preceded by prolonged cortical depressions (Dreier *et al* 2006).

1.4.7 Rebleeding

Rebleeding risk is high (20% to 40%) until the aneurysm is repaired (Brilstra *et al* 2002; Winn *et al* 1977). Risk is highest at day 1 (affecting around 4% of patients) (Kassell *et al* 1990) and 15% of re-bleeds occur within hours of the ictus (Brilstra *et al* 2000; Ohkuma *et al* 2001). However rebleeding risk remains moderate (1 to 2% per day) for 4 to 8 weeks (Kassell *et al* 1990; Macdonald *et al* 2007). Increased arterial blood pressure in response to raised ICP probably contributes to rebleeding. Reduced intracranial compliance following the initial bleed is likely to contribute to the poor outcome: 80% of these patients will die or suffer permanent disability (Bederson *et al* 2009; van Gijn *et al* 1985).

1.5 Management of subarachnoid haemorrhage

Management of SAH is focussed on securing the aneurysm to prevent rebleeding, maintaining cerebral perfusion in the presence of raised ICP or vasospasm, and preventing or alleviating vasospasm (Bederson *et al* 2000). High ICP may lead to impaired consciousness and intensive care management including respiratory support may be required. Current SAH management guidelines from the Stroke Council of the American Heart Association note that increased vascular permeability is linked to brain pathophysiology following SAH (Bederson *et al* 2009). However, it is unclear how such mechanisms contribute to outcome and there are no therapies that specifically target blood-brain barrier preservation.

1.5.1 Surgical management of subarachnoid haemorrhage

To prevent rebleeding, early aneurysm repair is recommended and may be achieved by surgical clipping of the aneurysm neck or by platinum coils inserted endovascularly into the aneurysm to induce thrombosis (Baltsavias *et al* 2000; Brilstra *et al* 2002; Dehdashti *et al* 2004; Frazer *et al* 2007). Cerebral vasospasm may be treated with cerebral angioplasty (endovascular balloon dilation) (Coenen *et al* 1998). Hydrocephalus may be managed with ventriculostomy in patients with diminished consciousness. Chronic hydrocephalus may be managed by temporary or permanent CSF diversion by ventriculoatrial, ventriculoperitoneal or lumboperitoneal shunt. In cases of high ICP that are unresponsive to non-surgical management, early craniectomy has been associated with improved outcome (Schirmer *et al* 2007).

1.5.2 Medical management of subarachnoid haemorrhage

Medical management includes blood pressure control with vasopressors and fluids to maintain CPP whilst balancing the hypertension-related risk of re-bleeding (Bederson *et al* 2009). Antifibrinolytics reduce re-bleeding risk and may be considered either ahead of surgery or where surgery is contraindicated (Hillman *et al* 2002; Roos 2000). Anticonvulsants may be administered prophylactically in the acute phase following SAH (Rhoney *et al* 2000).

Whilst SAH management guidelines do not address ICP management per se, the guidelines for the management of severe traumatic brain injury (Bratton *et al* 2007b; Bratton *et al* 2007c) make recommendations that are relevant to ICP and CPP management following SAH. These guidelines recommend treatment should be considered with ICP

above 20 mmHg, even if CPP can be maintained by blood pressure manipulation, due to the risk of brain herniation (Bratton *et al* 2007c). To prevent cerebral ischaemia, CPP should be maintained ≥ 50 mmHg, systolic blood pressure ≥ 90 mmHg, blood O_2 saturation ≥ 90% and PaO_2 ≥ 60 mmHg (Bratton *et al* 2007b; Bratton *et al* 2007d). However, due to the risk of adult respiratory distress syndrome, aggressive management with fluids and vasopressors to maintain CPP > 70 mmHg should be avoided (Bratton *et al* 2007b).

Intermittent hyperventilation is recommended with caution (Bratton *et al* 2007e) as the reduced $PaCO_2$, induces vasoconstriction, which results in reduced ICP but also reduces brain tissue oxygenation (Schneider *et al* 1998). Hyperosmolar therapy can be effective in reducing ICP, presumably by reducing brain oedema. Official guidelines recommend mannitol (Bratton *et al* 2007a). Evidence supporting use of hypertonic saline is increasing (Bermueller *et al* 2006; Tseng *et al* 2007a).

Oral nimodipine (a calcium channel blocker) appears to reduce symptomatic vasospasm but interestingly, does not impact on overall outcome at 3 months (Haley *et al* 1993). As hypovolaemia following SAH is associated with brain ischaemia (Hasan *et al* 1989b), isotonic fluids are recommended to maintain circulating blood volume (Bederson *et al* 2009). Cerebral vasospasm is commonly treated with triple-H therapy (induction of hypertension and blood volume expansion/ haemodilution) (Haley *et al* 1993; Lanzino *et al* 1999; Tseng *et al* 2007b). However recent clinical evidence questions the benefit of triple-H therapy over hypertension alone (Muench *et al* 2007). Induced hypertension (eg MAP 143 ± 10 mmHg) increases regional cerebral blood flow and brain tissue

oxygenation but with the addition of hypervolaemia/haemodiluation, brain tissue oxygenation is diminished (Muench *et al* 2007). Hyponatraemia (which affects some 10% to 30% of SAH patients) may be treated with hypertonic saline and fludrocortisone acetate to reduce the risk of poor outcome associated with fluid contraction (Brouwers *et al* 1993; Hasan *et al* 1989a; Qureshi *et al* 2002).

1.6 Substance P and brain pathophysiology

1.6.1 Substance P

Description

Substance P is an 11 amino acid peptide synthesised from the pre-protachykinin A gene (Carter and Krause 1990; Harrison and Geppetti 2001; Hokfelt *et al* 2000b). It was first isolated in the 1930's from equine brain and gut by von Euler and Gaddam and noted for its effects on vasculature (hypotensive) and gut kinetics (smooth muscle contraction) effects (Von Euler and Gaddam 1931). It is a member of the tachykinin family of peptides, named for their rapid onset of action in contrast to the slower acting bradykinins (Maggi 1995). Other tachykinins include neurokinin A (produced by alternate splicing of the same gene), neurokinin B, the endokinins and haemokinins (Harrison and Geppetti 2001).

Localisation

SP is found in nervous tissue throughout the body including in perivascular sensory neurons in the peripheral and central nervous systems, the dorsal root ganglia of the spinal cord, the gastrointestinal tract, the respiratory tract, blood vessels and many brain regions (De Biasi and Rustioni 1988; Severini *et al* 2002; Sternini *et al* 1995). In the brain, SP is widely distributed, especially in regions associated with emotion such as the

hypothalamus, amygdala and periaqueductal grey (Weidenhofer *et al* 2006; Yip and Chahl 2001). It is often co-localised with other neuropeptides including neurokinin A and calcitonin gene-related peptide, as well as other neurotransmitters including glutamate and serotonin (Hokfelt *et al* 2000a; Ribeiro-da-Silva and Hokfelt 2000).

Overview of functions

In the present study, the role of substance P as a promoter of inflammation (see 1.6.3) is a particular focus. However, substance P is also an excitatory neurotransmitter and neuromodulator a broad range of PNS and CNS functions including pain (De Felipe *et al* 1998; Zubrzycka and Janecka 2000), anxiety and depression (Ebner and Singewald 2006), nausea and emesis (Hesketh 2001), respiratory rhythm (Bonham 1995), learning and memory (Gerhardt *et al* 1992; Hasenohrl *et al* 2000; Huston *et al* 1993) and neurogenesis (Harrison and Geppetti 2001; Park *et al* 2007). Substance P may affect cardiovascular function via central excitation of the SNS (Unger *et al* 1981) and also exerts localised influence on vessel tone that may be vasodilatory or vasoconstrictive (Scotland *et al* 2004; Xie *et al* 2010), as discussed below (see 1.6.2).

Synthesis

SP is synthesised by cell body ribosomes, packaged into vesicles and transported to axon terminals where it undergoes final enzymatic processing to form the biologically active peptide (Harrison and Geppetti 2001; Hokfelt *et al* 2000b). In normal conditions, substantial amounts of SP are stored in large dense vesicles, ready for rapid release upon neuronal activation (Harrison and Geppetti 2001; Hokfelt *et al* 2000b). The SP in perivascular sensory neurons surrounding cerebral vessels mainly originates from the trigeminal nucleus, whilst the dorsal root ganglia of the upper 2 cervical nerve roots

produce SP in nerves surrounding vessels of part of the posterior circulation. In addition, SP found in the brain may be synthesised by endothelial cells, microglia and perivascular astrocytes.

Receptors

SP, neurokinin A and neurokinin B bind selectively but not exclusively to the tachykinin receptors NK1, NK2 and NK3 respectively (Regoli *et al* 1994). There is cross reactivity between various neurokinins (and indeed other tachykinins such as haemokinins) and their preferred receptors, depending on neuropeptide concentration and availability of receptors (Hardwick *et al* 1997; Regoli *et al* 1994). However, NK1 receptors predominate in the human brain (Dietl and Palacios 1991; Rigby *et al* 2005), and so are a major focus of research with potential application to human brain pathology.

NK1 receptors are G-protein coupled receptors (Maggi and Schwartz 1997). When SP activates NK1 receptors, G-protein stimulation results in a cascade of intracellular events leading to ion channel regulation, enzyme activity and gene expression changes (Ansar and Edvinsson 2008). The NK1-SP complex is rapidly internalised, dissociated and SP degraded, after which the NK1 receptor is recycled to the cell membrane over several hours (Lundy and Linden 2004). The rapidity of receptor internalisation and time required for receptor recycling results in a rapidly diminishing response to continued exposure of cells to SP.

As well as neurons, NK1 receptors are expressed by blood components including inflammatory cells and platelets, by endothelial cells and mast cells. SP can induce cytokines involved in induction of NK1 transcription factors (Cuesta *et al* 2002; Lotz *et al*

1988; Yamaguchi *et al* 2004), so that sensitivity to subsequent SP exposure may be enhanced. Such mechanisms may contribute to the role of substance P in the development of chronic pain (Lembeck and Donnerer 1981; Lembeck *et al* 1981; Urban and Gebhart 1999).

Metabolism

Inactivation of SP following its release occurs via proteolytic enzymes, which catalyse degradation of hydrolytic bonds so that the binding domain is cleaved. The primary enzymes involved appear to be angiotensin-converting enzyme (ACE) and neutral endopeptidase (Cyrino *et al* 2002; Wang *et al* 1991). Inhibition of these enzymes may exaggerate the effects of SP and other tachykinins (Harford-Wright *et al* 2010).

1.6.2 Influences of SP on vascular tone

SP exerts both vasodilatory and vasoconstrictive influences on vascular tone by a number of diverse mechanisms. As a major sensory neurotransmitter, SP is involved in a range of sensory reflexes, including those mediated by the SNS and parasympathetic nervous system (PSNS), in addition to exerting localised influences on vessel tone in certain circumstances. As discussed below, SP appears to exert a more dominant influence on vessel tone in pathological conditions whist other mediators are dominant in baseline conditions.

Low doses of SP applied intra-luminally leads to vasodilation and higher doses lead to vasodilation followed by vasoconstriction (Tsuji and Cook 1992). The vasodilation is endothelium-dependent: removal of endothelium (by saponin) results in decreased dilation and increased constriction in response to SP (Tsuji and Cook 1992). The vasodilation is

thought to occur by by local activation of endothelial NK1 receptors, stimulating nitric oxide release, which stimulates vascular smooth muscle relaxation (Mejia *et al* 1988; Stubbs *et al* 1992), possibly by K^+ channel opening and hyper-polarisation of smooth muscle cells (Faraci and Heistad 1998; Toda and Okamura 1998).

The constriction response produced by higher doses of intraluminal SP is related to opening of smooth muscle calcium channels and is prevented by the calcium channel antagonist nimodipine (Tsuji and Cook 1992). Potential implications in SAH are that high levels of circulating SP may induce vasoconstriction, while damaged cerebral endothelium may be susceptible to vasospasm in response to lower levels of circulating SP.

Another mechanism by which SP mediates vasoconstriction is related to the reflex response to increased intraluminal pressure. As noted above, this response is integral to the autoregulation of brain blood flow. In vitro studies have demonstrated that smooth muscle constriction in response to elevation of intraluminal pressure is suppressed by capsaicin desensitisation of sensory nerves, or by blockade of TRPV1 receptors (by capsazepine) and is almost completely abolished by NK1 receptor blockade. These effects have been demonstrated in both mesenteric (Scotland *et al* 2004) and cerebral (Xie *et al* 2010) arteries. An implication for SAH is that SP may mediate cerebral vessel constriction in response to increase blood pressure when ICP is elevated.

Experimental intracerebroventricular (icv) injection of SP causes a dose dependent blood pressure increase (Unger *et al* 1981). This response appears to be mediated by the SNS: it is accompanied by increases in plasma noradrenaline and adrenalin and is reversed by

blockade of peripheral α-receptors. Whilst icv injection of angiotensin II is more potent that SP in inducing a systemic BP increase (Unger *et al* 1981), the SP-induced in systemic BP may be protective in certain conditions, by maintaining perfusion of vital organs. This action of SP appears to be critical to outcome in a study of experimental hypotension (Wang *et al* 2008). Rats that received lipopolysaccharide injection demonstrated acute hypotension, followed by increases in plasma SP, norepinephrine and epinephrine and recovery of blood pressure within 1 hour. However, rats that received lipopolysaccharide injection plus NK1 receptor blockade with the selective antagonist RP-67580 demonstrated no BP recovery. Rats that received TRPV1 receptor blockade with capsazepine also had impaired BP recovery (Wang *et al* 2008). Both capsazepine and RP-67580 treated groups had increased mortality (Wang *et al* 2008), suggesting that SP mediated SNS stimulation that protected vital organ perfusion following lipopolysaccharide injection.

1.6.3 The role of SP in neurogenic inflammation

Neurogenic inflammation is an inflammatory response (including vasodilation, plasma extravasation and inflammatory cell infiltration) induced by neuropeptides released by sensory neurons. This mechanism is well recognised in the peripheral nervous system (Bayliss 1901). More recently, neurogenic inflammation in the central nervous system has been recognised and its importance in various CNS pathologies is increasingly evident (Donkin *et al* 2009; Nimmo *et al* 2004; Turner *et al* 2006; Vink *et al* 2003).

SP, calcitonin gene related peptide (CGRP) and NKA are released together from sensory neurons and can act synergistically to magnify each other's effects (Brain and Williams

1985). All three neuropeptides act as vasodilators whilst only SP and NKA increase vascular permeability. SP's overall impact on vessel tone appears to vary in normal versus pathophysiological conditions. SP anti-gammaglobulin does not affect the baseline diameter of cerebral arteries whilst CGRP anti-gammaglobulin decreases vessel diameter (Shiokawa *et al* 1993). This indicates that in baseline conditions CGRP is the dominant influence on vessel tone. In pathological conditions, the effect of SP appears more dominant, with noxious stimulation increasing release of SP both antidromically and centrally. SP applied directly to the cerebral cortex of anaesthetised cats increases arteriole calibre by 21% and calibre of veins by 16% (Edvinsson *et al* 1995). CGRP, a more potent vasodilator, increased arteriole calibre by 38% (Edvinsson *et al* 1995).

SP increases vascular permeability by activating NK1 receptors expressed by endothelial cells of post capillary venules (Bowden *et al* 1994; Hu *et al* 2005). In response, inter-endothelial cell gaps open in the walls of post-capillary venules. This enables protein extravasation, which is followed osmotically by water and ions. The effect of SP is limited by internalisation of ligand-bound NK1 receptors into endothelial cells, reducing sensitivity to further SP exposure and limiting plasma leakage (Bowden *et al* 1994). ACE inhibitors, which reduce SP metabolism and enable SP to accumulate in the CNS, are associated with increased plasma extravasation (Cyrino *et al* 2002; Harford-Wright *et al* 2010).

Plasma extravasation can be rapid following SP exposure. Evans blue leakage from postcapillary venules has been noted within seconds, peaking within two minutes then declining rapidly (Bowden *et al* 1994). A second dose of SP at three minutes has been

found to produce almost 50% less leakage of Evans Blue (due to receptor internalisation). The number of NK1 receptors on the surface of endothelial cells returned to normal at 120min, but it was 8 hours until the plasma leakage response to SP returned to the initial magnitude (Bowden *et al* 1994).

A number of triggers may stimulate SP release from sensory neurons including heat and acidosis, which activate transient receptor potential vanilloid type 1 (TRPV1) receptors (Caterina *et al* 1997). SP also stimulates mast cell degranulation, leading to the release of amines that enhance oedema (Cao *et al* 1999). Bradykinin, serotonin, prostaglandins and cytokines can in turn activate sensory neurons and trigger further SP release.

TRPV1 receptors are of particular interest in SP research as they are specific to SP containing neurons. They are activated by capsaicin, which is contained in chilli peppers. Both SP and capsaicin increase blood-brain barrier permeability in pial venular capillaries and NK1 receptor antagonists reduce the response (Hu *et al* 2005). Repeated capsaicin exposure can lead to depletion of SP from these neurons for a period. This can be used to assess the role of SP in various pathologies (Caterina *et al* 1997; Delgado-Zygmunt *et al* 1990; Nimmo *et al* 2004).

In addition to neurons, SP and NK1 receptors are also expressed by a range of inflammatory cells including leukocytes and mast cells (De Giorgio *et al* 1998; Lundy and Linden 2004; Maggi 1997). SP can stimulate mast cell degranulation with release of cytokines, and other proinflammatory agents including bradykinin, histamine and serotonin. These agents mediate may further SP release from sensory nerves. Cytokines

can also stimulate release of SP from sources other than neurons, such as brain endothelial cells and NK1 receptors on endothelial cells can facilitate leukocyte adhesion (Baluk *et al* 1995; Brain 1997; Lotz *et al* 1988; Yamaguchi *et al* 2004).

Figure 1.5 Overview of SP in neurogenic inflammation 1) Initial triggers may include mechanical and ischaemic tissue damage. 2) SP is released antidromically and centrally, mediating central conduction. 3) Vascular effects of peripherally released neurokinins include arteriolar dilation, increased venular permeability and inflammatory cell infiltration. 4) Chemicals released by mast cells (including tryptase, bradykinin, serotonin and prostaglandins) may act on sensory nerves to facilitate further SP release. Adapted from (Purves *et al* 2004)

1.6.4 Possible role in subarachnoid haemorrhage

Role of SP in acute brain injuries

Experimental studies have shown that SP is an important mediator of blood-brain barrier breakdown and brain oedema formation in traumatic brain injury (Donkin 2006; Nimmo *et al* 2004), ischaemic stroke (Turner *et al* 2006) and intracerebral haemorrhage (Kleinig *et al* 2010). Moreover, administration of the SP NK-1 receptor antagonist, n-acetyl-L tryptophan (NAT), reduces oedema in these models (Donkin 2006; Kleinig *et al* 2010; Turner *et al* 2006) and significantly improves outcome (Donkin 2006; Turner *et al* 2006). This intervention has not previously been tested in models of SAH, and the role of SP in SAH has not been fully characterised. Crucially, whilst blood-brain barrier breakdown and vasogenic brain oedema are considered important features of SAH pathophysiology, it is unclear if SP mediates oedema formation in SAH as it does in the other conditions noted above.

Increased SP release in SAH

SP is rapidly released from cerebral perivascular nerves following experimental SAH (Edvinsson *et al* 1990; Linnik *et al* 1989; Uemura *et al* 1987). Several studies report depletion of SP (reduced immunoreactivity) in perivascular fibres at 4 h, 24 h, 48 h and even 28 days (Edvinsson *et al* 1990; Hara *et al* 1986; Linnik *et al* 1989; Tran Dinh *et al* 1994; Uemura *et al* 1987). Results of these studies are summarised in Table 1.1. Increased levels of pre-protachykinin A mRNA and increased levels of SP protein (49% above baseline) have been noted in the trigeminal ganglia 2 days after experimental SAH (Linnik *et al* 1989).

Despite this, axonal SP levels take some time to be restored. They were not restored until day 63 in a canine SAH model (Uemura *et al* 1987), though normal levels were restored at seven days in a rabbit model (Linnik *et al* 1989). It is unclear if this was due to continued SP release throughout this period as levels of SP in brain tissue was not quantified. Interestingly, neuropeptide depletion was bilateral, though more marked on the side of the clot (Hara *et al* 1986). The rapid and widespread release of SP and its increased production early following SAH suggest that SP may contribute to early pathophysiological processes.

Table 1.1 Altered SP immunoreactivity following experimental SAH

Authors	Model	SP content following experimental SAH
(Tran Dinh *et al* 1994)	Rabbit injection	Increased in CSF at 30 min Slightly increased at 24 h
(Edvinsson *et al* 1990)	Rat injection	Decreased in brain perivascular sensory fibres at 48 h (late vasospasm phase)
(Linnik *et al* 1989)	Rabbit injection	Decreased^{+} in brain perivascular sensory fibres at 4 h and 48 h SP and PPTa mRNA increased in trigeminal ganglion at 48 h
(Uemura *et al* 1987)	Dog injection	Decreased^{+} in brain perivascular sensory fibres immediately
(Hara *et al* 1986)	Monkey clot placement	Decreased in cerebral perivascular nerves at 2 d, 7 d and 28 d

With SP depletion from perivascular nerves, a concurrent increase in SP within brain parenchyma and in CSF SP may be expected. None of these studies reported SP immunoreactivity within brain parenchyma (as opposed to perivascular nerves). CSF SP concentration was markedly increased 30 minutes after rabbit SAH, remaining high at 24 h

and returned to baseline at day 3 (Tran Dinh *et al* 1994). However, a clinical study found no change in CSF or venous SP concentration (Juul *et al* 1995). One possible explanation for this conflicting result is that a very acute increase in CSF SP following human SAH may have been undetected by the timing of sampling in the human study. CSF SP concentration can increase and decrease very rapidly, as data from post-traumatic stress disorder studies show (Geracioti *et al* 2006).

Effect of SP blockade in experimental SAH

Several studies have investigated the effect of SP blockade in experimental SAH and the results indicate SP may play a significant role in its pathophysiology. The outcome measures used in these studies (cerebral blood flow, arterial diameter and the phenotype of receptors in cerebral vessels) were all focussed on vasospasm. This is not surprising, since vasospasm has long been considered an important mechanism of secondary brain injury following SAH. The effect of SP blockade on oedema, ICP and functional outcome has never been characterised. However, the results of the studies that have been undertaken to date (summarised in Table 1.2) suggest that SP may influence mechanisms other than vasospasm following SAH.

Agents that block the actions of SP, either administered prior to experimental SAH or at 2 hours after SAH, have been found to eliminate acute vasospasm, eliminate delayed vasospasm and improve cerebral blood flow (CBF) compared to untreated animals (Delgado-Zygmunt et al 1993; Delgado-Zygmunt et al 1990; Shiokawa et al 1993). Likewise, capsaicin-induced depletion of SP from sensory neurons prior to experimental SAH prevents both acute and late vasospasm (Delgado-Zygmunt et al 1990).

Table 1.2 The effect of SP blockade in experimental SAH. Prevention of vasospasm and improvement in cerebral blood flow are consistent findings.

Authors	SAH model & treatment	Treatment effect
(Ansar *et al* 2007a)	Rat (injection) NK1R antagonist (L-822429) at 30 m + 24 h	Cerebral Blood Flow (CBF) preserved Vascular receptor phenotype preserved (Untreated: CBF reduced, ET and 5-HT1B receptors up-regulated)
(Delgado-Zygmunt *et al* 1993)	Squirrel monkey (injection) SP antagonist (Spantide) at 2h + 3 d	Delayed vasospasm reduced CBF preserved
(Delgado-Zygmunt *et al* 1990)	Rat (injection) Capsaicin pre-treatment	Acute vasospasm prevented Delayed vasospasm prevented
(Delgado-Zygmunt *et al* 1990)	Rat (injection) SP antagonist (Spantide) at 2h pre-SAH or at 2h post-SAH	 Acute vasospasm prevented Delayed vasospasm prevented Delayed vasospasm prevented
(Shiokawa *et al* 1993)	Squirrel monkey (injection) Gamma globulins against SP: pre-SAH and daily post-SAH x 5 d	Pre-SAH no effect Acute vasospasm prevented Delayed vasospasm prevented Cerebral blood flow improved

By preventing vasospasm, agents that block the actions of SP help to maintain normal vessel calibre following SAH, and consistent with this, animals treated with these agents have more normal CBF compared to untreated animals (Ansar *et al* 2007a; Shiokawa *et al* 1993).

As intraluminal SP has a vasodilation effect (Tsuji and Cook 1992), it is interesting to consider how SP blockade protected against vasospasm in these studies. Local and/or centrally mediated effects may have been involved. At the local level, blockade may have inhibited the SP mediated vasoconstriction in response to intraluminal hypertension (Scotland *et al* 2004) (Xie, 2010). With increased ICP following SAH, hypertension is also more likely, either as a physiological response or medically induced to maintain CPP. This may potentiate a vasoconstrictive response in cerebral vessels. Another localised mechanism of vasoconstriction that may be affected by SP blockade relates to the endothelium. Cerebral endothelial cell damage, including apoptosis, may be crucial in SAH pathophysiology, especially oedema formation (Park *et al* 2004). Hypothetically, endothelial cell damage may increase the susceptibility of underlying smooth muscle cells to the vasoconstrictive influence of circulating SP. Finally, SP blockade may also have inhibited vasoconstriction mediated by the SNS (Wang *et al* 2008). Lesions to the A2 nucleus of the medulla or the median eminence of the hypothalamus prevent acute and delayed vasospasm in squirrel monkey SAH (Svendgaard *et al* 1992). It has been hypothesised that SP conveys information to these brainstem centres to facilitate the regulation of cerebral blood flow and glucose metabolism (Shiokawa and Svendgaard 1994).

Nonetheless, more complex mechanisms are probably involved. Activation of NK1 receptors following experimental SAH in rats leads to up-regulation of receptors for vasoconstrictive agents (5-HT and ET receptors) in cerebral arteries (Ansar *et al* 2007a). The NK1 receptor antagonist L-822429 prevents this up-regulation and also prevents the post-SAH reduction in global and regional blood flow measured in non-treated animals during the late vasospasm period (Ansar *et al* 2007a). Regardless of the mechanisms underlying late development of vasospasm, the fact that it was completely prevented following experimental SAH in monkeys and in rats by interventions that blocked the action of SP, (Delgado-Zygmunt *et al* 1993; Delgado-Zygmunt *et al* 1990) even when administered up to 2 h following SAH, indicates that SP may play a critical role and that a therapeutic window may exist.

Several characteristics of SAH pathophysiology make it reasonable to hypothesise that SP plays a role beyond vasospasm development. Cerebral vasospasm following SAH is often a localised phenomena, but SP release is widespread (Hara *et al* 1986), consistent with SAH pathophysiology having global effects on the brain (Rabinstein *et al* 2005; Voldby *et al* 1985b). Blood-brain barrier (BBB) breakdown and oedema formation after SAH are also widespread (Doczi 1985; Nakagomi *et al* 1990), and whilst numerous mediators may be involved in BBB breakdown, SP has been shown to increases BBB permeability (Bowden *et al* 1994; Hu *et al* 2005).

1.7 Summary

SAH affects a relatively young population and mortality is high. Most patients die within 48 hours of the ictus, ahead of the period in which delayed vasospasm develops. Interventions that target early mechanisms of secondary brain injury following SAH are needed.

Intracranial hypertension is an important determinant of outcome following SAH, and brain oedema is well known to contribute to raised ICP in other conditions. Previous studies have shown that SP plays a critical role in oedema formation and the development of functional deficits following acute brain injury. However, no study has ever investigated whether SP contributes to brain oedema, ICP and poor outcome following SAH. It has been shown that SP is rapidly depleted from cerebral perivascular neurons following SAH (Edvinsson *et al* 1990; Hara *et al* 1986; Linnik *et al* 1989; Uemura *et al* 1987). Oedema formation following SAH has also been characterised (Doczi 1985; Nakagomi *et al* 1990) and has been linked to poor outcome (Thal *et al* 2009a). However it is unknown if SP contributes to the oedema formation and raised ICP after SAH, or if the oedema is associated with any increase in ICP after SAH. SP blockade, by administration of the NK1 receptor antagonists, reduces oedema and improves outcome in models of acute brain injury. SP blockade following experimental SAH inhibits vasospasm, normalises cerebrovascular receptor expression and maintains CBF (Ansar *et al* 2007a; Delgado-Zygmunt *et al* 1993). The focus of the current studies is therefore to characterise the impact of NK1 antagonists on blood-brain barrier permeability, oedema formation, ICP and the development of functional deficits after SAH.

1.8 Study aims

These studies aim to:

1. Characterise functional outcome, brain oedema, blood-brain barrier permeability and substance P immunoreactivity following injection and filament SAH; and

2. Determine the effect of the substance P antagonist NAT on these measures.

Rats were deemed the most suitable animal for use in the present study given the number of animals required, and the need for functional assessment. Neurological function can be readily assessed in rats following experimental SAH using a range of well established tests and other outcome measures, such as brain oedema, could be compared directly with previously published results. Both an injection SAH model and an arterial puncture SAH model were selected so that results could be informed by both a consistent haemorrhage volume and any biochemical cascade or pathophysiological phenomena particular to vessel puncture SAH

Chapter 2

Materials and methods

2 Materials and methods

2.1 Model selection

2.1.1 Overview of SAH models

Numerous experimental models of SAH have been developed using a range of animals including primates (Delgado-Zygmunt *et al* 1993), dogs (Sasaki *et al* 1985; Zubkov *et al* 2002), pigs (Mayberg *et al* 1990), cats (Brinker *et al* 1992), rabbits (Johshita *et al* 1990) and rats (Bederson *et al* 1995; Prunell *et al* 2002). Model selection is influenced by the model's ability to closely mimic human SAH (primates), the expression of key features (eg in models that require craniotomy, ICP elevation may be less pronounced), cost (rodents are relatively inexpensive) and ethical considerations (survival studies in monkeys and dogs generally induce more minor brain injury) (Megyesi *et al* 2000).

Two broad methods are used to induce experimental SAH. The first involves injection of fresh, autologous arterial blood into the subarachnoid space, for example into the prechiasmatic cistern (Prunell *et al* 2002) or into the cisterna magna (Shiokawa *et al* 1993). The alternative method involves endovascular puncture of the arterial circulation within the subarachnoid space, for example the circle of Willis (Bederson *et al* 1995).

Potential advantages of injection SAH models include a precise and reproducible haemorrhage volume, which can be adjusted to control SAH severity and model mortality. Multiple injections are used in some models to maximise features such as vasospasm (Foley *et al* 1994; Kai *et al* 2007). Arterial puncture SAH models reproduce many features

of human SAH including exposure of the vessel wall to the subarachnoid space and bleeding under arterial pressure until clotting occurs by normal physiological mechanisms. However, an associated disadvantage is variability in haemorrhage volume.

After reviewing the many SAH models that have been developed to date, we elected to use both the prechiasmatic cistern injection model of SAH (injection SAH) and the endovascular filament perforation model of SAH (filament SAH), using male Sprague-Dawley rats. The rationale for these selections is detailed below.

2.1.2 Species and strain

Sprague-Dawley rats were deemed the most suitable animal for use the present studies as they present the following advantages.

1. Cost effectiveness. The high number of animal required to complete the present studies is compounded by the high mortality rates associated with filament SAH (see 2.1.4)(Ostrowski *et al* 2005; Park *et al* 2004; Prunell *et al* 2003).
2. Functional outcome assessment. A range of neurological outcome tests for rats are well established in our laboratory (Cernak *et al* 2002; Donkin *et al* 2009; Kleinig *et al* 2010; Turner *et al* 2004) and used widely by others (Thal *et al* 2008). Sprague-Dawley rats have a calm disposition, which facilitates ease of handling.
3. Comparison of results with those reported elsewhere. Functional neurological outcome following experimental SAH in Sprague-Dawley rats has been reported by others (Germano *et al* 2007; Imperatore *et al* 2000; Thal *et al* 2009a; Thal *et al* 2009b). Brain water content following experimental SAH in this species and strain

has also been reported elsewhere, enabling direct comparison of our results (Ostrowski *et al* 2005; Park *et al* 2004; Thal *et al* 2009a; Yatsushige *et al* 2007). Albino rats have poorer visual acuity than pigmented rats (Prusky *et al* 2002) due to multiple factors including lack of iris pigment, fewer photoreceptors (Grant *et al* 2001), retinal degeneration in response to ambient light (Lanum 1978) and poor organization of visual pathways within the brain (Silver and Sapiro 1981). This can affect their performance in certain behavioural tests as detailed later in this chapter (see 2.3.7).

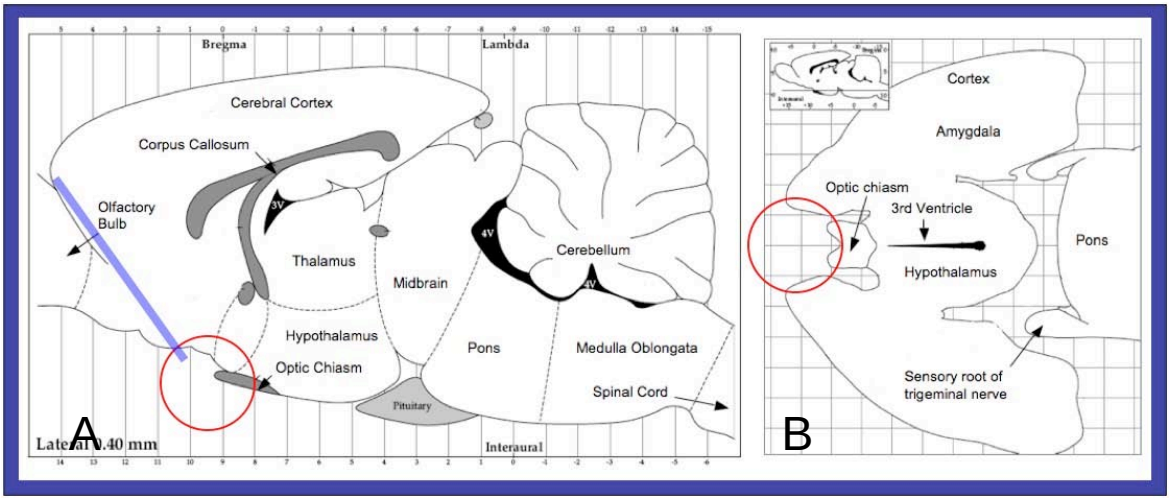
2.1.3 The prechiasmatic cistern injection model of SAH

Injection models of SAH provide a precise and reproducible haemorrhage volume. Several models have been developed in which fresh autologous arterial blood is injected into a subarachnoid cistern (Hara *et al* 1986; Prunell *et al* 2002; Svendgaard *et al* 1977). These cisterns are enlargements of the subarachnoid space formed where arachnoid and dura (conforming to the cranial cavity contours) stretch across prominences of the brain; blood pools in these cisterns following SAH.

The most widely used injection SAH models involve blood injection into the cisterna magna and this has been adapted to many species (Delgado *et al* 1985; Hara *et al* 1986; Tran Dinh *et al* 1994; Uemura *et al* 1987). As its name suggests, the cisterna magna (or cerebellomedullary cistern) is the largest cistern making access relatively easy. However, following cisterna magna injection, blood is found over the posterior cranial fossa and spinal canal (Prunell *et al* 2003). This is in contrast to human SAH, which most commonly arises from rupture of the anterior circulation and results in blood distribution more generally over the base of the brain (Kassell *et al* 1990; Velthuis *et al* 1998).

Another possible limitation of SAH involving injection into the cisterna magna relates to ICP changes at the ictus. An ICP rise to MABP level has been observed during human SAH (Nornes 1978) and it is hypothesised such a rise stems blood and facilitate clotting at the ictus (Nornes 1978). While an ICP rise to MABP level (and cerebral perfusion arrest) may not be essential for clotting (McCormick *et al* 1994), high ICP at the ictus, whether or not it produces a transient, global cerebral ischaemia, appears to be an important trigger of SAH pathophysiology (Nakagomi *et al* 1990). Cisterna magna injection SAH does not always produce a substantial ICP rise, possibly because of pressure transmission along the craniospinal axis.

This limitation of cisterna magna injection SAH has been addressed by the prechiasmatic cistern injection model developed by Prunell and colleagues (Prunell *et al* 2002). The prechiasmatic cistern lies just anterior to the optic chiasma at the base of the brain (Figure 2.1). A needle can be advanced between the cerebral hemispheres to access this cistern without causing trauma to the brain tissue. The procedure involved is detailed later in this chapter (see 2.1.3).



Adapted from (Paxinos and Watson 1998)

Figure 2.1 The prechiasmatic cistern injection model of SAH. A: Sagittal view of the rat brain 0.4 mm lateral to the midline (V = ventricle). The needle (blue) passes between the cerebral hemispheres to reach the cistern (circled). B: Horizontal view of the rat brain illustrating the boundaries of the prechiasmatic cistern (circled).

Blood can be injected at a rate that elevates ICP to MABP level, and most of the injected blood is subsequently distributed over the basal surface of the brain and the brain stem with sparse clots over the hemispheres and cerebellum (Prunell *et al* 2002). This blood distribution was confirmed in our pilot studies and parallels the distribution of blood commonly seen following human SAH (Figure 2.2).

In developing this model, Prunell and colleagues manually injected 300, 250 or 200 μL into the prechiasmatic cistern of subgroups of rats (Prunell *et al* 2002). At 300 μL, mortality was close to 100%, at 250 μL mortality was 50% and at 200 μL mortality was

considered acceptable at 25%. Therefore, for these studies we elected to use a 200 µL injection volume.

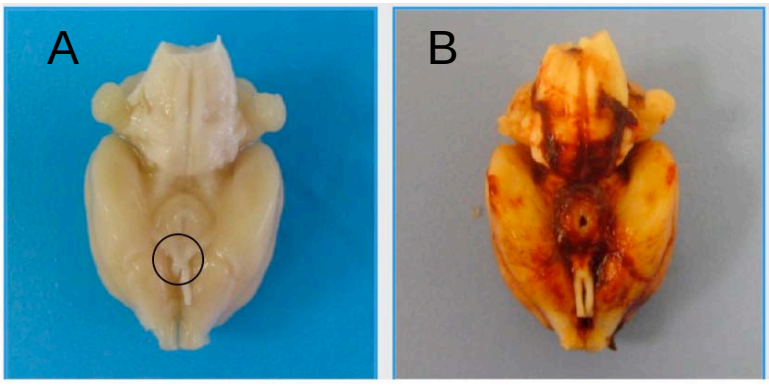


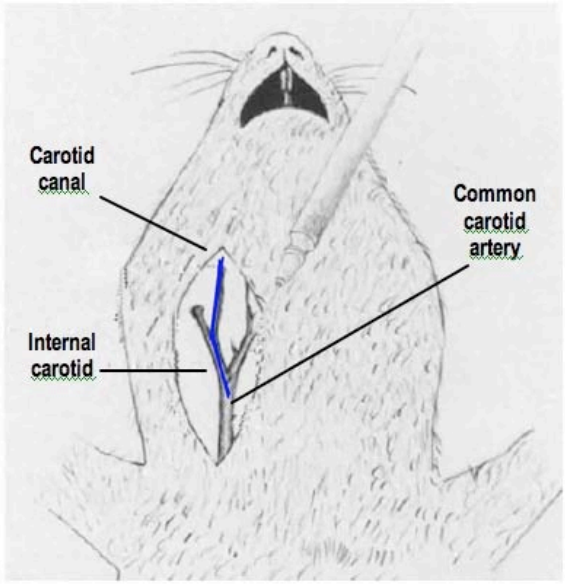
Figure 2.2 SAH produced by the prechiasmatic cistern injection model. A: The prechiasmatic cistern into which the needle tip is placed is circled. B: Blood distribution over the basal cisterns 5 hours following blood injection. Both brains have been perfuse fixed with formalin.

2.1.4 The endovascular filament perforation model of SAH

In addition to the above injection model, an arterial puncture SAH model was also selected to investigate the biochemical cascades and pathophysiological phenomena triggered by vessel puncture. The most widely utilised perforation model is the endovascular filament model, which was initially described by Bederson and colleagues (Bederson *et al* 1995). In this model a surgical stump is made from the external carotid artery into which a piece of nylon suture is threaded and advanced to the circle of Willis near the junction of internal carotid artery and the middle cerebral artery, where arterial puncture leads to haemorrhage into the subarachnoid space (Figure 2.3).

Those who developed the model reported it produced extensive SAH, with blood distributed throughout the basal cisterns and to a lesser extent the ventricular system, resulting in a 24 hour mortality rate around 50% (Bederson *et al* 1995). Refinements of the technique have subsequently been published, including variations in vessel occlusion and puncture technique, and the use of various diameter endovascular filaments in attempt to control haemorrhage volume (Schwartz *et al* 2000).

NOTE:
This figure is included on page 46 of the print copy of the thesis held in the University of Adelaide Library.



B

(van Gijn *et al* 2007)

Figure 2.3 Endovascular filament perforation model of SAH. A: Arrow indicates the site of arterial rupture. Note the proximity to the common sites of aneurysm formation (blue circles) and to the prechiasmatic cistern (black circle). B: The surgical filament (blue) is inserted into the internal carotid artery in the neck and advanced through the carotid canal to reach the circle of Willis.

If ICP is monitored during SAH in this model, the acute ICP peak has been found to correlate with the degree of haemorrhage (Schwartz *et al* 2000). This may provide some control of haemorrhage volume but is obviously more invasive as burr holes need to be drilled and a probe placed into brain parenchyma (see 2.3.3). Another consideration is the need to have the animal positioned prone for the surgical procedure, as weight of abdominal contents on the inferior vena cava in this position is likely slow venous return and reduce ABP. This may compromise CPP if SAH is extensive causing ICP to be high.

Despite the potential disadvantages of filament SAH, including high mortality and the necessity to undertake the procedure with the animals positioned supine, the endovascular filament perforation model of SAH was chosen for use in this study because it mimics human aneurysmal SAH in the following ways:

1. The bleed occurs at the site where aneurysm formation is common clinically;
2. Vessel rupture occurs, exposing the vessel wall to the subarachnoid space;
3. Bleeding occurs under arterial pressure until clotting; and
4. The acute ICP elevation is a physiological reflection of the haemorrhage and not controlled externally by injection rate.

2.2 Animal care

2.2.1 Ethics

Approval for all experiments was obtained from the University of Adelaide Animal Ethics Committee (approval number M-061-2008) and from the Institute of Medical and Veterinary Science Animal Ethics Committee (approval numbers 9-08 and 47-09). Experiments were performed within the guidelines established by the National Health and Medical Research Council of Australia.

2.2.2 General

Adult male Sprague-Dawley rats (350-420g) were obtained from the Australian Research Council Animal Resource Centre (Canning Vale, Western Australia) at least 4 days prior to experimental studies. They were group housed in a conventional rodent room that was maintained at 24°C with a 12 hour light-dark cycle. Animals had free access to food and water. They were randomly assigned to treatment and control groups in experimental studies and the numbers of animals used per group in each study are detailed in the relevant chapters.

2.3 Experimental procedures

2.3.1 Anaesthesia

Isoflurane and ventilation

Isoflurane was obtained from Independent Veterinary Supplies (Melrose Park, South Australia) and was stored in a safe at room temperature away from light. For induction of general anaesthesia, animals were placed in a transparent chamber (approximately 5.5 L

capacity) into which 4% Isoflurane was delivered in 100% oxygen via a calibrated vaporiser at a flow rate of 1.5 L/min. A satisfactory level of anaesthesias was achieved at 4 to 5 minutes, with absent pain reflexes and pilot testing confirming normal levels of blood oxygen saturation despite reduced respiratory rate.

Animals were removed from the induction chamber and suspended by the incisors in an intubation frame. The larynx was visualised using a rat laryngoscope and animals were intubated with a 13G plastic cannula. Tilting the animal approximately 15° in the sagittal plane aided visualisation of the larynx. Animals were then connected to a rodent ventilator (Harvard Instruments) and the cannula base sutured to the cheek to prevent dislodgement during surgery.

Anaesthesia was maintained using 1.8% Isoflurane in 30% O2 and 70% N_2O, with a ventilation rate of between 60 and 85 breaths per minute (adjusted according to blood pCO2), a tidal volume of 2.5ml and 2cm H_2O of positive end expiratory pressure. To ensure a surgical level of anaesthesia was maintained, pain withdrawal reflexes were tested at least every 15 minutes and the level of Isoflurane adjusted as indicated. Ventilatory parameters were adjusted as required to maintain normal arterial blood gases (see 2.3.3).

Urethane

Urethane can provide a surgical level of anaesthesia whilst preserving a number of cardiovascular reflexes that are inhibited by Isoflurane and other general anaesthetics (Maggi and Meli 1986). It therefore presents advantages for studying physiological responses to SAH. Urethane has also demonstrated carcinogenic properties and is therefore not used in survival studies. Thus two different anaesthetic regimes were used in this

study. Non-survival animals were used for continuous ICP and ABP monitoring. These animals received Urethane (Sigma, Australia) anaesthesia during and following SAH or sham procedure and were sacrificed at the end of the monitoring period. Survival animals, which were allowed to wake from surgery for later assessment of neurological function, brain histology and brain oedema, received general anaesthesia with Isoflurane only.

Animals used in ICP and ABP monitoring experiments received initial anaesthesia with Isoflurane for intubation, establishing ventilation and insertion of femoral and arterial lines. Once arterial blood pressure monitoring was in place, Isoflurane was withdrawn and Urethane (1g/kg, prepared 2.5 g per 100 ml solution) was administered (iv) through a cannula positioned in the right femoral vein. The cannulation procedure is described later in this chapter (2.3.2). The initial dose was administered gradually (over approximately 40 minutes), so that normal blood pressure was retained. Pain withdrawal reflexes continued to be assessed at least every 15 minutes throughout the remainder of the surgery and post-surgery monitoring period and additional Urethane was administered in 0.1 ml increments as required.

Lignocaine

Lignocaine solution (Lignocaine hydrochloride 2%, MAVLAB, Australia) was stored at room temperature in a drug safe. As a local anaesthetic, Lignocaine (0.2 ml) was administered subcutaneously prior to all incisions and again prior to wound closure. Applied to the external aspect of exposed vessels, Lignocaine has a vasodilation effect, and so was used as required (0.1 ml) to facilitate femoral vessel cannulation.

Pentobarbital

Pentobarbital (pentobarbitone sodium, 60 mg/ml, Rhone Merieux) was stored at room temperature in a drug safe. It was used as a general anaesthetic prior to perfuse fixation in preparation for histology studies (2.3.9). Following rapid induction (5% Isoflurane in 100% O_2 for 3 minutes), Pentobarbital was administered 60 mg/kg IP using a 25-G, 12.5 mm needle. Perfuse fixation was initiated when pain withdrawal and corneal reflexes were absent (approximately 10 minutes following Pentobarbital injection).

2.3.2 Femoral vessel cannulation

The right femoral artery and vein were cannulated as described below. The arterial line was used for monitoring arterial blood pressure, sampling blood for gas analysis and for harvesting blood for injection SAH. The venous line was used for administration of Urethane anaesthesia and administration of treatment (NAT or saline vehicle).

Cannulae were prepared using polyethylene tubing (Biocorp, Australia) with an inner diameter of 0.58 mm and an outer diameter of 0.96 mm. This size tubing was too large to insert into the vessel lumen, but minimised problems associated with smaller cannulae including small clotting and blood pressure signal loss. To accommodate the small intraluminal size of femoral vessels, a 2 cm section of smaller diameter tubing (outer diameter mm, inner diameter mm) was inserted 2mm into the tubing at one end. At the free end of the larger tubing, a blunted 23 G needle was inserted for attachment to a syringe or blood pressure transducer. Lines were flushed with saline to remove air and to ensure no leakage occurred at junctions.

The right femoral region was shaved, swabbed with alcohol and following injection of local anaesthetic, an incision was made to expose the femoral artery and vein. These were cleaned and tied distally using 4.0 surgical silk (Dynek, Australia). A proximal section of each vessel was transiently occluded, a small incision was made using microscissors and a cannula was inserted and secured with surgical silk. Lines were periodically flushed with saline to maintain patency, also providing fluid replacement for animals.

2.3.3 Physiological monitoring

Temperature

Temperature was monitored using a rectal thermometer and maintained at 37.0 ± 0.5°C using a heat pad and lamp.

Arterial blood gases

Blood samples (0.2 ml) were periodically taken from the femoral arterial line and analysed using an Osmetech OPTI blood gas analyser (CCA, Helena Laboratories Pty Ltd Australia). Ventilatory rate and oxygen were adjusted to maintain normal arterial blood gases (pH between 7.4 and 7.5, pO_2 between 100 and 150mmHg and pCO_2 between 35 and 45mmHg).

Intracranial pressure

ICP was continuously monitored before, during and after SAH or sham procedure by a Codman ICP Express monitoring system (Codman and Shurtleff Inc, USA) and digitally recorded using LabChart data acquisition software (ADInstruments).

With the animal prone, a midline scalp incision was made, the skull exposed and a 2mm burr hole drilled 1mm posterior to bregma and 2mm to the left of the midline. After calibration, the Codman Microsensor probe was inserted so that the tip was positioned just below the dura into the parenchyma of the left parietal cortex.

Injection SAH animals were initially placed in the stereotaxic frame and the lead of the ICP probe was fixed to a carrier arm. The probe could then be positioned with minimal brain tissue trauma (Figure 2.4). In filament SAH animals, the probe was positioned manually and fixed in place with bone wax and super glue. Filament SAH animals were then placed supine on a foam pad, which accommodated the ICP probe and surgery for filament SAH was performed as detailed below (see 2.3.4).

Arterial blood pressure

Arterial blood pressure was continuously monitored using a Stantham-type pressure transducer connected via polyethylene tubing to the femoral arterial catheter. Data was digitally recorded using LabChart data acquisition software (ADInstruments).

2.3.4 Induction of subarachnoid haemorrhage

Injection SAH induction

Autologous blood (200µL) was injected into the prechiasmatic cistern, according to the method devised by Prunell and colleagues (Prunell *et al* 2002). Once anaesthesia and ventilation were established, the rat was placed in a stereotaxic frame (Kopf Instruments). A 2mm burr hole was drilled in the midline, 6.5mm anterior to bregma and sealed with bone wax to prevent bleeding from the underlying venous sinus. A blunt 28G needle with a side hole pointing to the right (Grace Davidson, Australia) was placed in a metal guide

cannula attached to a carrier on the frame, angled 30° in the sagittal plane (Figure 2.4). The needle was advanced through the burr hole at the 30°angle until the needle tip lay in the prechiasmatic cistern (approximately 11.5 mm at this angle from the superior surface of the burr hole).

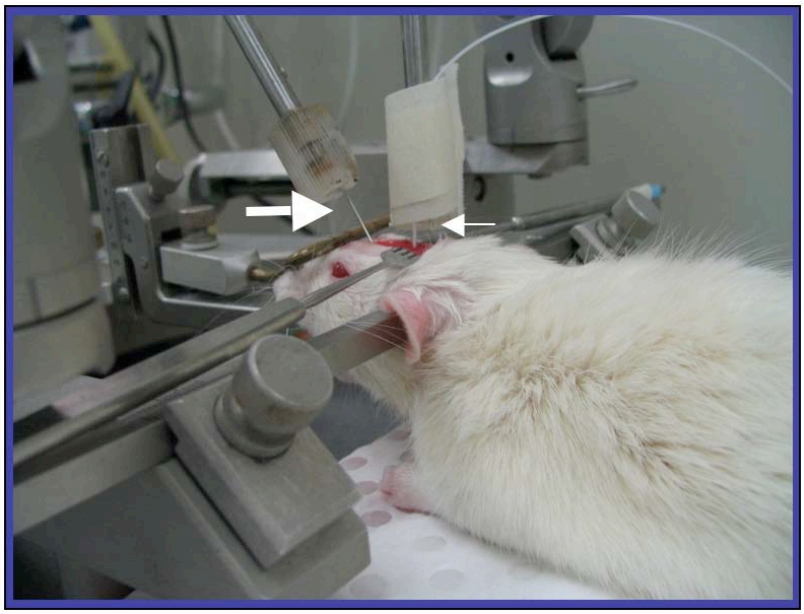


Figure 2.4 Rat positioned in stereotaxic frame for injection SAH. Note needle (large arrow) positioned at 30° in the sagittal plane, passing between the cerebral hemispheres to reach the prechiasmatic cistern with no needle trauma to brain tissue. The lead of the Codman microsensor probe (small arrow) is taped to a carrier of the frame for stable positioning within brain parenchyma 1mm below the dura.

Pilot studies indicated haemorrhage was localised to the subarachnoid space (and subdural or intracerebral haemorrhage avoided) if the needle rested in this position for 30 minutes prior to blood injection. Possibly this period enabled normal CSF flow to return to the cistern that may have collapsed during needle insertion. As non-heparinised blood within

the needle and syringe clots within approximately 3 minutes, it was necessary to insert the needle some time before blood was harvested for injection and keep the needle completely stable during blood injection to avoid potential disruption of the prechiasmatic cistern.

To achieve this, a 20mm length of polyethylene tubing was attached to the base of the needle prior to needle insertion. The tubing and needle were filled with artificial CSF (aCSF, volume = 20 µL). After 30 minutes, blood was taken from the femoral artery, drawn up into a 250 µL syringe attached to a 28G needle with a bevelled tip. The bevelled tip was inserted into approximately 4 mm into the tubing, forming a seal impermeable to air or fluid, and 200 µL of blood, together with the 20 µL of aCSF, was injected over 30 seconds into the prechiasmatic cistern. Injection sham animals had all procedures including needle insertion but no blood injection. In addition, some sham animals received an injection of 220 µL of aCSF (n = 2) or normal saline (n = 2) into the prechiasmatic cistern.

Filament SAH induction

Once anaesthesia and ventilation were established, the rat was placed supine, the neck was shaved, swabbed with alcohol and following injection of local anaesthetic, an incision was made to expose the carotid region. A surgical stump was made from the external carotid artery. A 3-0 nylon filament was introduced through the stump into the internal carotid artery and advanced (around 22mm) to perforate the internal carotid artery near the middle cerebral artery junction. The filament was then immediately withdrawn and the stump tied. Sham operated animals had all procedures including advancement of the endovascular filament, but not vessel rupture.

2.3.5 Drug treatment

Animals were randomly assigned to receive treatment with either N-acetyl-L-tryptophan (2.5 mg/kg) or equal volume normal saline (vehicle). NAT (A-6376, Sigma, Australia) was prepared 2 to 5 hours prior to use. It was dissolved in normal saline at a concentration of 2.5 mg/ml and the pH adjusted to 7.5. The dosage regime was guided by the following:

1. A dose-response study undertaken previously in this laboratory found 2.5 mg/kg to be the optimal NAT dose to preserve blood-brain barrier integrity following traumatic brain injury in male Sprague-Dawley rats (Donkin *et al* 2009).
2. The blood-brain barrier is open in the acute phase following SAH (Doczi *et al* 1986), as it is following brain trauma, enabling NAT to cross the blood-brain barrier.
3. NAT administered 30 minutes following reperfusion in an ischaemic-reperfusion model of stroke (also previously undertaken in this laboratory using male Sprague-Dawley rats) reduced brain oedema and improved outcome (Turner 2007).

NAT or normal saline was injected IV into the cannulated right femoral vein at 30 minutes following SAH. We considered this dose a reasonable baseline dose for SAH studies, with additional dose-response studies to be undertaken as guided by results.

2.3.6 Post-surgery recovery

Following application of Lignocaine, wounds were sealed with wound clips and Isoflurane was ceased. When animals resumed breathing spontaneously (usually around 5 minutes after Isoflurane was ceased), mechanical ventilation was ceased and the endotracheal tube

removed. Animals were placed alone, in the recovery position, (on their side) in a recovery box, which was positioned on a heating pad. Once ambulating, animals were returned to their home cage.

2.3.7 Assessment of functional outcome

Functional deficits were assessed daily following SAH or sham procedure in both models for up to 7 days following the ictus, as detailed below. For consistency, all tests were conducted in the same order, at the same time of day and by the same assessor using the same room and equipment. The assessor was blinded as to whether the rat had undergone SAH or sham procedure.

Neurological deficit score

The general condition of rats and their neurological function were assessed by a neurological deficit score (Neuroscore). This Score that was developed from previously published neurological assessment tools that have been used to measure outcome in rat models of SAH and ischaemia brain injury (Bederson *et al* 1986; Garcia *et al* 1995; Germano *et al* 2007; Thal *et al* 2008). We undertook pilot testing of naive, SAH and sham operated animals using these previously published assessment tools and modified the protocol to improve reliability and sensitivity to injury.

Components of previously published tests that relied on a visual response showed poor reliability in our pilot testing. Turning the head in response to sound (Thal *et al* 2009a) or a moving object (Thal *et al* 2009a) are reported to be normal responses of Sprague-Dawley rats in studies conducted internationally. Turning the head in response to touch has been

Table 2.1 The Neuroscore score used to assess functional outcome following SAH or sham procedure. Higher scores indicated more profound neurological deficits.

Weight loss past 24h	**0-20g** **20-40g** **40-60g**	**0** **1** **2**
Respirations	**Normal** **Rapid** **Laboured**	**0** **1** **2**
Startle response	**Present** **Absent**	**0** **1**
Coat	**Normal** **Fine spiking** **Ruffled**	**0** **1** **2**
Stress marks	**Absent** **Mild** **Marked**	**0** **1** **2**
Spontaneous activity (bench top)	**Explores environment, approaches objects in > 3 directions** **Movement slightly limited but reaches and explores objects** **Limited movement and no rearing** **Minimal or no spontaneous movement**	**0** **1** **2** **3**
Tail suspension	**All 4 limbs extended symmetrically** **Reduced extension 1 limb** **Minimal movement 2 or more limbs** **No limb movement**	**0** **1** **2** **3**
Forepaw walking	**Walks symmetrically on forepaws** **Reduced outstretching one forelimb** **Minimal forelimb movement one or both sides** **No forelimb movement**	**0** **1** **2** **3**
Vibrissae evoked forepaw response	**Brisk and equal left and right** **Reduced response one side** **No response one side** **Absent bilaterally**	**0** **1** **2** **3**
Olfactory	**Sniffs food** **No response**	**0** **1**
Gait	**Normal and symmetrical** **Persistent forelimb flexion/adduction** **Non-symmetrical resistance to lateral push** **Circles if pulled by tail** **Circles spontaneously** **No spontaneous walking**	**0** **1** **2** **3** **4** **5**
Ledged beam walking	**Reaches home box with 0 - 6 foot faults** **Reaches home box with 7-20 foot faults** **> 20 faults or unable to traverse beam**	**0** **1** **2**
	TOTAL	**/ 29**

reported in Wistar rats (Garcia *et al* 1995). Naive animals from our breeding colony did not consistently turn their head (eyes) towards any stimulus. However, Wistar rats from our animal care facility performed better in visual tests.

Olfactory and proprioceptive tests were very reliable. An example is the vibrissae-elicited forepaw placement test, which demonstrates a sensorimotor reflex involving the basal ganglia. The rat is held with one forepaw hanging freely. The vibrissae are brushed lightly against the corner of a table and the normal response is for the rat to rapidly place its ipsilateral forepaw on the table. Three trials were conducted each day following surgery and the reflex was graded as normal if brisk and present both sides, diminished if only elicited by repeated stimulation or absent.

Rotarod

The rotarod assesses motor function including strength, speed, balance and coordination. It has been used extensively in this laboratory (Harford-Wright *et al* 2010; O'Connor *et al* 2007; Thornton *et al* 2006) and elsewhere to assesses functional deficits associated with brain lesions, including experimental SAH (Thal *et al* 2008). The rotarod consists of a rotating assembly of 18 metal rods each 1 mm in diameter. Rats were placed on the device, which initially remained stationary for 10 seconds, and are then required to increase walking speed to remain on the frame. The device begins to rotate at a speed of 3 revolutions per minute and increases by 3 revolutions per minute every 10 seconds. The test was complete when rat walked at the maximum speed for 10 seconds (30 revolutions per minute) or if the animal was unable to continue (ie the device completes 2 full revolutions without the rat walking or the rat fell from the device). The time (0 to 120

seconds) was recorded from when the rat was first placed in the frame until the test was complete.

Adhesive paper removal

This test assesses both sensory and motor function as the rat removes adhesive tape that has been placed on each forepaw. Whilst particularly sensitive to sensory neglect or sensory loss, the test also relies on balance and coordination, as animals generally shift weight to the hind limbs and bring both forepaws to the mouth simultaneously. The rat was briefly restrained and a 1 cm x 1 cm section of adhesive vinyl tape was attached to each forepaw (right or left first in random order). The animal was then placed on a bench top and the time taken (in seconds) to remove each tape was recorded with a stopwatch. The test was ceased at 180 seconds if the rat had not yet removed the tape.

2.3.8 Assessment of brain oedema

Brain water content was assessed by the wet weight-dry weight method at 5, 24 and 48 hours after injection SAH or sham procedure, and at 24 and 48 hours after filament SAH or sham procedure. At the appropriate time points, rats (n=5 per group) were decapitated under Isoflurane anaesthesia and brains were rapidly removed. The left and right cerebral hemispheres, cerebellum and brain stem were carefully separated and placed in glass vials. After weighing, brains were dried at 100°C for 24 hours. The dry weight was then obtained and brain water content calculated according to the formula

$$\%water = \frac{wetweight - dryweight}{wetweight} \times 100$$

For comparison to studies elsewhere in which the brain was not divided into sections, we used the wet and dry weights of all 4 sections of each brain to also calculate whole brain water content of each brain in this study.

2.3.9 Assessment of brain histology

Perfusion fixation and brain sectioning

At 5, 24 and 48 hours after SAH or sham surgery, rats (n=5 per group) were anaesthetised with Lethobarb and perfused transcardially with 10% formalin. Ten minutes prior to perfusion rats received 0.5 ml Heparin (5000 IU/ml) intraperitoneally. When a surgical level of anaesthesia with Lethobarb was established, the thorax was opened and retracted to expose the heart. The apex of the left ventricle was stabilised with a towel clip, and a blunted 19G, 37 mm needle was inserted through the left ventricle and advanced to the ascending aorta. An incision was made in the right atrium to enable blood and perfusate to escape. The rat was then perfused with 10% neutral buffered formalin driven at approximately 130 mmHg until the fluid draining from the right atrium was clear. Brains were left in-situ for 1 hour following perfusion to minimise artefactual damage. Animals were then decapitated and brains removed and stored in 10% neutral formalin buffered formalin for at least 24 hours before further processing. Brains were photographed to record the extent and distribution of subarachnoid blood.

Brains were placed in a rodent brain blocker (Kopf, PA002) and cut into 2mm coronal slices. These were placed in cassettes in normal buffered formalin and processed over night for 20 minutes each in ethanol baths of increasing concentration (50%, 70%, 80%, 95%, 100%, 100%), followed by 2 xylene baths (each for 90 minutes), and finally paraffin

baths of increasing duration (30, 60, 60 and 90 minutes). The following morning the processed brain slices were embedded in paraffin wax and 5μm sections were cut using a microtome (Microm, HM330). Sections were mounted on glass slides (Menzel-Glaser, Superfrost Plus), dried in hot air and stored in slide boxes in a humidified oven at 37°C until staining.

Haematoxylin and eosin staining

Haematoxylin and eosin staining was undertaken to enable visualisation of cell morphology within brain tissue following SAH or sham procedures in both models. Slides were placed in metal racks and de-waxed by heating and then passing through 2 changes of xylene (1 minute each) and 2 changes of ethanol (1 minute each). Slides were then placed in haemotoxylin for 5 minutes, washed in running water and placed in acid alcohol (10 seconds), washed in water, placed in lithium carbonate (10 seconds) and washed in water again. They were then paced in eosin (1 minute), and then into 2 changes of alcohol (1 minute each) and 2 changes of histolene (1 minute each). Slides were then mounted in DePex (BDH, UN1993) and assessed with light microscopy (Olympus, BX45) ahead of high resolution scanning (detailed later).

Albumin immunohistochemistry

Albumin staining was undertaken to mark the presence of albumin within brain parenchyma following SAH or sham procedure. Albumin is a large molecular weight plasma protein that is not normally present within brain tissue. Breakdown of the blood-brain barrier may allow albumin to cross the walls of microvessels within the brain and enter brain parenchyma. The perfusion process undertaken prior to staining removes blood from brain vessels, while any blood in the subarachnoid space does not contribute to the

parenchymal albumin staining. Therefore, parenchymal albumin staining represented albumin that had crossed the blood-brain barrier prior to perfuse fixation, indicating that a loss of blood-brain barrier integrity has occurred.

Sections were de-waxed by heating and being passed though 2 changes each of xylene and ethanol (3 minutes each). They were then placed in 0.5% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase activity. Sections were then washed twice in phosphate buffered saline (PBS), for 3 minutes each time, incubated in 3% normal horse serum in PBS for 30 minutes and then incubated overnight in the primary antibody (polyclonal goat anti-albumin 1:20 000, Cappel Cappel Laboratories, Malvern, PA). On the following day, slides were washed twice in PBS (3 minutes each) and the secondary biotinylated anti-goat antibody was applied (vector, Burlington, CA) for 30 minutes. After 2 further PBS washes (each for 3 minutes), slides were incubated in streptavidin peroxidase complex (1:1000, Pierce, Rockford, IL) for 60 minutes and washed in PBS again (3 minutes x 2). Antigen was visualised by precipitation of 3, 3' diaminobenzidone tetrahydrochloride (DAB; Sigma, Australia) in the presence of hydrogen peroxide.

After washing in running water, sections were lightly counterstained with haematoxylin to visualise cell morphology. Slides were placed in haematoxylin for 1 minute, washed in water, placed in acid alcohol for 10 seconds, washed in water and placed in lithium carbonate for 10 seconds and washed in water again. Slides were then passed twice through ethanol (1 minute each), twice through histolene (1 minute each) and mounted in DePex. Slides were viewed with light microscopy and scanned as detailed below.

Substance P immunohistochemistry

Sections were stained for substance P to assess if SP immunoreactivity and its association with type of SAH model used, the amount of oedema produced and/or the severity of functional deficits. Sections were de-waxed by heating and being passed through 2 changes of xylene and ethanol for 3 minutes each as described above. They were then placed in 0.5% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase activity and washed twice (for 3 minutes each time) in PBS. Antigen retrieval was achieved by heating in EDTA at close to boiling point for 10 minutes in a microwave oven.

Once slides were cooled to below 40°C, they were washed in PBS (twice for 3 minutes) and incubated in 3% normal horse serum in PBS for 30 minutes. Sections were then incubated overnight in the primary antibody (polyclonal goat anti-SP 1:2 000 N-18, Santa Cruz Biotechnology inc., Santa Cruz, CA). Immunolabelling was then completed as described above, using biotinylated anti-goat secondary antibody (vector, Burlington, CA) and strepavin peroxidase complex (Pierce, Rockford, IL). As with albumin immunolabelling, SP antigen was visualised with 3, 3’ diaminobenzidone tetrahydrochloride (DAB) (Sigma, Australia) and sections were counterstained with haematoxylin.

Fluoro Jade C

Sections we de-waxed by heating and passed though 2 changes each of xylene and ethanol as described above. Slides were place in 70% alcohol for 2 minutes and then washed twice for 2 minutes each time in distilled water. Potassium permanganate (240 mg in 400 L distilled water, or 0.6%) was then applied for 10 minutes following which slides were

washed in water and placed in water for 2 minutes. Flouro-Jade C staining solution was prepared by adding 1 mL of flouro-Jade C stock (1 mg in 10 mL distilled water) to 99 mL of 0.01% glacial acetic acid solution (396 µL in 396 mL distilled water). The following steps were then carried out in the dark. Sections were incubated in flouro-Jade C for 20 minutes, then washed 3 times (1 minutes each) in distilled water. Slides were then dried at 45°C for 30 minutes, then placed in histolene and mounted in DePex. Sections were scanned and viewed as above with NDP.view as described above.

Digital imaging and semiquantification

To quantify the amount of a protein (such as albumin or substance P) within a sample (e.g. brain tissue), enzyme-linked immunosorbent assay (ELISA) is considered the gold standard. Fresh tissue samples, and therefore additional animals, are required. Power calculations and the results of previous work in this laboratory indicated that $n \geq 10$ animals per group would need to be sacrificed at each time point to detect a difference between the treatment groups at the 0.05 significance level.

Immunohistology enables staining of a particular protein but does not provide quantification as ELISA does. The advantage of histology is that it provides an image of SP or albumin staining throughout various cross sections of the brain. The intensity of protein staining can be compared in highly localised regions of the brain. Counter staining enables visualisation of the protein of interest within the context of various cell populations. ELISA analyses protein within a sample of homogenised brain tissue, and so does not provide the same degree of localisation.

Very high-resolution digital images of immuno-stained sections can be mathematically processed to assess the intensity of protein staining in various brain regions. Whilst not providing the precise quantification offered by ELISA, this comparatively inexpensive process can be used to gauge relative levels of albumin or SP in highly localised regions of the brain. It may also reduce bias in the assessment of staining intensity in the brains of animals from various sub-groups and guide decision-making about further experiments. We termed this process 'semi-quantitation' to distinguish from true quantification as performed with ELISA.

Digital images of all sections were obtained via a Nanozoomer Digital Pathology scanner at a magnification of 40 x (NDP Scan U10074-01, Hamamatsu Photonics K.K, Japan). Slides were viewed with the associated software (NDP.view v1.2, Hamamatsu) and images of regions of interest were exported as jpeg2000 files (a lossless image format). These images were digitally processed using Ruifrok and Johnston's colour deconvolution method (Ruifrok and Johnston 2001) (Figure 2.5).

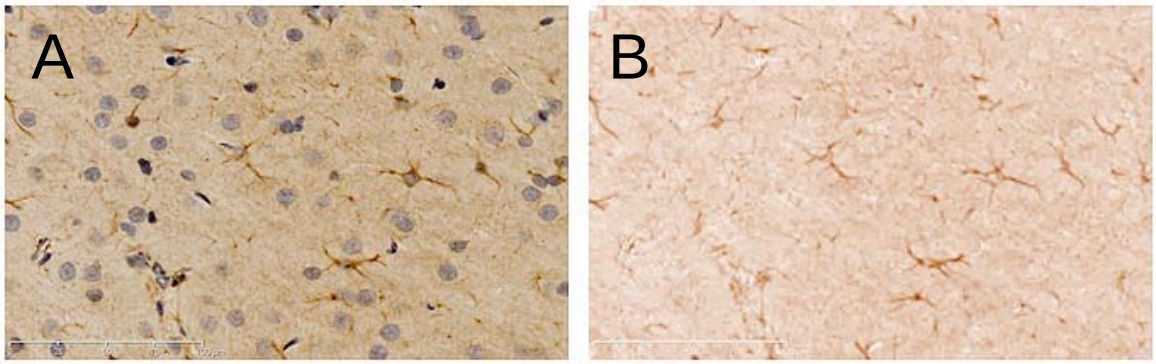


Figure 2.5 A: SP staining within the motor cortex (including light counterstaining to show cell morphology) at 5 hours following injection SAH. B: The same region with counterstaining removed by colour deconvolution processing.

This processing removed counter staining from images so that antibody staining can be viewed in isolation (B). This processing was done within ImageJ (NIH, USA) using software code obtained online (Landini 2008) that was imported to run as a macro. Background staining was subtracted using the "rolling ball" method of Castle and Keller (Castle and Keller 2007). The deconvolved DAB channel (representing the antigen content of the whole image) was semi-quantitated by performing a histogram analysis and a percentage calculated as the summation of pixel frequency as a product of pixel intensity. This has the effect of weighting the histogram to make more frequent pixels 'darker' and so improve the signal to noise ratio. Hence antibody content of an image is expressed as DAB weight %. A more detailed explanation of this method has been submitted for publication (Helps *et al* 2010). Previous experiments in this laboratory have shown the

reliability of this method, demonstrating a linear correlation ($r^2 = 0.89$, $p < 0.0001$) between increasing antibody concentration and DAB weight % (Kleinig 2010).

2.3.10 Statistical analysis

Mortality was analysed by Fisher's exact test. Parametric data was analysed by 2-tailed unpaired students t-test or analysis of variance (ANOVA; one-way, two-way and/or repeated measures, as appropriate) followed by Bonferroni post-tests for multiple comparisons. Non-parametric data was analysed using Kruskal-Wallis or Friedman's ANOVA followed by Dunn's multiple comparisons test. Correlation was assessed by Pearson's Correlation (for parametric data) or Spearman's Correlation test. All data was analysed using PRISM (Graphpad Software, San Diego) and are expressed as mean ± standard error of the mean (SEM). A *p* value less than 0.05 was considered significant.

Chapter 3

Substance P expression, brain oedema and functional outcome following injection and filament subarachnoid haemorrhage

3 Substance P expression, brain oedema and functional outcome following injection and filament subarachnoid haemorrhage

3.1 Introduction

Blood brain barrier breakdown and vasogenic brain oedema following SAH have been demonstrated both in the clinical setting and in experimental models (Doczi 1985; Doczi *et al* 1986). Moreover, the extent of this brain oedema has been linked to the severity of functional deficits following experimental SAH (Thal *et al* 2009a). The mechanisms involved in blood-brain barrier breakdown following SAH are not fully understood and the possible role of SP in the genesis of brain oedema and the development of functional deficits has not yet been investigated.

SP mediates oedema formation in models of acute brain injury including occlusive stroke (Turner *et al* 2006), intracerebral haemorrhage (Kleinig *et al* 2010) and traumatic brain injury (Donkin *et al* 2009), and its actions appear linked to the development of functional neurological deficits in these conditions. Elevated SP following experimental intracerebral haemorrhage precedes the development of neuronal death and infiltration/activation of inflammatory cells in brain parenchyma (Kleinig 2010). Conversely, depletion of SP from perivascular sensory nerves prior to reversible-occlusive stroke is also associated with reduced oedema formation and improved functional outcome (Turner 2007).

Rapid and widespread release of SP from perivascular sensory neurons following experimental SAH is well documented (Edvinsson *et al* 1990; Hara *et al* 1986; Linnik *et al* 1989; Tran Dinh *et al* 1994; Uemura *et al* 1987) but the role of SP in SAH pathophysiology is unclear. SP immunoreactivity within brain parenchyma following SAH is yet to be characterised, despite neuronal levels of SP taking 2 months to be restored after canine SAH (Uemura *et al* 1987). Depletion of SP from perivascular sensory nerves prior to experimental SAH prevents the development of vasospasm (Delgado-Zygmunt *et al* 1990) but it is unclear if SP contributes to brain oedema or functional deficits following SAH.

This study used both an injection and a vessel perforation model of SAH to investigate the potential role of substance P in secondary injury, SP expression, oedema formation and functional neurological outcome following SAH.

3.2 Methods

3.2.1 Experimental groups

Adult male Sprague-Dawley rats (n = 110; 350-450g) were randomly allocated to receive injection SAH, filament SAH or the corresponding sham procedure as described in Chapter 2. Animals that died were replaced and are included in mortality data only. Functional outcome was assessed in all surviving animals. A summary of subgroups assessed for brain oedema and histology is provided below in Figure 3.1 and Figure 3.2.

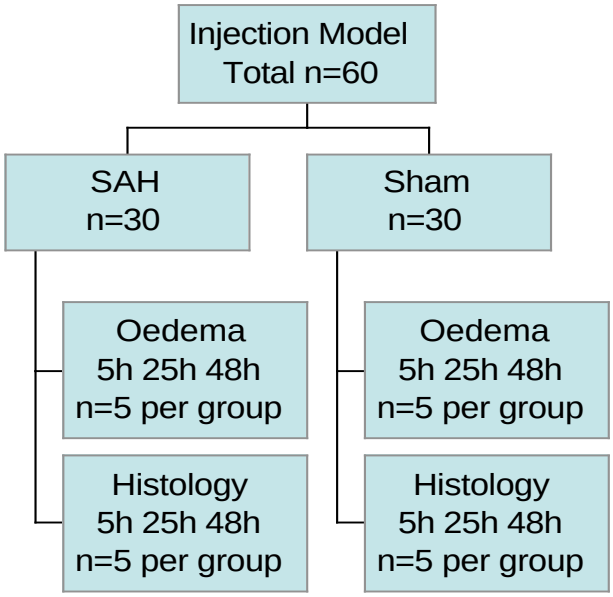


Figure 3.1 Summary of injection SAH animals in this study. Animals were sacrificed following functional assessment for additional outcome measures at 5, 24 and 48 hours.

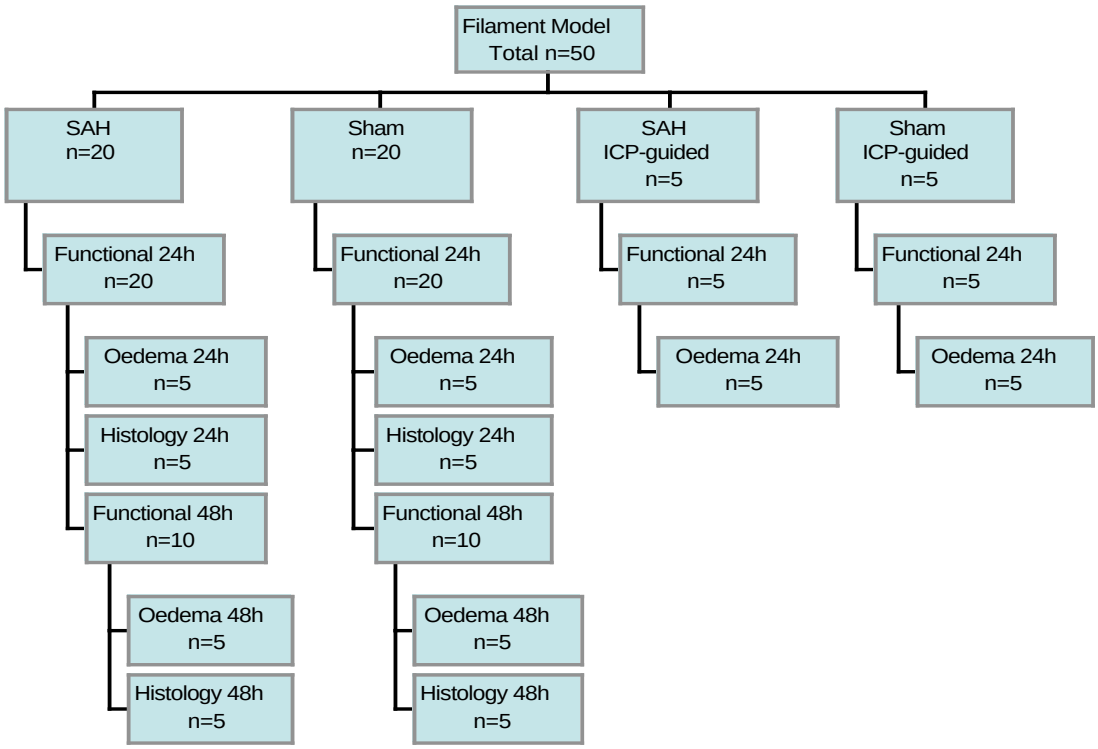


Figure 3.2 Summary of filament SAH animals in this study. Animals were sacrificed following functional assessment for oedema and histology studies at 24 and 48 hours.

For histology studies, animals were sacrificed at 5 h, 24 h and 48 h after injection SAH or sham procedure (n = 5 per group at each time point) and at 24 and 48 hours after filament SAH or sham procedure (n = 5 per group at each time point). For brain oedema studies, animals were sacrificed at 5 h, 24 h and 48 h after injection SAH or sham procedure (n = 5 per group at each time point) and at 24 and 48 hours after filament SAH or sham procedure (n = 5 per group at each time point).

An additional ICP-guided filament SAH group (n = 5 SAH and n = 5 sham) was included because pilot studies indicated that the variation in haemorrhage volume inherent in filament SAH may reduce the power of tests to detect differences between groups. ICP was monitored during SAH in this group to confirm that haemorrhage was large enough to produce an ICP rise above 30 mmHg for at least 30 seconds at the ictus. If this threshold was not achieved initially, the filament was advanced to puncture the artery again. This group was sacrificed at 24 hours for brain oedema studies so that results could be compared with previous reports of oedema at 24 hours following filament SAH (Ostrowski *et al* 2005; Park *et al* 2004; Thal *et al* 2009a; Thal *et al* 2009b; Yan *et al* 2008; Yatsushige *et al* 2007).

3.2.2 Intracranial pressure monitoring

With the animal prone, a 2 mm burr hole was drilled 1 mm posterior to bregma and 2 mm left of the midline. After calibration, a Codman Microsensor probe (Codman and Shurtleff Inc, USA) was inserted so that the tip was positioned just below the dura into the parenchyma of the left parietal cortex. To minimize movement of the probe when turning the animal supine for surgery and prone again prior for removal of the probe at the completion of surgery, the probe was stabilised as follows. A hollow cone was fashioned from the plastic base cut from of a 23G hypodermic needle. The lead of the probe was threaded through the cone and taped at the apex so that when the base of the cone was flat against the skull, the tip of the Codman probe was correctly positioned in the parenchyma. After calibration, the probe was inserted and the base of the cone was fixed to the skull with glue.

3.2.3 Functional outcome

Functional outcome was assessed by Neuroscore, rotarod and sticky label removal tests at 24 and 48 hours after SAH or sham procedure, according to the procedures described in Chapter 2. Neuroscore indicates the degree of functional deficits (more severe deficits result in higher Neuroscore). Rotarod score demonstrates motor deficits and is reduced by functional deficits (walking time on rotarod is reduced), while sticky label removal latency demonstrates sensorimotor deficits and is increased by functional deficits (the time between removing the first and second label is increased, generally due to the asymmetrical nature of sensorimotor deficits).

3.2.4 Brain oedema

Brain water content was assessed by the wet weight-dry weight method as described in Chapter 2. The water content of each of 4 regions (left and right hemisphere, cerebellum and brain stem) was assessed in each brain. To enable results to be compared to those of studies in which whole brain water content was assessed (ie brains were not divided prior to drying), raw data was used to calculate whole brain water content for each brain in the present study.

3.2.5 Histology and immunohistochemistry

At the time points noted above, animals were sacrificed by perfusion and brains processed for histology as described in Chapter 2. After staining for H & E, albumin or substance P immunoreactivity, sections above the injection site (~ 0.5 mm anterior to bregma) and sections enabling visualisation of the hippocampus (~ 4.0 mm posterior to bregma) were scanned and high-resolution digital images were viewed using NDP.view software.

3.2.6 Semi-quantification of albumin and SP immunostaining

Following immunostaining of histological sections, areas of albumin extravasation or substance P immunoreactivity were identified by the characteristic brown staining of the reaction product when viewed with light microscopy or after digitally scanning. Regions of interest in the motor cortex and in basal regions adjacent to the haemorrhage, as well as whole brain coronal sections, were isolated from digital images and intensity of staining in these regions was semi-quantified using the colour deconvolution and analysis methods described in Chapter 2.

3.2.7 Statistical analysis

Mortality data was analysed using Fisher's exact test. Non-parametric data (Neuroscore) was analysed using Kruskal-Wallis one-way ANOVA with selected Dunn's post-tests or by using the Mann Whitney U test as indicated. Parametric data (other functional tests, oedema and histology data) were analysed using two-way ANOVA and Bonferroni post-tests for inter-group (SAH vs sham) comparisons at each time point. Repeated measures ANOVA was used where possible. Correlation was assessed using the Spearman rank correlation test where non-parametric data was included and Pearson r for parametric data. For clarity, the statistical test applied in each instance and the number per group is detailed with each chart in the results section.

3.3 Results

3.3.1 Mortality

As expected, all sham animals of both models survived surgery and exhibited no mortality. One animal that underwent injection SAH failed to wake from surgery and died (3%).

However, following filament SAH, mortality was significantly higher than injection SAH (26% at 5 hours, $p < 0.01$; 33% at 24 hours, $p < 0.01$).

3.3.2 Functional outcome

Injection SAH animals and shams of both groups displayed no functional deficits. In contrast, deficits were common following filament SAH and were demonstrated by increased neuroscore, reduced rotarod time and increased sticky paper removal latency. Pooled data (including filament SAH with and without ICP-guidance) clearly demonstrates the increased neuroscore at 24 hours ($p < 0.001$) (Figure 3.3A). The sub-group assessed at both 24 and 48 hours after SAH demonstrated minimal change in the severity of deficits over this period, with neuroscore consistently above sham levels ($p < 0.01$) (Figure 3.3B).

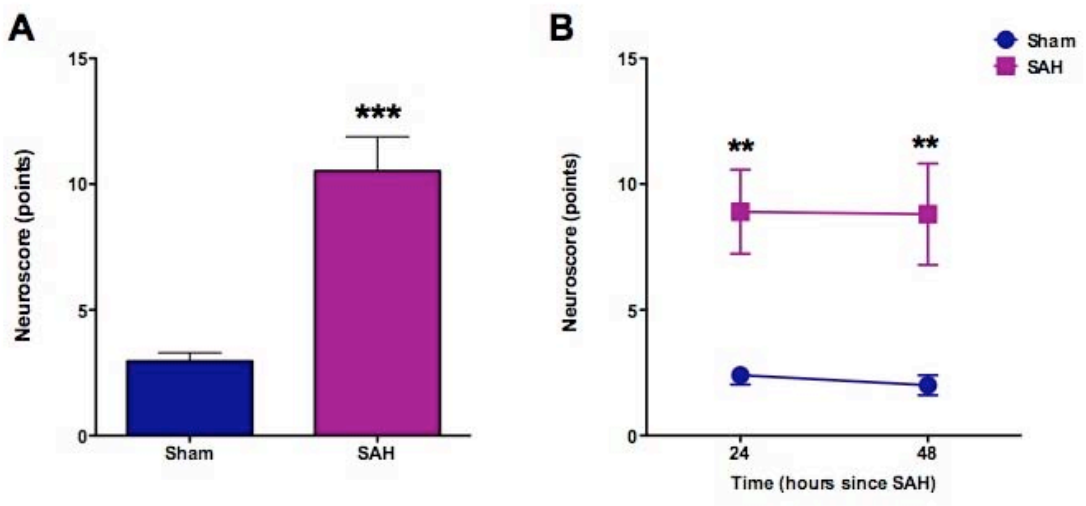


Figure 3.3 Functional deficits demonstrate by Neuroscore following SAH. A: Filament SAH animals had increased Neuroscore at 24 hours compared to shams (n = 25 per group, *** $p < 0.001$). B: Neuroscore changed minimally from 24 to 48 hours after SAH (n = 10 per group, repeated measures one-way ANOVA followed by Dunn's Multiple Comparison Test, ** $p < 0.01$ versus shams).

The impact of ICP-guidance on neuroscore after filament SAH is shown above, by separation of the pooled neuroscore data at 24 hours into ICP-guided and non-ICP guided groups (Figure 3.4). Neuroscore was slightly increased in the ICP-guided group (B).

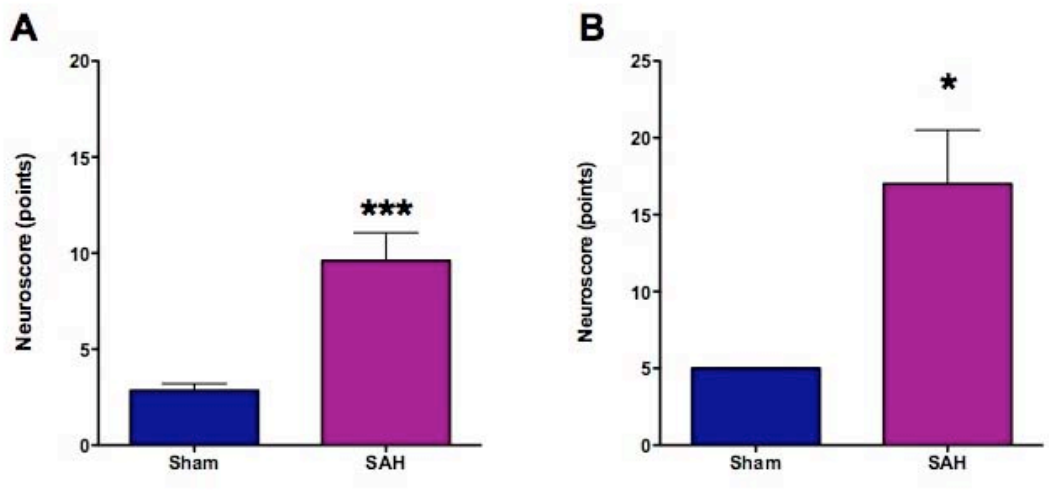


Figure 3.4 The impact of ICP-guidance on neuroscore after filament SAH. A: Neuroscore was increased at 24 hours after SAH in the group without ICP guidance (n = 20 per group, *** p < 0.001). B: Neuroscore was slightly higher in the ICP-guided SAH group, and was increased compared to shams (n = 5 per group, * p < 0.05).

Rotarod also demonstrated functional deficits following filament SAH (Figure 3.5) with SAH animals showing reduced endurance compared to shams at 24 hours (A). Sham animals demonstrated the expected improvement (training effect) when re-tested at 48 hours, but SAH animals demonstrated no improvement (B).

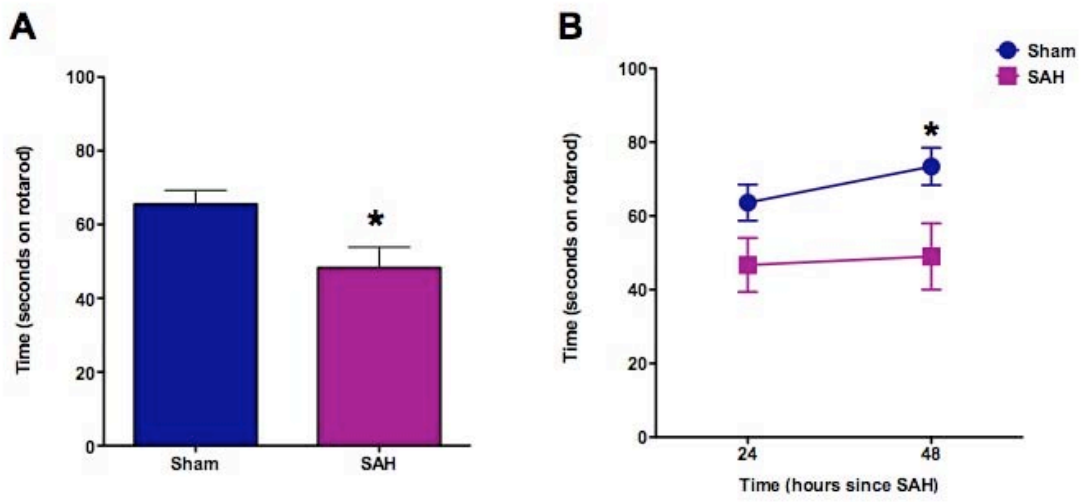


Figure 3.5 Rotarod endurance score following filament SAH. A: SAH animals had poorer rotarod performance at 24 hours after SAH (pooled data, n = 25 per group, * p < 0.05 versus shams). B: SAH animals had reduced rotarod performance at 48 hours compared to shams, and their performance did not improve between 24 and 48 hours (n = 10 per group, 2 way repeated measures ANOVA followed by Bonferroni posttests, * p < 0.05).

The sticky label removal test also demonstrated functional deficits at 24 hours after filament SAH (Figure 3.6A). However several shams demonstrated increased latency when reassessed at 48 hours, so that this test was not sensitive to detecting differences between SAH and sham animals at 48 hours (Figure 3.6B).

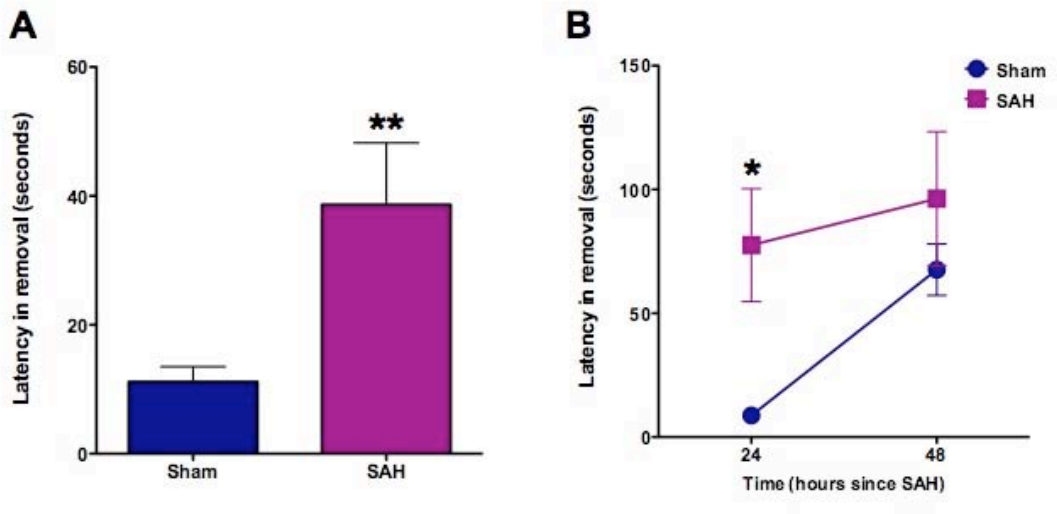


Figure 3.6 Sticky label removal latency following filament SAH. A: Latency (the time between paper removal from left and right forepaws) was increased following SAH (n = 25 per group, ** p < 0.01 versus shams). B: Latency at 24 and 48 hours after filament SAH (n = 10 per group, * p < 0.05 versus shams).

3.3.3 Brain oedema

Injection SAH caused a small increase in brain water content in all brain regions at all time points (Figure 3.7). This increase compared to shams was approximately 0.46% at 5 hours, 0.31% at 24 hours and 0.31% at 48 hours (ANOVA right hemisphere $p < 0.05$, whole

brain $p < 0.01$). However, post-hoc tests indicated that the increase was not significant at any individual time point for this small number of animals in each group.

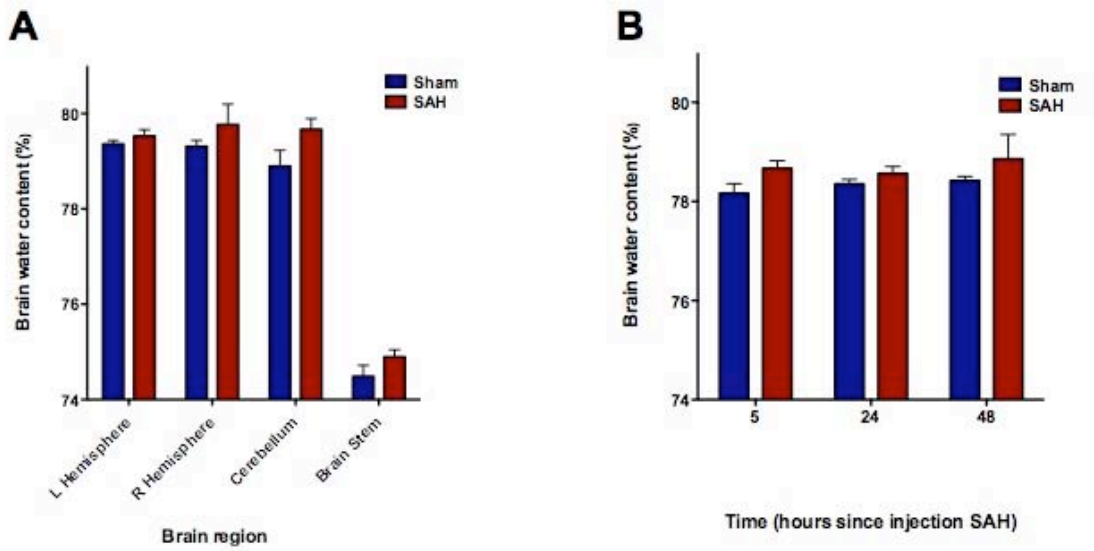


Figure 3.7 Brain water content following injection SAH. A: At 48 hours, note regional differences (highest in right cerebral hemisphere, lowest in brain stem). B: Right cerebral hemisphere water content at 5, 24 and 48 hours after injection SAH.

As noted below in Figure 3.8, brain water content was assessed in 3 sub-groups of filament SAH animals (non-ICP guided filament SAH at 24 and 48 hours and ICP-guided at 24 hours). A consistent trend towards an increase in brain water content was seen at 24 and 48 hours after filament SAH without ICP guidance. This trend was demonstrated in all brain regions and in the brain as a whole.

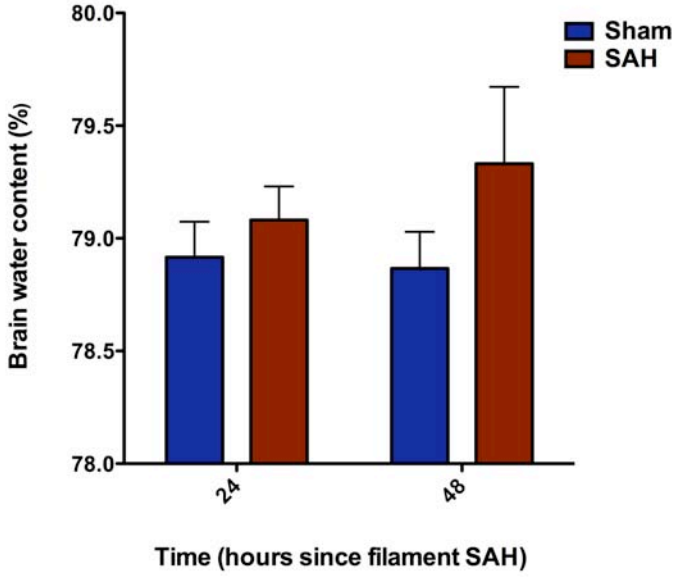


Figure 3.8 Whole brain water content at 24 and 48 hours following non-ICP guided filament SAH. A non-significant increase is seen at both time points (n = 5 per group).

ICP-guided SAH produced significant brain oedema at 24 hours, with whole brain water content increased by 0.87% (SAH 79.68 ± 0.17 vs sham 78.81 ± 0.13; Figure 3.9A). Water content increased in all regions but oedema was most prominent in the right cerebral hemisphere and the brain stem (Figure 3.9B).

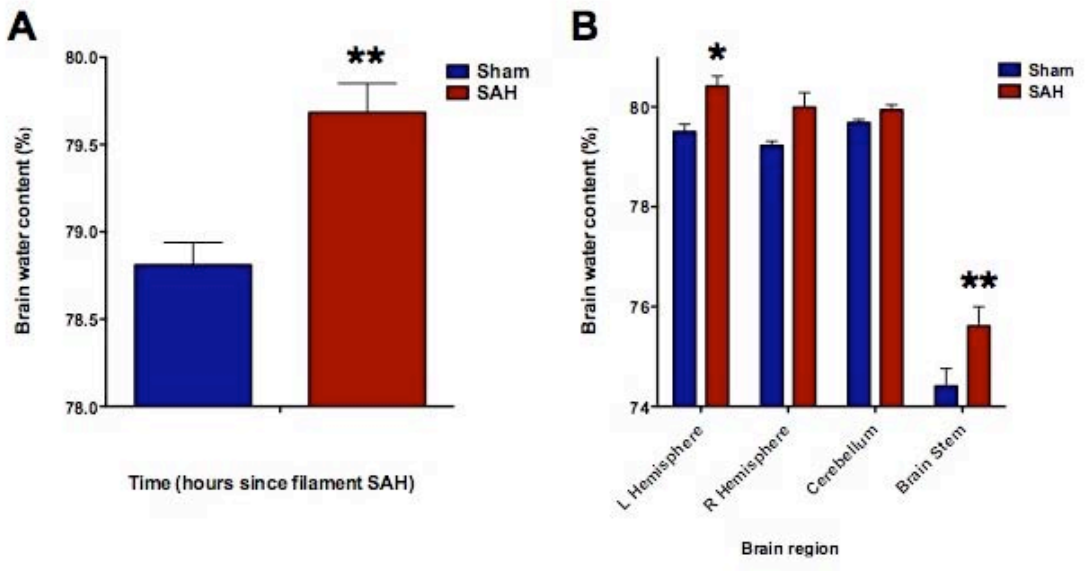


Figure 3.9 Brain oedema following ICP-guided filament SAH. A: Whole brain water content was significantly increased at 24 hours compared to shams (n = 5 per group, ** p < 0.01). B: Regional increases in brain water content at this time point (* p < 0.05, ** p < 0.01 versus shams).

The severity of functional deficits measured by neuroscore correlated with brain water content at 24 hours after filament SAH (Figure 3.10), even though the absolute increase in brain water content was modest. As there were only 5 animals per group at each time point this graph shows pooled data (ICP-guided and non ICP-guided filament SAH).

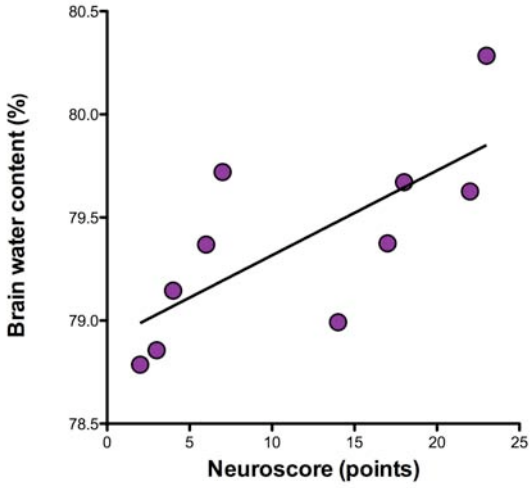


Figure 3.10 Correlation between neuroscore and brain water content at 24 hours after filament SAH (Spearman r = 0.81, p < 0.01, α = 0.05, n = 10).

3.3.4 Histology

Haematoxylin and Eosin

Haematoxylin and eosin staining confirmed that in both SAH models blood was distributed widely throughout the subarachnoid space (Figure 3.11). The section shown above (B) was taken from an animal with a neuroscore of 14 at 24 hours after SAH.

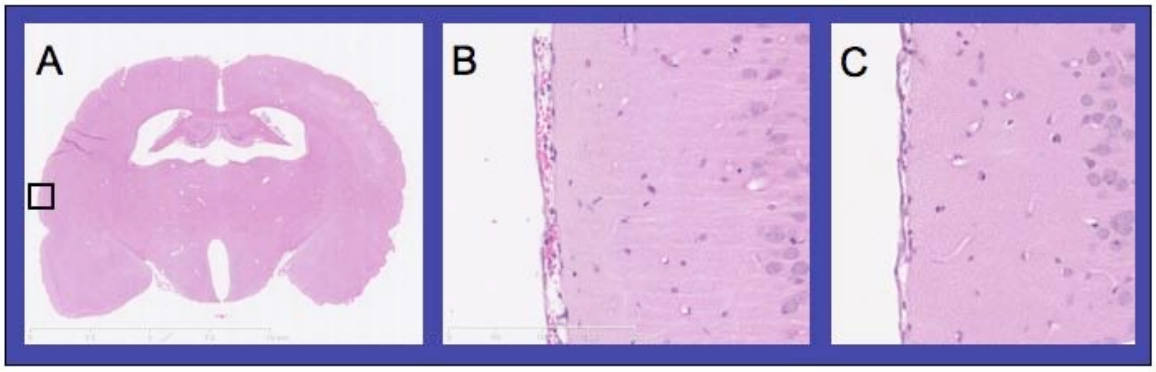


Figure 3.11 Haematoxylin and eosin staining at 24 hours after filament SAH. A: Coronal section ~ 2 mm posterior to bregma indicating region enlarged in B. B: Blood is clearly visible in the subarachnoid space. C: At 24 hours following sham procedure the subarachnoid space is clear of red corpuscles.

The hippocampal region appeared normal in injection SAH animals and in sham operated animals of both models, with neurons appearing plump and closely packed (Figure 3.12A). However filament SAH animals often demonstrated extensive damage throughout this brain region (Figure 3.12B). The section shown above (B) was taken from an animal with a neuroscore of 18 at 24 hours after SAH.

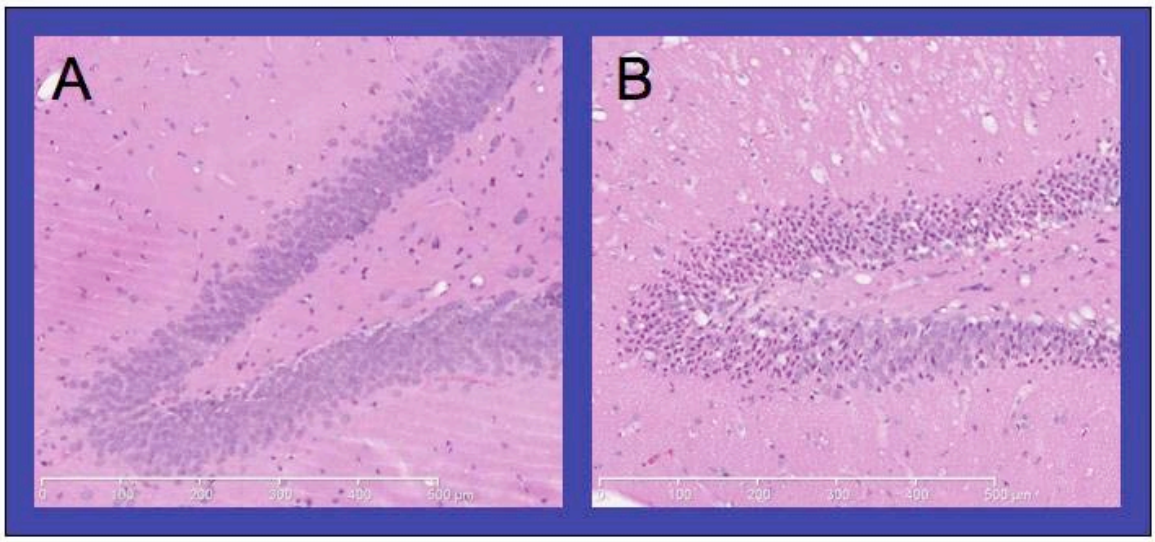


Figure 3.12 Haematoxylin and eosin staining in the dentate gyrus region of the hippocampus at 24 hours. A: Following sham procedure. B: Following filament SAH, showing darkly stained, shrunken neurons, and perineuronal vacuolation, consistent with ischaemic damage.

The motor cortex also revealed histological changes consistent with ischaemic damage following filament SAH (Figure 3.13). In contrast to the round and healthy appearance of cells in the motor cortex of sham operated animals (A), filament SAH animals demonstrated dark cell change, vacuolation of the parenchyma and tissue pallor (B).

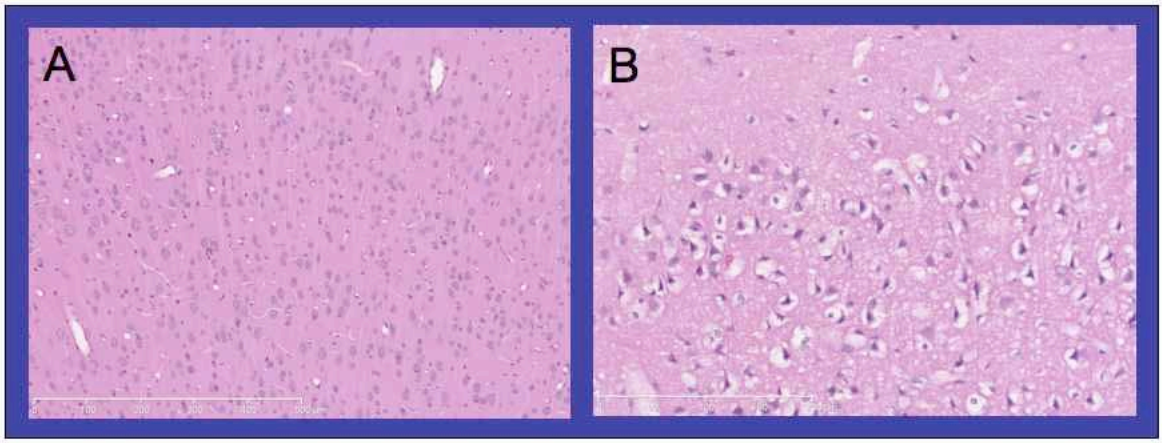


Figure 3.13 Haematoxylin and eosin staining in the motor cortex at 24 hours. A: Following sham procedure. B: Following filament SAH, showing dark cell change and vacuolation.

Flurojade

Fluoro-jade staining confirming the presence of multiple degenerating neurons in the hippocampus and motor cortex following filament SAH (Figure 3.14).

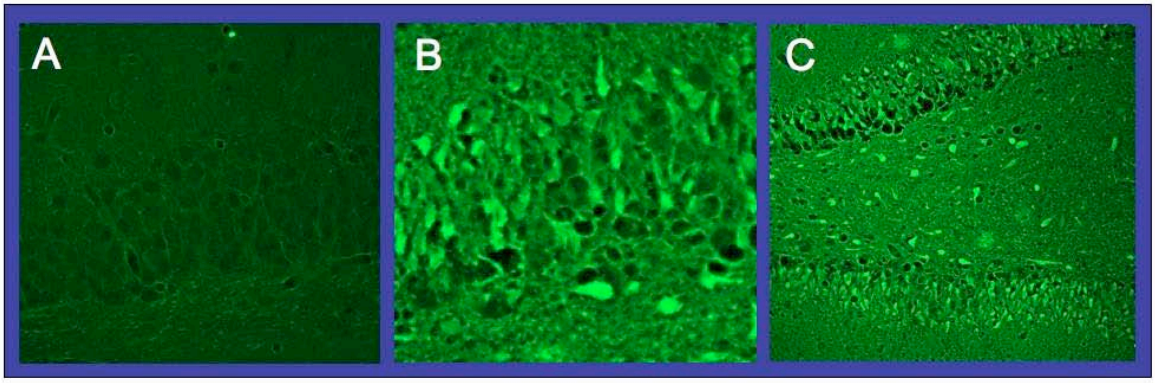
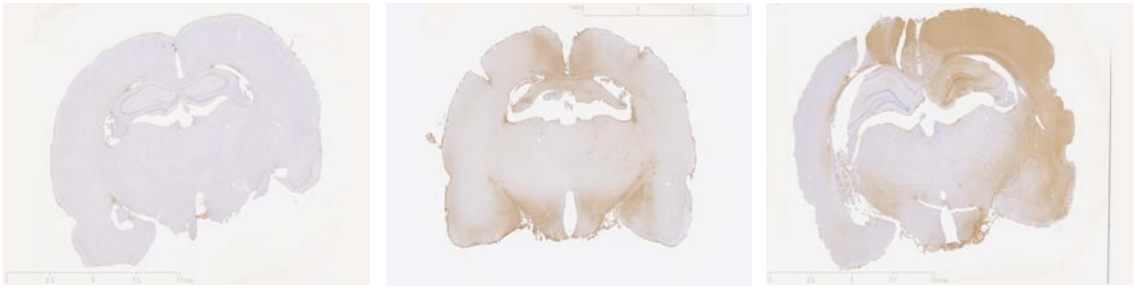


Figure 3.14 Fluoro-jade staining in the hippocampus. A: At 24 hours following sham procedure no fluoro-jade positive cells were visible. B: At 24 hours following filament SAH. C: Lower magnification demonstrating numerous fluoro-jade positive cells.

Albumin

Albumin influx into brain parenchyma followed both injection and filament SAH (Figure 3.15). As these scanned images show, sham procedure did not result in albumin staining within brain parenchyma and only a small amount of albumin is visible within the subarachnoid space and the ventricles only. In contrast, both SAH models led to blood-brain barrier breakdown enabling albumin to enter brain tissue. Albumin staining following injection SAH is relatively symmetrical and following filament SAH, albumin staining is more prominent in the middle cerebral artery territory. Staining is also more intense and more extensive after filament SAH, consistent with more severe blood-brain barrier disruption in this model.



Sham **Injection SAH** **Filament SAH**

Figure 3.15 Coronal rat brain sections immuno-stained for albumin (brown) following sham procedure (left), injection SAH (centre) and filament SAH (right).

When the intensity of albumin staining following injection SAH was analysed by colour deconvolution, a consistent trend towards an increase compared to shams was seen at all time points and was significant at 48 hours (Figure 3.16).

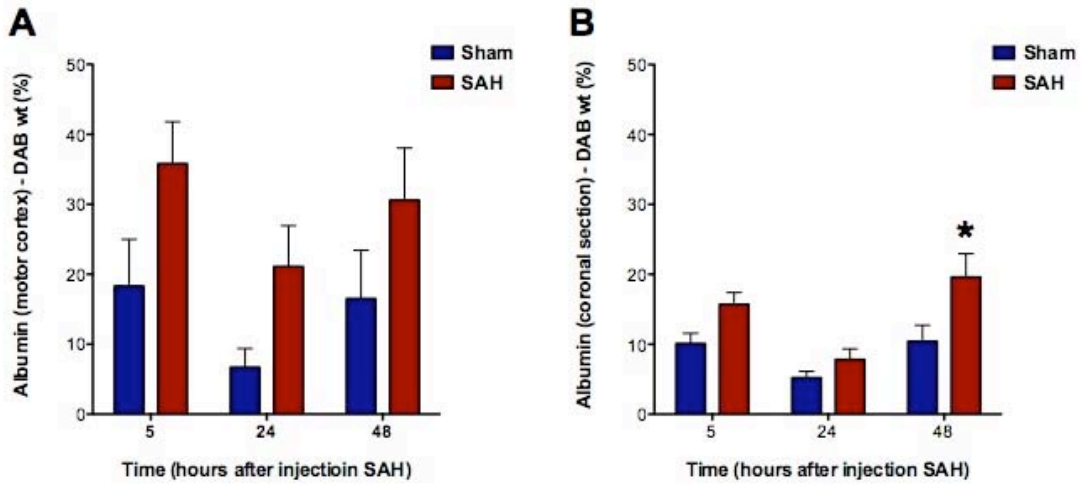


Figure 3.16 The intensity of albumin immunostaining following injection SAH. A: Motor cortex staining. B: Staining throughout whole coronal sections (* $p < 0.05$ versus shams).

Albumin staining was more intense and widespread following filament SAH compared to injection SAH. After filament SAH, motor cortex albumin staining was 7 times higher than sham levels at all time points (Figure 3.17A). Analysis of entire coronal sections also revealed significantly increased staining at 5 hours (B).

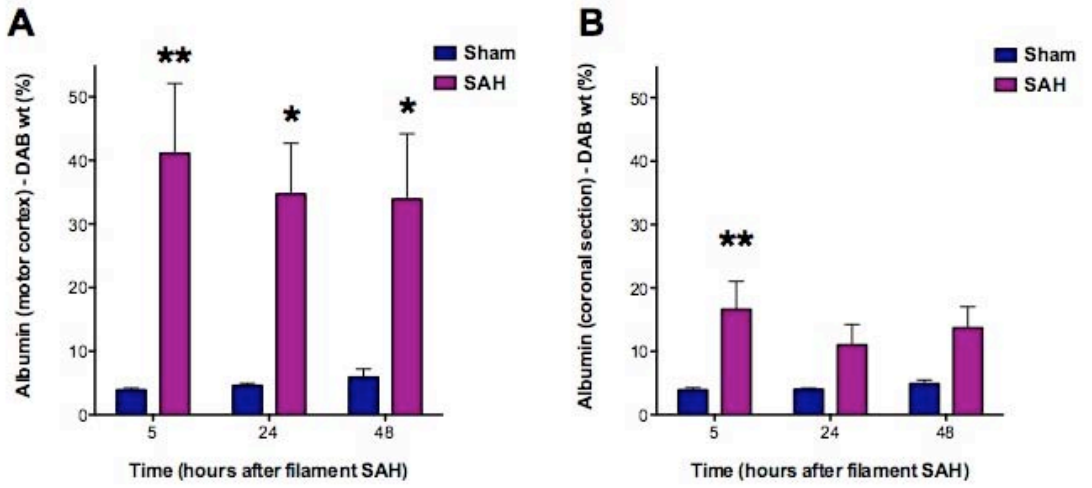


Figure 3.17 Albumin immunostaining following filament SAH. A: Motor cortex B: Whole coronal sections (n = 5 per group at each time point, * p < 0.05, ** p < 0.01).

Animals with more severe functional deficits (higher Neuroscore) had more intense albumin immunostaining. There was a strong correlation between Neuroscore and motor cortex albumin reactivity at 24 hours after filament SAH (Figure 3.18).

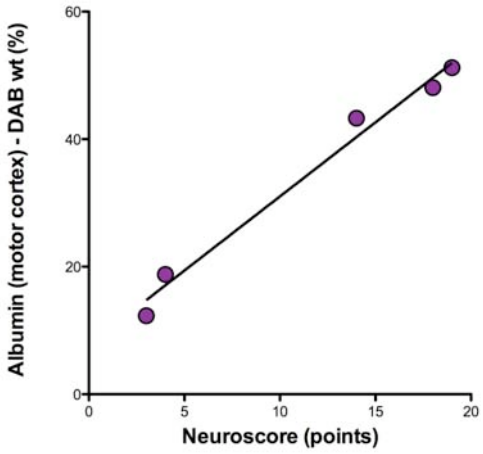


Figure 3.18 Neuroscore correlated with motor cortex albumin immunoreactivity at 24 hours after filament SAH (Spearman r = 1.0, p < 0.05, α = 0.05).

Substance P

A mild, diffuse increase in SP immunoreactivity was apparent following injection and filament SAH (Figure 3.19).

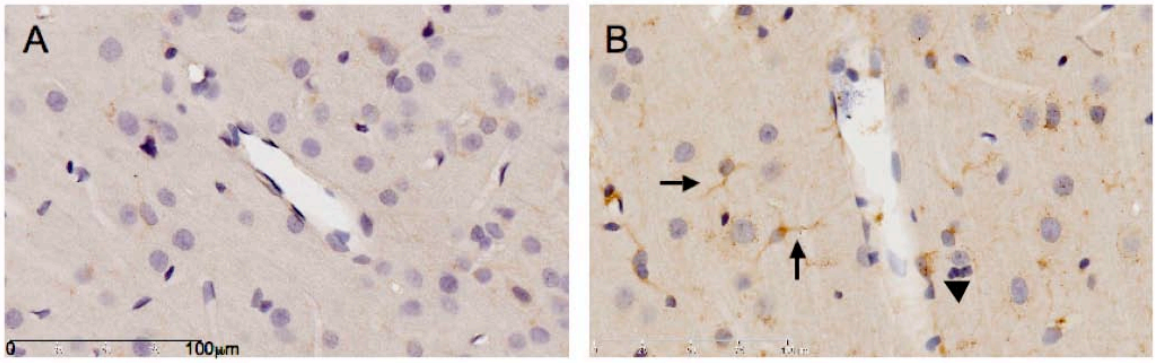


Figure 3.19 Substance P immunostaining within the motor cortex at 5 hours. A: Following filament sham procedure. B: Following filament SAH. Note staining along cellular processes (arrows) and vessel margins (arrowhead).

Assessment of colour deconvoluted images indicated that injection SAH was followed by a trend towards increased in SP immunostaining at all time points (Figure 3.20A). The increase compared to sham levels was more pronounced following filament SAH (B), but did not reach significance at any time point for this number of animals.

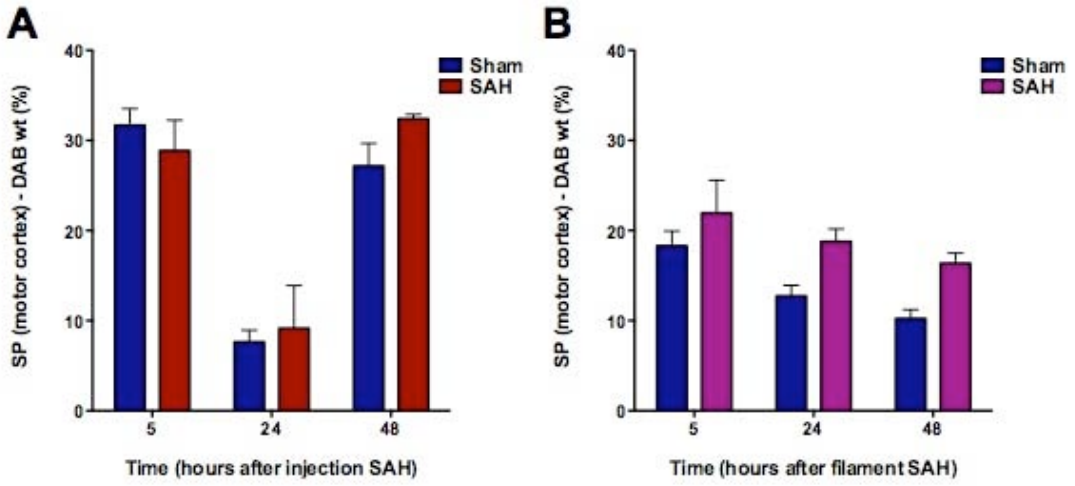


Figure 3.20 The intensity of SP immunoreactivity within the motor cortex following SAH. A: Injection SAH. B: Filament SAH. (n = 5 per group at each time point)

Because SP mediates vasogenic brain oedema in experimental brain trauma and ischaemic stroke, we were interested to see if there was correlation between SP and albumin immunostaining, ie did animals with more intense albumin immunostaining within the motor cortex also have altered SP staining?

There appeared to be a positive correlation between albumin and SP immunostaining intensity in the motor cortex (ie., animals that exhibited greater SP immunostaining intensity also tended to exhibit greater albumin staining intensity (Figure 3.21). However the limited sample size at each time point prevented a significant relationship being demonstrated.

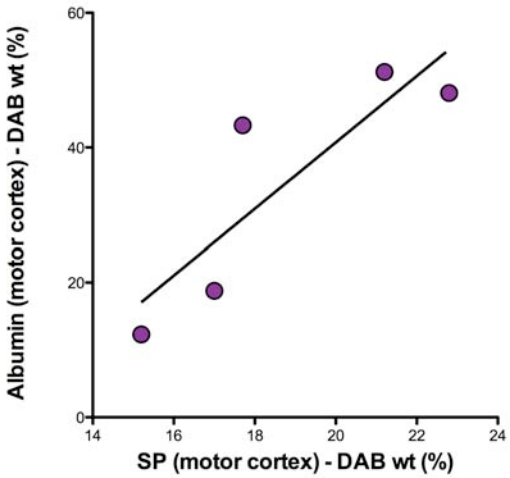


Figure 3.21 Correlation between Albumin and SP immunoreactivity in the motor cortex at 24 h following filament SAH. (Pearson r = 0.86, $\alpha \neq 0.05$, n = 5).

3.4 Discussion

In recent years there has been a renewed interest in secondary brain injury following SAH, particularly as interventions to prevent delayed vasospasm have failed to improve outcome in clinical trials (Macdonald *et al* 2007). Brain oedema, in particular, has attracted considerable attention given the close association between oedema, mortality and morbidity after acute brain injuries.

In the present study, slight but significant brain oedema was demonstrated after filament SAH and a trend towards increased brain water content was observed following injection SAH. ICP-guided SAH, the group with the most pronounced functional deficits, demonstrated an increase in whole brain water content of less than 1%. This is similar to

levels of oedema reported by others using the same species, strain and SAH model (Ostrowski *et al* 2005; Yatsushige *et al* 2007).

Despite the small increases in brain water content, functional deficits correlated with oedema magnitude. This finding is also consistent with what has been reported elsewhere (Thal *et al* 2008). The importance of this finding is that oedema may be a useful therapeutic target, even though the magnitude of the oedema is small.

This oedema was at least partly vasogenic in origin given the simultaneous appearance of albumin immunoreactivity within brain parenchyma. A substantial amount of albumin appears to have crossed the blood-brain barrier prior to perfuse fixation. Increased SP immunoreactivity within brain parenchyma following SAH is consistent with previous reports of SP depletion from cerebral vessels following SAH (Delgado-Zygmunt *et al* 1990; Edvinsson *et al* 1994; Hara *et al* 1986; Juul *et al* 1995; Uemura *et al* 1987). It also indicates that neurogenic inflammation may have contributed to blood-brain barrier breakdown in these models. It was interesting to note that in regions of increased SP reactivity, albumin reactivity was also increased.

Correlation between oedema and functional deficits does not infer that brain oedema was the cause of these deficits. Nor does correlation between albumin and SP immunoreactivity infer that SP mediated the blood-brain barrier breakdown and albumin extravasation. However, the correlations demonstrated in this study support the value of further investigations to further characterise the role of SP in SAH.

The colour deconvolution method provided a measure of albumin staining that was not only consistent with impressions gained by light microscopy, but also correlated with measures including brain water content and functional outcome. Colour deconvolution may provide a valuable, preliminary indicator of protein expression and guide decision-making ahead studies that require additional animals and other resources.

3.5 Conclusions

In summary, this study supports previous reports that filament SAH produces vasogenic brain oedema, and extends this observation to include the prechiasmatic cistern injection SAH model of SAH. Filament SAH produced greater brain oedema and greater functional deficits than injection SAH. In both models, blood-brain barrier breakdown is evidenced by albumin immunoreactivity within brain tissue following SAH. Brain oedema was significant at 24 hours following ICP-guided SAH and the severity of functional deficits correlated with both brain oedema and with immunoreactivity for albumin. Increased substance P immunoreactivity and correlation with albumin immunoreactivity indicate that neurogenic inflammation may contribute to the blood-brain barrier breakdown, oedema and poor outcome following filament SAH. Further studies are warranted to determine the effect of SP blockade on brain oedema, albumin immunoreactivity and functional deficits following filament SAH, and this will be pursued in subsequent chapters.

Chapter 4

Characterisation of intracranial pressure and cerebral perfusion pressure in experimental subarachnoid haemorrhage

4 Characterisation of intracranial pressure and cerebral perfusion pressure in experimental SAH

4.1 Introduction

Raised ICP following SAH is a known predictor of poor outcome (Citerio *et al* 2007; Czosnyka *et al* 2005; Ryttlefors *et al* 2007; Soehle *et al* 2007), with haemorrhage volume causing an immediate (primary) ICP rise, and mechanisms such as brain oedema, hydrocephalus and increased intracranial blood volume all potentially contributing to a secondary increase in ICP. Even the most aggressive management currently available, including craniotomy, often delivers only temporary improvement in ICP (Nagel *et al* 2009). Understanding which mechanisms are involved is important in the identification of new therapeutic targets.

The results of the study detailed in Chapter 3 support the view that substance P mediated vasogenic brain oedema may contribute to poor outcome following SAH. Breakdown of the blood-brain barrier and development of brain oedema was demonstrated, consistent with findings reported by a number of research groups using various SAH models (Gao *et al* 2008; Johshita *et al* 1990; Ostrowski *et al* 2005; Park *et al* 2004; Scholler *et al* 2007; Yatsushige *et al* 2006; Yatsushige *et al* 2007). Furthermore, the extent of brain oedema was shown to correlate with the degree of functional deficits, which is also consistent with the findings of others (Thal *et al* 2009a).

However, it is unclear if brain oedema contributes to an increase in ICP in these models. Previous experimental SAH studies appear to have only focused on the primary (ictal) ICP increase only (Bederson *et al* 1998; Bermueller *et al* 2006; Prunell *et al* 2002) and to the best of our knowledge, secondary increases have not previously been reported. Therefore the present study investigated ICP and CPP before, during and for 5 hours following both injection and filament SAH, with a particular interest in evidence of secondary ICP increases following the ictus.

4.2 Materials and methods

4.2.1 Experimental groups

Adult male Sprague-Dawley rats (n = 21; 350-450g) were randomly allocated to receive injection SAH (n = 5), filament SAH (n = 5) or the corresponding sham procedure (injection sham n = 6, filament sham n = 5), as detailed in Chapter 2. To ascertain the impact of subarachnoid blood versus fluid injection on ICP, two injection sham animals had 220 μL of normal saline injected into the prechiasmatic cistern, two had 220 μL of aCSF injected, whilst the remaining two injection sham animals had a needle inserted into the prechiasmatic cistern but had no fluid injected.

4.2.2 Intracranial pressure monitoring

ICP was monitored continuously from 30 minutes prior to induction of SAH to 5 hours following SAH or sham procedure. In injection SAH and sham animals, a calibrated Microsensor probe was inserted through a 2 mm burr hole drilled 1 mm posterior to bregma and 2 mm left of the midline, so that the tip was positioned just below the dura, into the parenchyma of the left parietal cortex, as described in Chapter 2. In filament SAH

and corresponding sham animals, an additional procedure enhanced stability of the probe when the animal was turned supine for surgery and throughout the prolonged monitoring period. A 5 mm burr hole was drilled in the left parietal bone keeping the underlying dura intact. The Microsensor probe was passed through a 5 mm metal bolt that fitted firmly into the burr hole, and the tip was positioned just below the dura as described above. The bolt was then filled with bone wax.

4.2.3 Cerebral perfusion pressure monitoring

MABP was monitored by a polyethylene catheter inserted into the right femoral artery as described in Chapter 2. CPP was then calculated from the formula

$$CPP = MABP - ICP$$

We elected to keep injection model animals positioned in the stereotaxic frame (in a sphinx like position) and filament model animals supine, throughout the monitoring period, for 2 reasons. Firstly, to avoid movement that might impact on the integrity of ICP data (for example minor movement of the probe within brain parenchyma may result in a local tissue response). Second, to avoid the additional cardiovascular stress that repositioning an animal soon after SAH would entail (exemplified by a pilot filament SAH animal that had a cardiac arrest while being turned from supine).

As noted in Chapter 1, ICP varies with position, being slightly lower if the head is elevated (as in the sphinx-like position of injection model animals). MABP may also be become slightly lower in supine animals, as the weight of abdominal contents on the inferior vena cava may diminish venous return. Therefore ICP and CPP results for each SAH group can be directly compared to their respective shams, but positional differences should be considered when comparing SAH groups of different models.

4.2.4 Statistical analysis

ICP and CPP data was analysed using two-way ANOVA and Bonferroni post-tests for inter-group (SAH vs. sham) comparisons, and one-way repeated measures ANOVA followed by Bonferroni post-tests at selected time points for intra-group comparisons. All data was analysed using PRISM (Graphpad Software, San Diego, CA, USA) and are expressed as mean ± standard error of the mean, with the exception of baseline physiological data which is expressed as mean ± standard deviation. A p value less than 0.05 was considered significant.

4.3 Results

4.3.1 Baseline physiological parameters

There were no differences amongst the groups with respect to physiological variables prior to SAH including baseline CPP, arterial blood gases, arterial pH and temperature (Table 4.1). A non-significant difference in baseline CPP between injection and filament model animals reflects the sphinx vs. supine position of the animals during monitoring.

Table 4.1 Baseline physiological variables prior to induction of SAH.

Baseline physiological parameters: mean (SD)				
	Injection SAH	Injection Sham	Filament SAH	Filament Sham
Baseline CPP (mmHg)	119 (11)	108 (17)	104 (6)	98 (17)
PO2 (mmHg)	128 (19)	146 (7)	141 (10)	135 (15)
PCO2 (mmHg)	40 (3)	40 (4)	40 (3)	39 (3)
pH	7.4 (0.02)	7.5 (0.02)	7.4 (0.03)	7.4 (0.04)
Temperature at time of SAH (°C)	36.7 (0.2)	36.7 (0.3)	36.7 (0.2)	36.6 (0.1)

4.3.2 Mortality

All animals survived the monitoring period, although two filament SAH animals (40%) developed a sustained decline in CPP that was catastrophically low (~ 0 mmHg). Brain death would most likely to have ensued had mechanical ventilation been ceased in these two animals. Fishers exact test did not reveal significantly increased incidence of brain death following filament SAH compared to injection SAH ($p = 0.4$).

4.3.3 Intracranial pressure

ICP data is summarised in Table 4.2. SAH animals demonstrated 3 patterns of ICP increase; an *ictal increase* (a peak followed by a decline to an elevated baseline), a *secondary increase* (developing gradually within 2 hours) and *delayed ICP waves* (characteristic of plateau waves). These ICP patterns are detailed in the following pages.

Filament shams demonstrated no ICP change throughout the monitoring period with needle insertion into the prechiasmatic cistern having no impact on ICP. Injection of either saline or aCSF produced a peak ICP determined primarily by injection rate (rapid injection produced ICP > 100 mmHg), followed by a rapid decline to baseline consistent with compensation of fluid along the craniospinal axis.

Table 4.2 Summary of ICP data from baseline to 5 hours after SAH

Intracranial Pressure (mmHg) mean (SEM)				
	Injection SAH	Injection Sham	Filament SAH	Filament Sham
Baseline (pre-SAH)	7.2 (1.1)	6 (0.9)	8.8 (1.2)	6.6 (1.0)
Peak	100.4 (4.9)	49.8 (15.0)	92.8 (56.6)	7.8 (1.6)
Post-SAH 15 min	11.6 (1.0)	5.0 (1.4)	27.2 (3.7)	6.6 (1.4)
60 min	14.8 (1.3)	6.8 (1.5)	30.2 (3.3)	6.6 (1.5)
90 min	15.6 (1.2)	7.0 (1.4)	34.2 (3.8)	6.6 (1.4)
300 min	15.0 (1.6)	4.0 (1.2)	39.2 (4.5)	7.2 (1.7)

Ictal ICP increase in injection SAH

During injection SAH, ICP rose to a peak of 100.4 ± 4.9 mmHg but only briefly maintained this high level of ICP. This peak ICP is consistent with guidelines for this model, which aims to elevate ICP during SAH to the level of MABP. ICP rose and fell sharply at the ictus (Figure 4.1A). Most of the decline from peak ICP to post-ictal baseline occurred within one minute, though a gradual decline continued for 10 minutes, representing various compensatory mechanisms.

The graph summarising acute ICP changes in the injection SAH group (Figure 4.1B) illustrates the consistent haemorrhage volume produced by this model with error bars being small or obscured at all time points.

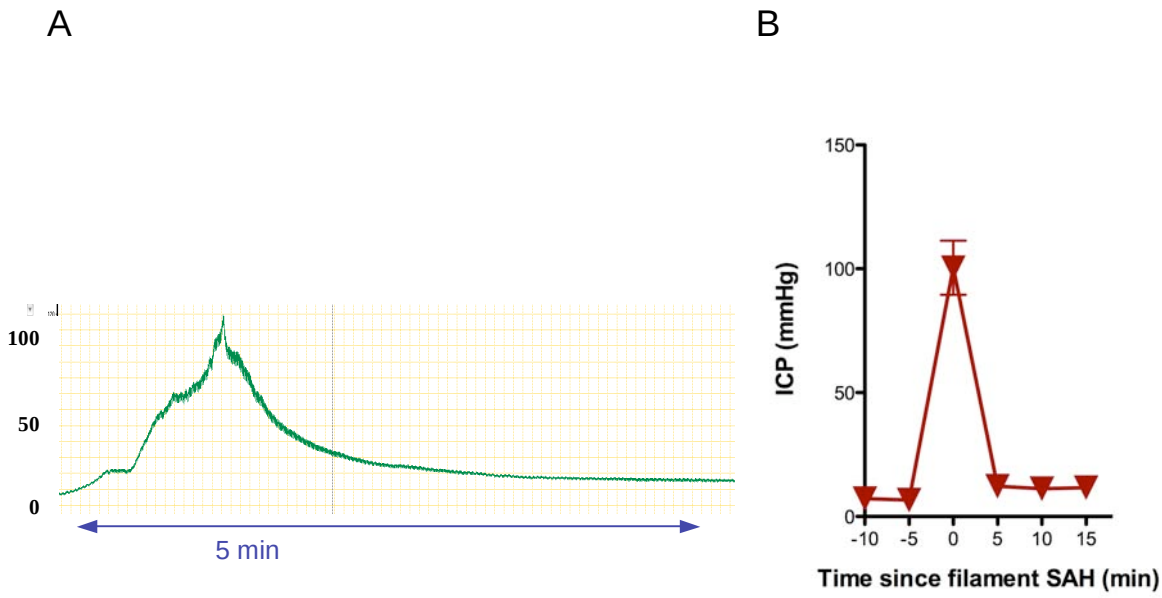


Figure 4.1 Ictal ICP increase during injection SAH. A: ICP trace of a single animal demonstrating a transient rise at the ictus. B: Graph summarising ICP in the acute phase of injection SAH (n = 5).

Ictal ICP increase in filament SAH

During filament SAH, ICP rose more gradually and reached a peak of 49.8 ± 15 mmHg. Whilst this is lower than peak levels observed during injection SAH, the slower ICP decline following filament SAH, as illustrated by the trace below (Figure 4.2A), indicates a greater potential for ischaemic brain injury at the ictus in this model.

From peak levels, ICP declined gradually over 15 minutes to reach the post-ictal baseline (Figure 4.2B). ICP was higher at 15 minutes after filament SAH (27.2 ± 3.7 mmHg) compared to ICP at this time point after injection SAH (11.6 ± 1.0 mmHg). This is

consistent with a greater haemorrhage volume produced by filament SAH. The wider error bars at each time point following injection SAH illustrate variability of ICP due to variable haemorrhage volumes in this model.

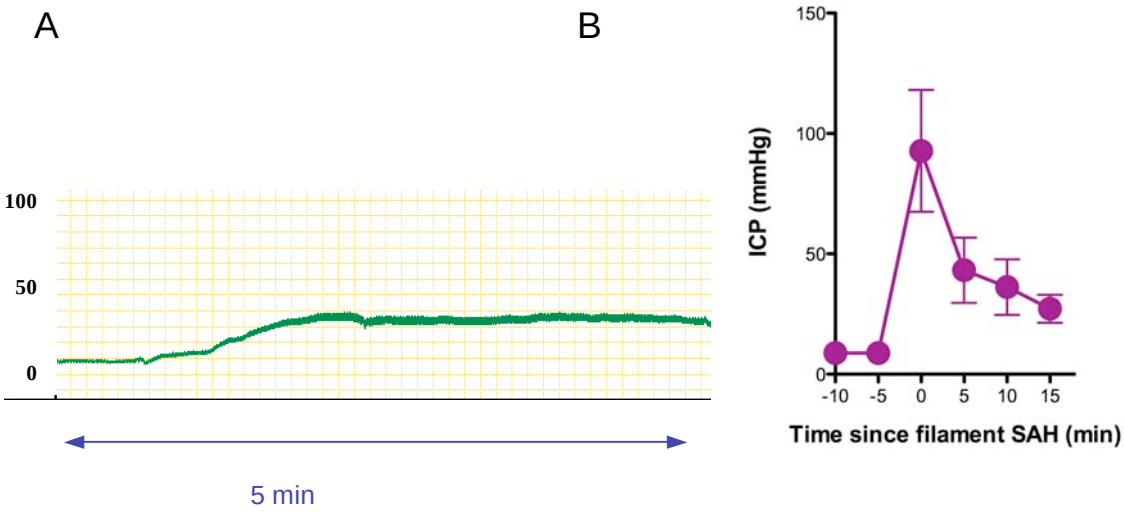


Figure 4.2 Ictal ICP increase in filament SAH. A: ICP trace of a filament SAH animal demonstrating a prolonged rise at the ictus. B: Graph summarising ICP in the acute phase of filament SAH (n = 5).

Secondary ICP increases

All SAH animals of both models demonstrated a secondary ICP increase within 2 hours of the ictus. Specifically, injection SAH produced a secondary ICP increase between 10 and 90 minutes after the ictus (Figure 4.3A). In this period ICP rose from 11.2 ± 0.4 mmHg to 15.6 ± 1.2 mmHg. Needle insertion into the prechiasmatic cistern had no impact on ICP at this (or any) time point, while injection of normal saline or aCSF produced a small, non-

significant ICP increase at this time point, with no difference between the two (Figure 4.3A).

The filament SAH group demonstrated a secondary ICP increase from 15 to 120 minutes after the ictus (Figure 4.3B). All animals in this group demonstrated this increase (independently of plateau waves, as detailed below). In this period mean ICP rose from 27.2 ± 3.7 to 41.4 ± 7.9 mmHg. Sham animals did not demonstrate a secondary ICP increase.

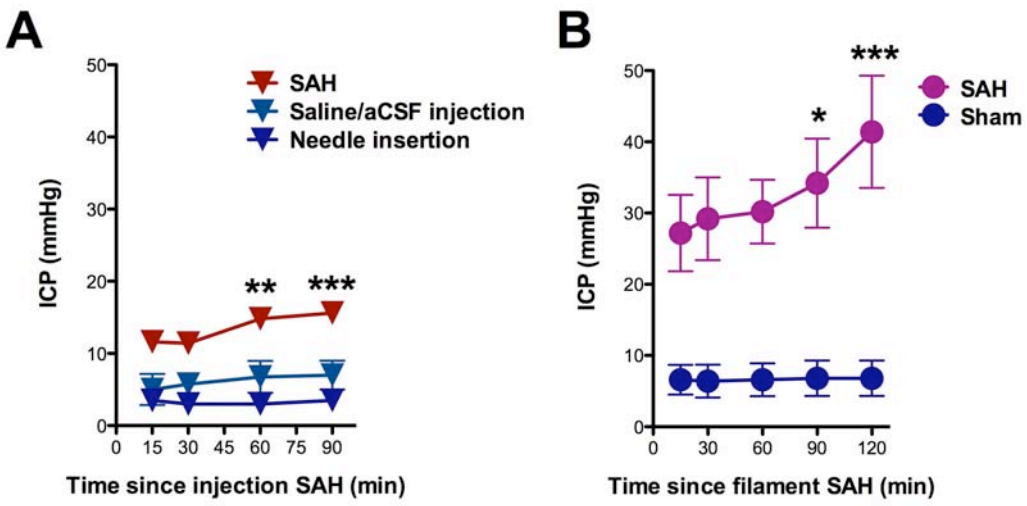


Figure 4.3 Secondary ICP increases observed following A: injection SAH and B: filament SAH. Asterisks denote increases above post-ictal baseline (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

From 2 to 5 hours following SAH no further change in ICP was demonstrated in either group (Figure 4.4A and B).

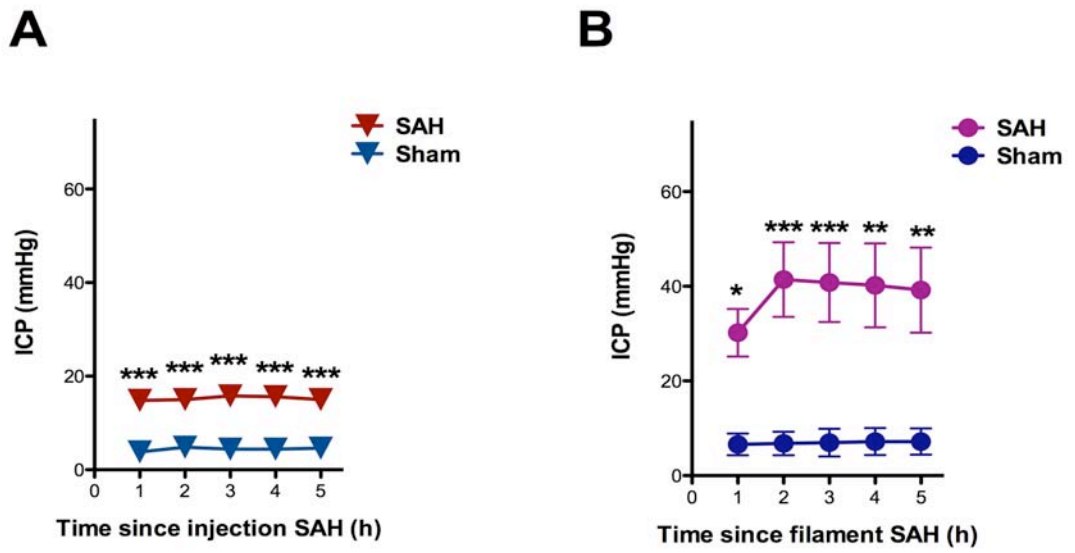


Figure 4.4 ICP changes after SAH. Asterisks represent increase above sham levels at each time point (* p < 0.05, ** p < 0.01, *** p < 0.001).

Delayed ICP waves

Multiple delayed ICP waves were observed in 2 of 5 filament SAH animals whilst none were observed in injection SAH or sham animals. These waves generally had the characteristics of plateau waves (Rosner and Becker 1984), with the phase of elevated ICP (average duration 9.6 minutes) followed by a decline to previous ICP levels (Figure 4.5). However, in both filament SAH animals that exhibited plateau waves, the repeated ICP elevations were eventually followed by the onset of arterial hypotension, with a decline in ABP to near ICP levels. The negligible CPP in these circumstances inevitably leads to brain ischaemia and brain death. The scenario, which commences with low CPP, is discussed in more detail below.

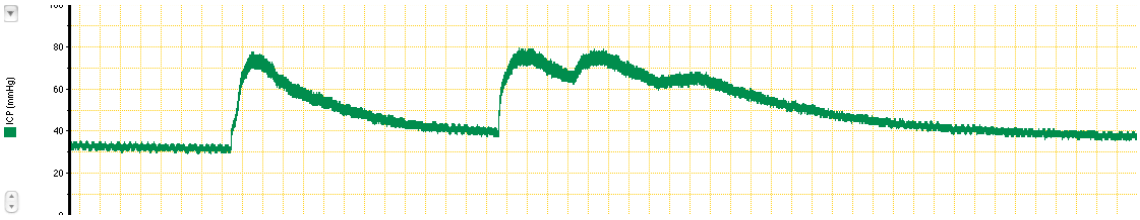


Figure 4.5 ICP trace at 15 min to 30 min following filament SAH demonstrating late waves of ICP increase and partial recovery.

4.3.4 Cerebral perfusion pressure

Cerebral perfusion pressure was maintained following injection SAH and was not different to that of injection sham animals at any time point (Figure 4.6A). In contrast, CPP was diminished following filament SAH and was significantly below sham levels at 3 and 4 hours (Figure 4.6B).

The relationships observed between low CPP and physiological responses including plateau waves, the Cushing response and systemic arterial hypotension is summarised in Figure 4.7.

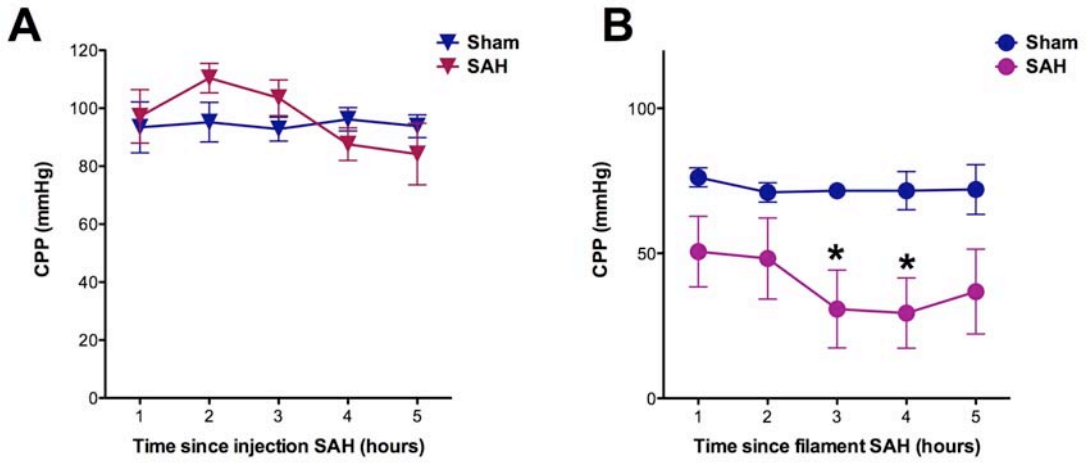


Figure 4.6 CPP following injection SAH (A) and filament SAH (B) (* p < 0.05).

Both of the animals that demonstrated delayed ICP waves eventually developed arterial hypotension with CPP at or near ICP levels. The physiological responses that led to arrest of the cerebral circulation are illustrated in the ABP and ICP traces above, recorded around one hour after filament SAH (Figure 4.7). The increase in MABP (4) that immediately preceded the onset of arterial hypotension seen above appears to represent the commencement of a Cushing response (tachycardia and increased cardiac contractility) preceding the development of cerebral circulatory failure (with vascular baroreflexes triggering vasodilation and hypotension).

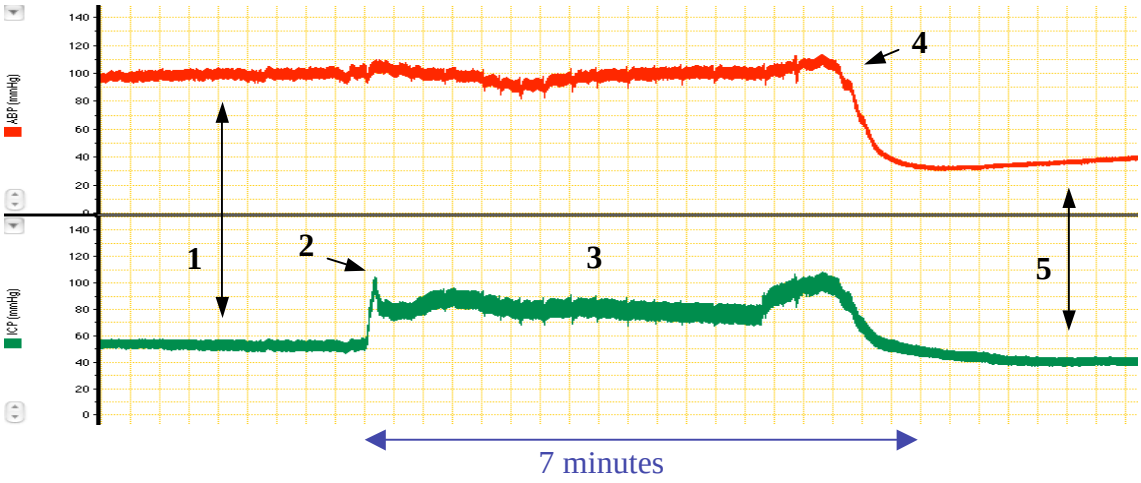


Figure 4.7 Physiological events leading to cerebral circulatory failure after filament SAH. Trace recordings of ABP (top, red) and ICP (bottom, green) demonstrate: 1) ABP is initially within normal range but high ICP compromises CPP; 2) ICP rises steeply, typical of plateau wave commencement; 3) A sustained period of elevated ICP; 4) Transient ABP rise followed by onset of arterial hypotension; 5) ABP is near ICP level so that CPP is minimal.

4.4 Discussion

The present study has shown that both experimental SAH models produced a profound ICP increase at the ictus followed by a secondary ICP increase within 2 hours. The secondary increase was demonstrated in all SAH animals and was even more pronounced following filament SAH as opposed to the prechiasmatic cistern injection model of SAH. The secondary increase was substantial, particularly in view of the reduced compliance following the initial haemorrhage. Oedema may have contributed to the secondary increase in ICP, however vasodilation and/or hydrocephalus may also have contributed.

This study also found that filament SAH produced a longer period of high ICP at the ictus compared to injection SAH. Thus, brain ischaemia at the ictus may also have contributed to the poorer outcome demonstrated after filament SAH in the previous study (Chapter 3). The present study also described low CPP following filament SAH, which may also impact deleteriously on outcome. Critically low CPP can trigger cerebrovascular vasodilation (Rosner and Becker 1984), which increases brain blood volume and thus further increases ICP, establishing a vicious cycle. Plateau waves represent this vasodilation response (Rosner and Becker 1984) and as autoregulation is altered but not lost during plateau waves, their duration is usually limited so that ICP falls, CPP improves and normal/intact autoregulation returns (Czosnyka *et al* 1999; Rosner and Becker 1984). Understanding the clinical significance of plateau waves is still evolving and they are not inevitably associated with poor outcome (Czosnyka *et al* 1999). However, the plateau waves observed in this study lasted from 3 to 18 minutes, and represented substantial periods of high ICP and brain ischaemia. They also preceded the development of catastrophic CPP loss in 2 animals. Filament SAH may provide an excellent animal model for further investigating plateau waves.

It is possible that the deleterious effects of a secondary ICP increase (or plateau waves) may be mistakenly attributed to rebleeding. A study of the incidence of rebleeding before hospitalisation reported that 14% of patients suffered rebleeding in the ambulance and that 77% of rebleeds occurred within 2 hours of SAH (Ohkuma *et al* 2001). As these patients did not under go CT assessment (confirming haemorrhage volume) before hospital arrival, it is possible that a secondary ICP increase without rebleeding may have caused clinical deterioration in some of these patients.

4.5 Conclusions

In summary, this study found that injection and filament SAH produced both primary (ictal) and secondary ICP increases. The primary ICP increase most likley was related to haemorrhage volume while the secondary ICP increase remains of uncertain aetiology. The impact of secondary ICP increases may be magnified following the primary increase due to reduced compliance (compensatory mechanisms for additional intracranial volume have been partially exhausted) and reduced CPP. Brain oedema may contribute to the secondary ICP increase and a relatively small volume of oedema may have a substantial impact on outcome. The impact of the secondary ICP increase may also have been particularly deleterious to the filament SAH group, which demonstrated a longer period of brain ischaemia at the ictus and reduced CPP subsequently, leaving them vulnerable any further increase in ICP. These findings, together with evidence that SP may contribute to vasogenic brain oedema after experimental SAH (Chapter 3), support further studies to determine the effect of SP blockade in injection and filament SAH.

Chapter 5

The impact of substance P blockade on oedema, histology and functional outcome following experimental subarachnoid haemorrhage

5 The effect of substance P blockade on brain oedema, histology and functional outcome following experimental SAH.

5.1 Introduction

The experimental findings detailed in Chapters 3 and 4 have demonstrated a secondary ICP increase following experimental SAH and significant brain oedema following filament SAH, implying that oedema may contribute to raised ICP. Albumin extravasation into the brain parenchyma indicated a vasogenic mechanism contributing to oedema formation, with the magnitude of the oedema correlating with the severity of functional deficits. Since SP expression was enhanced following experimental SAH, and has been previously associated with the development of vasogenic oedema following acute brain injury (Nimmo et al., 2004; Turner et al., 2006; Donkin et al., 2009), it is feasible that SP mediated neurogenic oedema may be involved. Further investigation is thus required to determine if a causal relationship exists between increased SP expression and oedema development in filament SAH.

A recent study has supported a causal relationship between oedema formation and functional outcome in this model of SAH (Thal *et al* 2009b). These investigators demonstrated that inhibition of bradykinin B2 receptors reduced oedema formation and improved functional outcome after rat filament SAH. However, only animals treated prior to induction of SAH demonstrated improvement. This indicates that in pre-treated animals,

B2 receptor blockade maintained BBB integrity and perhaps prevented the initiation of cascades leading to oedema formation and poor outcome after filament SAH. However, in post-treated animals, the B2 receptor antagonist was ineffective, and a mechanism independent of B2 receptors was proposed as mediating ongoing oedema formation and poor outcome following SAH.

SP mediated neurogenic inflammation promotes significant oedema formation in various models of acute brain injury (Donkin *et al* 2009; Kleinig *et al* 2010; Turner *et al* 2006), but it is unclear if this mechanism makes a significant contribution to oedema formation and outcome after filament SAH. . One approach to resolving this question is to inhibit the SP NK1 receptors, which can be achieved by receptor antagonists such as N-acetyl-L tryptophan. Moreover, their delivery to the CNS may be facilitated by blood-brain barrier opening following filament SAH (Scholler *et al* 2007).

The aim of this study is therefore to characterise the effects of the substance P antagonist N-acetyl-L-tryptophan (NAT), on brain oedema formation and functional outcome following filament SAH.

5.2 Materials and methods

5.2.1 Experimental groups

Adult male Sprague-Dawley rats (350 – 450 g, n = 40) underwent filament SAH (as described in Chapter 2) and at 30 minutes after SAH, received treatment with either NAT (2.5 mg/kg) or an equivalent volume of normal saline vehicle via the right femoral vein. The rationale for the dosage regime is detailed in Chapter 2.

For oedema studies, 20 animals were killed at 24 and 48 hours after SAH (n = 5 per group). For histology studies, 10 animals were used, and were killed at 24 hours after SAH (n = 5 per group). All animals underwent functional assessment prior to being sacrificed, so that 10 animals were assessed in each treatment group at 24 hours after SAH.

5.2.2 Functional outcome

Functional outcome was assessed by Neuroscore, rotarod and sticky label removal tests at 24 and 48 hours after SAH, according to procedures detailed in Chapter 2.

5.2.3 Brain oedema

Brain oedema was assessed at 24 and 48 hours following SAH by the wet weight-dry weight method as previously described in detail previously in Chapter 2.

5.2.4 Histology and semiquantification of immunostaining

Histology including haematoxylin and eosin staining, albumin and substance P immunostaining was assessed following perfuse fixation with formalin at 24 hours after SAH. All processes were undertaken as detailed in Chapter 2. The intensity of albumin and SP immunostaining within the motor cortex was semiquantified as detailed in Chapter 2.

5.2.5 Statistical analysis

Mortality data was analysed using Fisher's exact test. Non-parametric data (Neuroscore) was analysed using Kruskal-Wallis one-way ANOVA with selected Dunn's post-tests or by using the Mann Whitney U test as indicated. Parametric data (other functional tests, oedema and histology data) were analysed using two-way ANOVA and Bonferroni post-

tests for inter-group (SAH vs sham) comparisons at each time point. Repeated measures ANOVA was used where possible.

5.3 Results

5.3.1 Mortality

At 24 hours after filament SAH, mortality of NAT treated animals was 46% and saline treated animals was 27%. This represents a non-significant increase in mortality in the NAT-treated group ($p = 0.17$).

5.3.2 Functional outcome

NAT treatment had no impact on functional outcome measures following SAH, including Neuroscore, rotarod endurance or latency in removal of adhesive paper from each forepaw at 24 hours. (Figure 5.1). Groups assessed at both 24 and 48 hours also demonstrated no differences between groups at either time point and no significant change over time.

With respect to brain water content, NAT treatment had no significant impact on brain water content at 24 or 48 hours after filament SAH (Figure 5.2). If anything, NAT-treated animals demonstrated a non-significant increase in brain water content at 24 hours (A), and this increase was more pronounced in the right hemisphere and the brain stem (B).

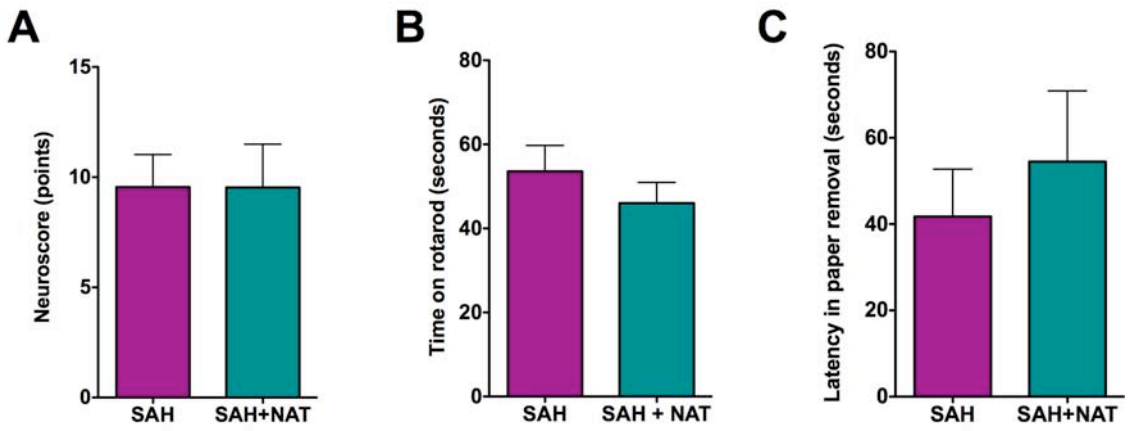


Figure 5.1 The impact of NAT treatment on functional outcome at 24 hours after filament SAH. A: Neuroscore. B: Rotarod. C: Sticky paper removal latency. (n = 10 per group for all measures).

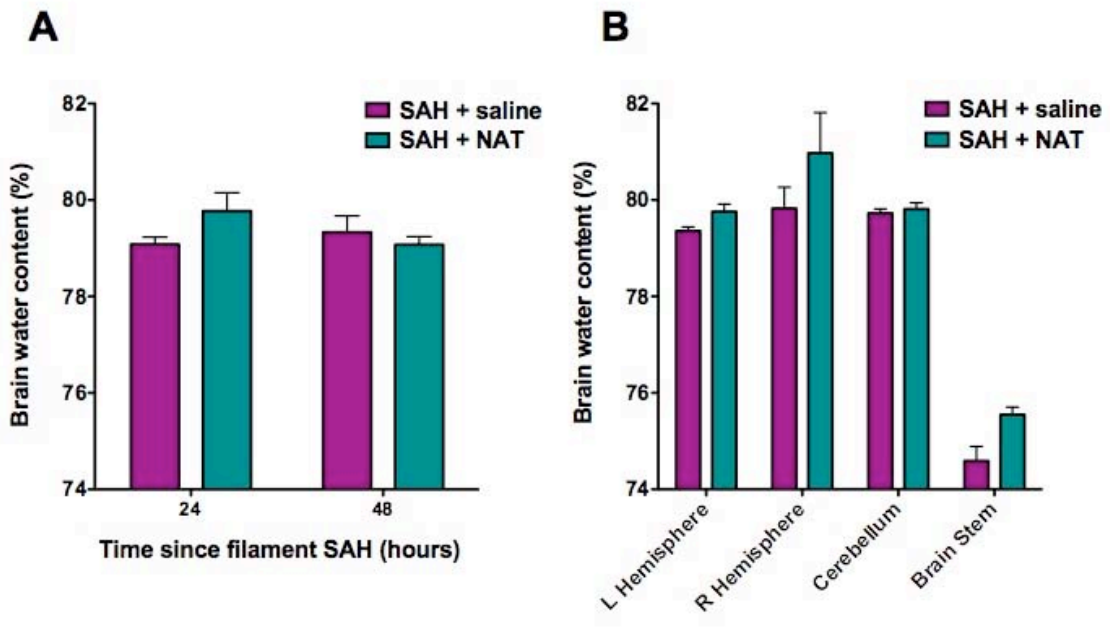


Figure 5.2 The impact of NAT treatment on brain water content after filament SAH. A: Brain water content at 24 and 48 hours. B: Water content in each of 4 brain regions.

5.3.3 Histology

Three NAT treated animals were killed at 5 hours after filament SAH due to poor neurological condition. Histology demonstrated reduced motor cortex SP immunoreactivity in these animals compared to non NAT-treated filament SAH animals at this time point (Figure 5.3). Consistent with this, colour deconvolution processing indicated that NAT treatment was associated with significantly reduced intensity of SP immunostaining at 5 hours (Figure 5.4A). However, by 24 h, SP immunoreactivity was similar in NAT treated and vehicle treated animals (Figure 5.4B).

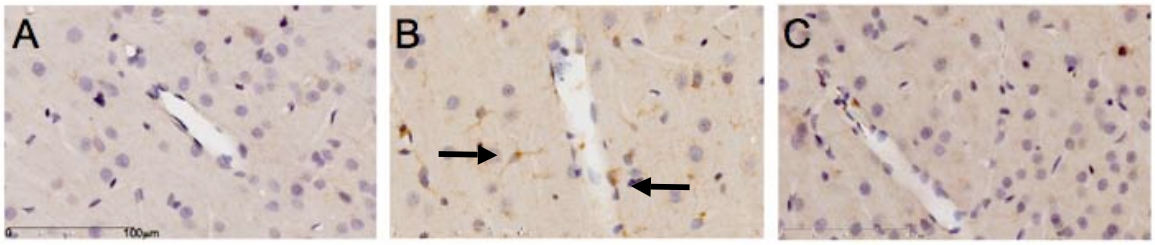


Figure 5.3 The impact of NAT treatment on SP immunostaining in the motor cortex. A: Minimal staining at 5 hours following filament sham procedure. B: SP staining at 5 hours following filament SAH. C: NAT treatment was associated with reduced SP staining in the motor cortex at 5 hours after filament SAH.

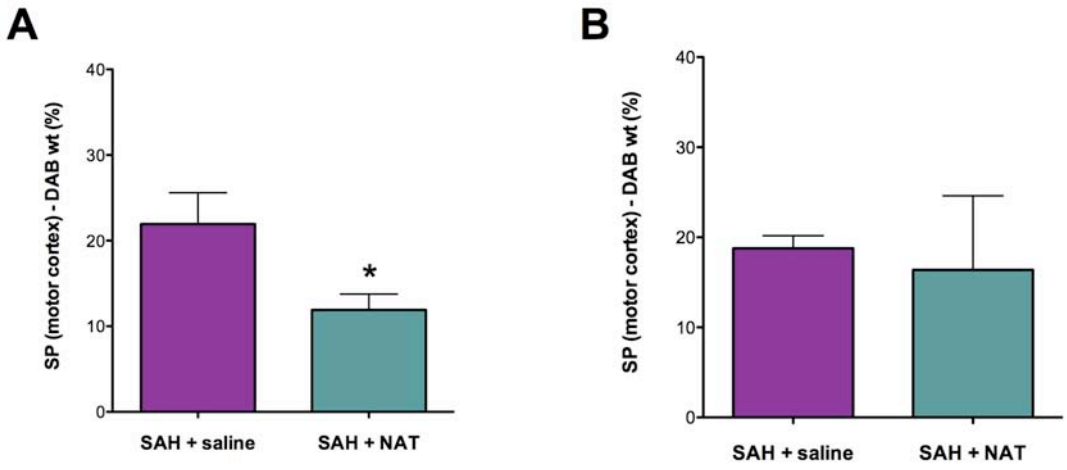


Figure 5.4 The impact of NAT treatment on motor cortex SP immunoreactivity following filament SAH. A: At 5 hours (* $p < 0.05$). B: At 24 hours (n = 5 per group except SAH + NAT at 5 hours, n = 3).

NAT treatment had no impact on albumin immunoreactivity in the motor cortex at 5 or 24 hours after SAH (Figure 5.5). Data at 5 hours is based on n = 3 NAT-treated animals sacrificed early due to poor neurological condition. Larger errors bars for NAT-treated

groups reflect greater variability of immunoreactive staining demonstrated by these animals compared to the saline-treated group.

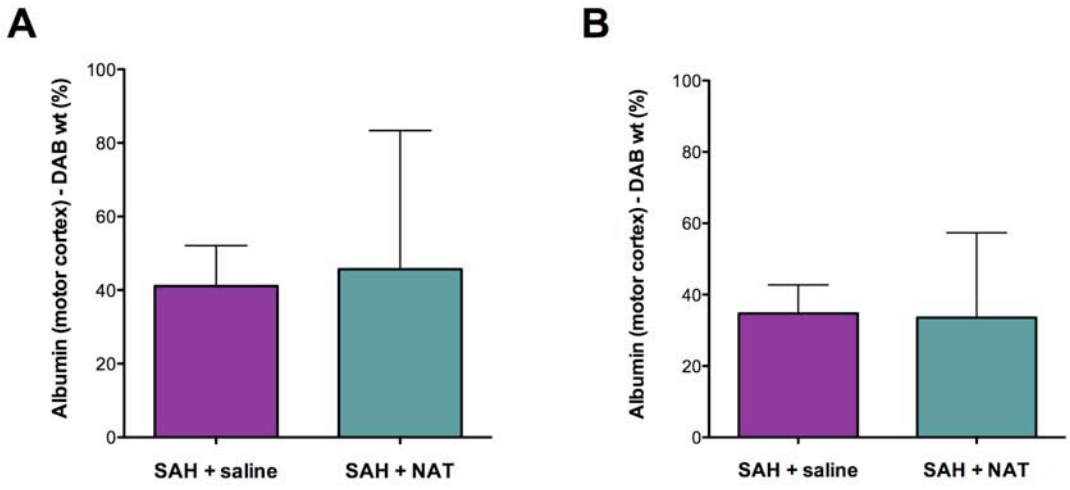


Figure 5.5 The impact of NAT treatment on motor cortex albumin immunoreactivity. A: At 5 hours after filament SAH. B: At 24 hours after filament SAH.

5.4 Discussion

This study found that treatment with a SP NK1 receptor antagonist did not significantly improve neurological outcome following filament SAH or reduce brain oedema or blood-brain barrier permeability to albumin. This suggests that these events have little association with SP-mediated neurogenic inflammation. The lower SP immunoreactivity of NAT-treated animals with poor neurological condition at 5 hours supports this view, that is that their poor condition was due to factors other than SP mediated effects.

Despite the correlation observed between brain oedema and neurological deficits in Chapter 3, the extent of oedema following filament SAH was modest even when the functional deficits were severe. Greater increases in brain oedema have been reported following experimental filament occlusive stroke (3.6%)(Turner *et al* 2006), intracerebral haemorrhage (3.2%)(Li *et al* 2009), endothelin stroke (1.3%)(Huang *et al* 2007) and brain trauma (2.2%) (Nimmo *et al* 2004). These other acute brain injury models produced 2 to 5 times more oedema than filament SAH, but have substantially lower mortality rates. NAT treatment in these acute brain injury studies also results in a significant reduction in oedema formation that is associated with significant improvements in neurological outcome (Donkin 2006; Turner *et al* 2006; Vink and van den Heuvel 2010). This is in contrast to the present study where increases in SP immunoreactivity were only marginal and NAT treatment did not reduce brain oedema or improve functional outcome, despite reducing early (5h) SP immunoreactivity. Thus, SP mediated neurogenic inflammation does not appear to play a crucial role in filament SAH.

Nonetheless, brain oedema may still be an important mechanism of secondary injury in experimental SAH, despite the increase in intracranial volume associated with oedema being small. Brain compliance becomes limited after SAH because compensatory mechanisms are rapidly exhausted following the initial bleed and the primary ICP increase. Therefore, relatively modest oedema may impact on ICP and subsequent outcome. This mechanism may explain why administration of a bradykinin B2 receptor antagonist prior to induction of filament SAH reduced oedema and improved outcome as opposed to administration after induction of SAH (Thal *et al* 2009b). Administration of the antagonist prior to SAH would prevent oedema formation and improve outcome whereas

administration after SAH, when brain compliance is already limited and any existing oedema would be highly deleterious, would have limited effects.

An appropriate therapeutic target for SAH therefore remains elusive. The lack of benefit of NAT treatment on BBB permeability, oedema formation and functional outcome would seem to exclude neurogenic inflammation as a potential therapeutic target, although effects of NAT on ICP and CPP are yet to be determined.

5.5 Conclusions

Treatment with the SP NK1 receptor antagonist NAT did not demonstrate benefit following filament SAH. The amount of oedema produced in filament SAH may have been too small for NAT to demonstrate a significant benefit. However, the lack of improvement in survival or functional outcome in NAT-treated animals suggests that other secondary injury mechanisms, such as impaired cerebral autoregulation, may be more important in SAH than SP mediated neurogenic oedema. Non-neurogenic mechanisms may contribute to blood-brain barrier breakdown and oedema formation in this model.

Chapter 6

The effect of substance P antagonism on intracranial pressure and cerebral perfusion pressure following experimental subarachnoid haemorrhage

6 The effect of substance P antagonism on ICP and CPP following experimental SAH

6.1 Introduction

Raised intracranial pressure and brain oedema have both been found to be independent predictors of poor outcome following SAH (Claassen *et al* 2002; Ryttlefors *et al* 2007). The study detailed in Chapter 3 demonstrated that filament SAH produced significant brain oedema and that the magnitude of oedema correlated with functional deficits. Consistent with this association between brain oedema and functional outcome, injection SAH produced smaller increases in brain water content and no functional deficits. The study detailed in Chapter 4 demonstrated that both SAH models produced a secondary increase in ICP of unknown aetiology. The late ICP increase may be attributed to brain oedema, but also might be associated with hydrocephalus and increased intracranial blood volume.

With respect to brain oedema, the neuropeptide SP mediates blood brain barrier breakdown and vasogenic brain oedema formation, by acting on vascular NK1 receptors (Donkin *et al* 2009). This action of SP contributes to secondary injury in experimental brain trauma and ischaemic stroke (Donkin *et al* 2009; Turner 2007). Vasogenic oedema is evident in both injection and filament SAH, as outlined in Chapter 3, but the study detailed in Chapter 5 indicated that SP mediated vasogenic oedema did not contribute significantly to BBB breakdown and brain oedema after filament SAH. While ruling out a significant role in vasogenic oedema formation, the results of Chapter 5 did not exclude potential

beneficial effects of the SP antagonist on other factors that may impact on outcome after SAH, such as hydrocephalus and blood volume, both of which would impact on ICP and CPP.

The present study therefore investigated the potential role of SP in both injection and filament SAH by observing the impact of the SP NK1 receptor antagonist, NAT, on ICP and CPP.

6.2 Methods

6.2.1 Experimental groups

At 30 minutes after induction of injection (n = 10) or filament SAH (n = 10) as detailed in Chapter 2, adult male Sprague-Dawlet rats (350 - 450 g) were treated with either N-acetyl-L-tryptophan (2.5 mg/kg) or an equivalent volume of normal saline vehicle (n = 5 per group) via the right femoral vein.

6.2.2 Intracranial pressure monitoring

ICP was monitored continuously in all animals from 30 minutes prior to induction of SAH to 5 hours following SAH, as detailed in Chapters 2 and 4.

6.2.3 Cerebral perfusion pressure monitoring

In all animals MABP was monitored by a polyethylene tube inserted into the right femoral artery as detailed in Chapter 2 and CPP was then calculated according to the formula

$$CPP = MABP - ICP$$

Injection model animals were positioned in the stereotaxic frame throughout the monitoring period, while filament model animals were positioned supine, as detailed in Chapter 4.

6.2.4 Statistical analysis

Mortality data was analyzed by Fisher's exact test while ICP was assessed using repeated analysis of variance (ANOVA) followed by Bonferroni post-tests. All data was analyzed using PRISM (Graphpad Software, San Diego) and are expressed as mean ± standard error of the mean with the exception on baseline physiological data which is expressed as mean ± standard deviation (SD). A p value less than 0.05 is considered significant.

6.3 Results

6.3.1 Baseline physiological parameters

There were no significant baseline differences between groups regarding baseline CPP, arterial blood gases or temperature at the time of SAH induction, as shown in Table 6.1.

Table 6.1: Baseline physiological parameters. Measures are expressed as mean (SD).

	Injection SAH		Filament SAH	
	+ saline	+ NAT	+ saline	+ NAT
Baseline CPP (mmHg)	110.9 (12.0)	95.4 (18.5)	103.8 (6)	86.4 (14.5)
PO2 (mmHg)	128 (19)	143 (9)	141 (10)	140 (16)
PCO2 (mmHg)	40 (3)	41 (2)	40 (3)	40 (3)
pH	7.44 (0.02)	7.43 (0.04)	7.44 (0.03)	7.47 (0.02)
Temperature at time of SAH (°C)	36.7 (0.2)	36.9 (0.2)	36.7 (0.2)	36.7 (0.2)

6.3.2 Mortality

Two of the 5 saline treated filament SAH animals developed sustained, very low CPP, consistent with arrest of the cerebral circulation and brain death. Similarly, 1 of the 5 NAT-treated filament SAH animals demonstrated cerebral circulatory arrest. This difference did not represent a statistical difference in mortality between groups (p = 1.0). No injection SAH animals of either treatment group demonstrated catastrophic CPP loss.

6.3.3 Intracranial pressure

Secondary ICP increase

NAT treatment had no impact on the significant increase in ICP that was demonstrated within 2 hours of SAH in either the injection or filament model of SAH (Figure 6.1).

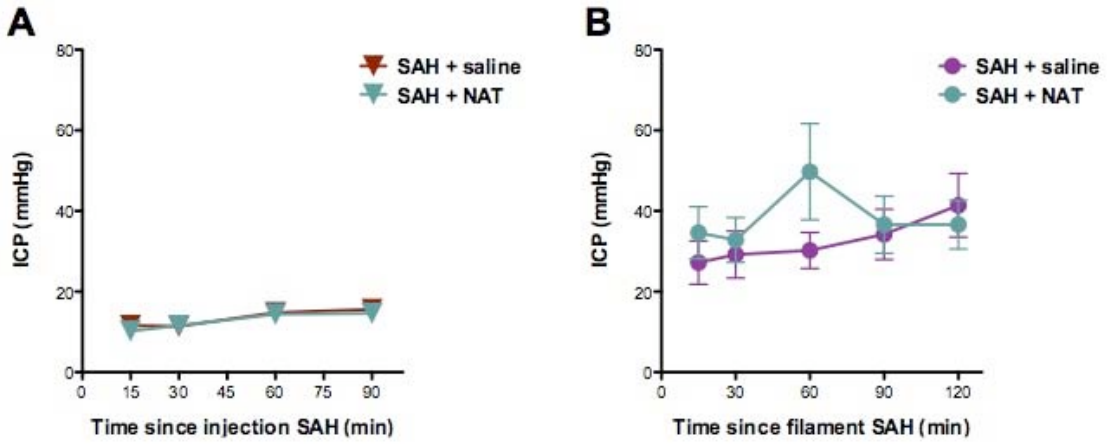


Figure 6.1 The impact of NAT treatment on secondary ICP increase following SAH. A: Injection SAH. B: Filament SAH.

Similarly, when analysing the first 5 h of ICP data, NAT treatment had no impact on ICP up to 5 hours after induction of SAH in either model (Figure 6.2). In the injection SAH graph (A), small or obscured symbols and error bars at all time points indicate consistent haemorrhage volume in saline and NAT-treated groups.

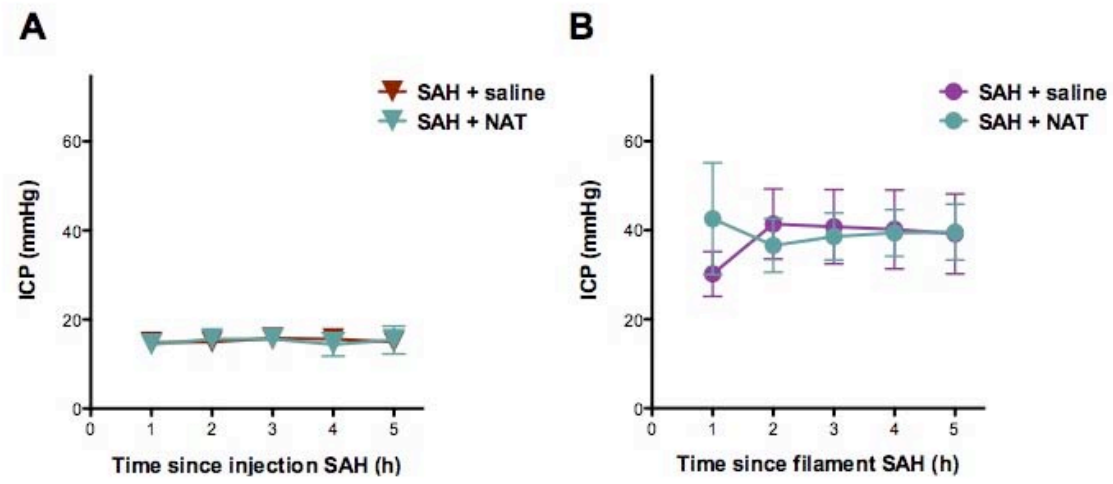


Figure 6.2 The impact of NAT treatment on ICP at 1 to 5 hours after SAH. A: Injection SAH. B: Filament SAH.

Plateau waves and cerebral autoregulation

Injection SAH animals of both treatment groups demonstrated no plateau waves, which is consistent with the findings in Chapter 4. However, after filament SAH, 2 saline-treated filament SAH animals demonstrated plateau waves, with both developing loss of cerebral autoregulation with systemic arterial hypotension and CPP ~ 0 mmHg. in contrast, 3 NAT-treated animals demonstrated plateau waves, but only one of these developed sustained, catastrophic CPP loss. The other 2 animals appeared to regain normal autoregulation, despite prolonged periods of relatively low CPP (often below 40 mmHg).

6.3.4 Cerebral perfusion pressure

At 2 and 3 hours after injection SAH, CPP was significantly reduced in NAT-treated injection SAH animals compared to the saline-treated group ($p < 0.05$) (Figure 6.3, A). As a whole, the filament SAH group demonstrated no response to NAT treatment (Figure 6.3, B).

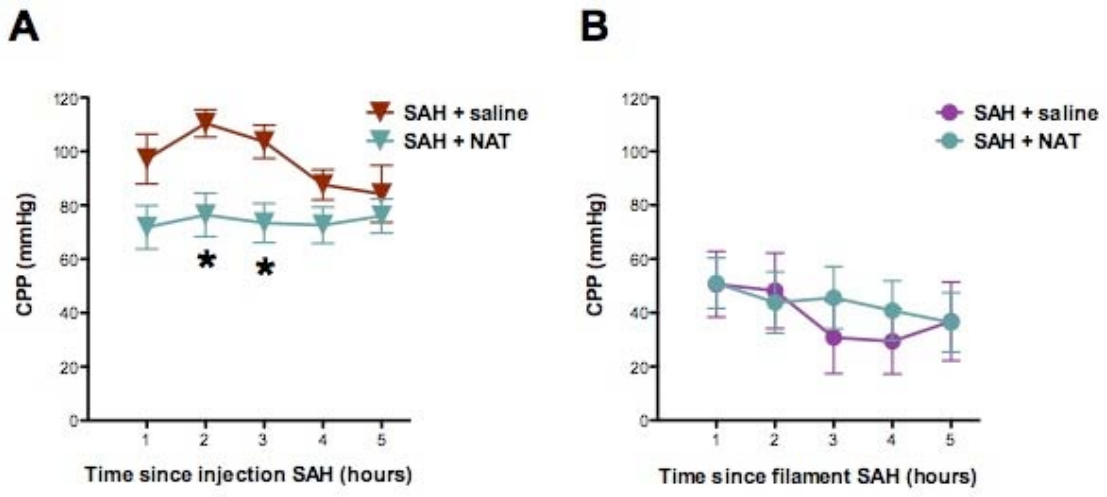


Figure 6.3 The impact of NAT treatment on CPP following experimental SAH. A: Injection SAH (n = 5 per group, * p < 0.05). Asterisk indicates a significant decrease in CPP below the level of the saline-treated group. B: Filament SAH (n = 5 per group).

As Figure 6.4 demonstrates, a similar CPP pattern is seen following SAH if data excludes animals with brain death due to sustained, catastrophic CPP loss. SAH + saline animals of both injection and filament groups demonstrated a CPP increase in the early hours

following SAH while NAT-treated animals of both models did not demonstrate this early CPP increase.

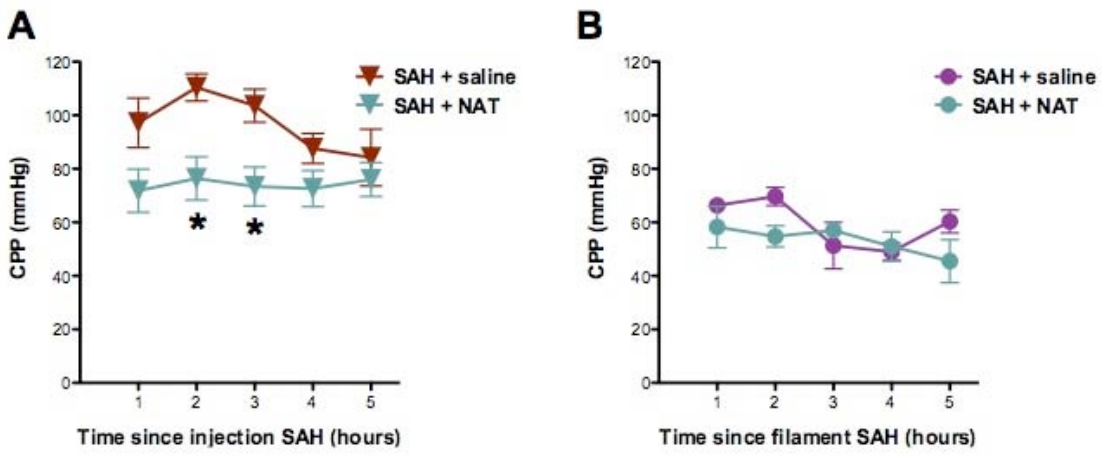


Figure 6.4 Impact of NAT on CPP following SAH after excluding "brain dead" animals. A: Injection SAH (n = 5 per group, as above). B: Filament SAH excluding animals that suffered brain death (Filament SAH + saline n = 3, filament SAH + NAT n = 4).

6.4 Discussion

This study has shown that NAT administration did not reduce the secondary ICP increase that followed injection and filament SAH. This finding is consistent with the results from the previous study (Chapter 5) demonstrating that NAT does not reduce increased BBB permeability or brain oedema in these models. Moreover, it establishes that NAT has little impact on other factors that may influence the secondary rise in ICP following SAH. We can only conclude that the bulk of the brain oedema after SAH is formed by a mechanism other than SP-induced neurogenic inflammation.

Interestingly, NAT-treated filament SAH animals did demonstrate better recovery of CPP following plateau waves compared to the saline treated group. Despite ongoing periods of relatively low CPP that might have been expected to trigger further vasodilation, these animals exhibited surprisingly few plateau waves. A possible explanation is that a small, localised perivascular oedema in the brains of non-NAT treated animals, too small be detected by brain water content or ICP measurements, may have decreased local brain tissue oxygenation and increased the tendency for a vasodilation response (and therefore plateau waves). By this mechanism, SP may play a deleterious role on cerebral autoregulation following brain insults leading to reduced CPP. The impact would potentially be greater for insults such occlusive stroke where a greater amount of oedema may be involved.

Any beneficial effects of NAT treatment that might be mediated through reducing the incidence of plateau waves may have been obscured by other deleterious events associated with filament SAH. For example, the relatively long period of brain ischaemia at the ictus that this model produces is likely to cause a substantial primary injury, and secondary injury due to plateau waves may be minor in relation to this. However, the plateau waves observed in this study involved periods of negligible CPP lasting long enough to cause substantial secondary injury due to brain ischaemia. So if NAT treatment prevented plateau waves following filament SAH, why did animals treated with NAT fail to demonstrate improved functional outcome or reduced mortality in the previous study (Chapter 5)?

There are two responses to this question based on the results of the present study. One possibility is that SP antagonism inhibited not only the global cerebral vasodilation responses that are involved in the development of plateau waves, but that NAT treatment also inhibited regional vasodilation responses in response to regional metabolic demands, and that this had an overall deleterious impact. As noted previously, cerebral autoregulation is altered, but not lost, before and during plateau waves, and so we cannot assume that a treatment that prevents the development of plateau waves inevitably also improves brain metabolism. Studies that include monitoring of brain tissue oxygenation in addition to CPP may be useful to investigate this further. Another possibility is that SP antagonism did have a beneficial effect through preventing plateau wave development, but some other effect of NAT treatment offset this benefit.

Elevated CPP in the early hours following SAH appears to be a protective response, despite the associated high ABP and the increased potential for rebleeding and cardiac complications. Epidemiological studies have found that high CPP (> 100 mmHg) or high ABP (> 180 mmHg) after SAH are associated with decreased risk of clinical deterioration (Ryttlefors *et al* 2007). NAT -treated animals did not demonstrate a period of elevated CPP that saline treated animals demonstrated at 2 and 3 hours after injection SAH and (to a lesser extent) at 2 hours after filament SAH, because they failed to generate an increased MABP in this period. Animals with loss of cerebral autoregulation, who subsequently developed a negligible CPP, also failed to demonstrate this early MABP increase, regardless of their treatment. This effect of NAT is consistent with previous studies that have shown that SP mediates recovery from systemic hypotension (Wang *et al* 2008), and mediates blood pressure elevation in response to stimulation of the gall bladder (Pan *et al*

1995). Our studies indicate that SP may play a similar role in the pressor response to SAH. Blocking this pressor response may be deleterious to outcome.

6.5 Conclusion

Treatment with the SP, NK1 receptor antagonist NAT had no impact on ICP following SAH, suggesting that SP-mediated neurogenic oedema may not contribute to secondary ICP increase in these models. NAT treatment may have impacted positively on cerebral autoregulation, as these animals exhibited fewer plateau waves and appeared to have improved ability to regain CPP following plateau waves. Paradoxically, NAT treatment may have had a dampening effect on the positive sympathetic (MABP) response to CPP loss following SAH. There may be scenarios in which beneficial effects of SP antagonists, mediated through effects on impaired cerebral autoregulation and sympathetic drive may be clinically useful. However, in SAH, NAT treatment did not address the major components of the overall injury

Chapter 7

General discussion

7 General discussion

7.1 Overview

7.1.1 Principle findings

The principle findings from the experiments detailed in this thesis are that experimental SAH produced increased levels of cerebral SP immunoreactivity, blood-brain barrier breakdown, vasogenic brain oedema, functional deficits that correlated with the magnitude of oedema, and both primary and secondary increases in ICP. SP blockade by treatment with the NK1 receptor antagonist NAT had no impact on oedema, ICP or functional outcome, suggesting that SP mediated neurogenic oedema was not a critical factor in the development of brain oedema or intracranial hypertension following SAH. Other factors, not involving neurogenic inflammation, are therefore more important in determining functional outcome following SAH.

The oedema demonstrated in this study, as measured by increased brain water content in the presence of albumin extravasation into brain parenchyma, is consistent with vasogenic oedema, and has been demonstrated by a number of research groups in clinical SAH (Doczi 1985) as well as various animal models of SAH (Doczi 1985; Gao *et al* 2008; Johshita *et al* 1990; Ostrowski *et al* 2005; Park *et al* 2004; Scholler *et al* 2007; Yatsushige *et al* 2006; Yatsushige *et al* 2007). However, whereas SP facilitates vasogenic brain oedema formation following brain trauma, occlusive stroke and intracerebral haemorrhage, other mechanisms of oedema formation appear to be more important in the current SAH experiments. The lack of benefit from NAT treatment in this study is in contrast to its

effects in traumatic brain injury and ischaemic stroke, where a significant reduction in oedema formation was associated with significant improvements in neurological outcome (Donkin 2006; Turner *et al* 2006; Vink and van den Heuvel 2010).

It follows then that in experimental SAH, the observed brain oedema may be caused by mechanisms that do not involve substance P, or that cellular/ cytotoxic mechanisms of oedema also play an important role. Indeed, the oedema development and the BBB disruption following SAH may not be causally related. Various SAH models point to multiple factors that trigger and facilitate blood brain barrier opening following subarachnoid haemorrhage. High ICP during induction of experimental SAH triggers blood-arterial wall barrier breakdown in large cerebral arteries (Nakagomi *et al* 1990), and probably also triggers barrier breakdown in small cerebral vessels. Clot placement models of SAH that do not produce high ICP or high ABP also cause blood-brain barrier breakdown, indicating that vasoactive blood products play an important role (Doczi 1985). Other important factors in the disruption of the BBB in SAH include occludin degradation and opening of tight junctions between endothelial cells (Doczi 1985; Yan *et al* 2008), endothelial cell pinocytosis (Doczi 1985), basal lamina degradation (Scholler *et al* 2007; Yan *et al* 2008) and apoptosis of endothelial cells (Park *et al* 2004; Yatsushige *et al* 2007). Thus the formation of oedema after SAH may not be solely dependent on the disruption of the blood brain barrier, generating what is known as classic vasogenic oedema, but rather be one of the multiple consequences. Indeed, delayed vasospasm in the absence of BBB opening is a potential cause of oedema following SAH.

7.1.2 Areas for further research

Cytotoxic oedema may thus be an important mechanism of secondary injury in the acute phase following SAH. Loss of consciousness at the ictus predicts global cerebral oedema after clinical SAH (Claassen *et al* 2002), and as brain ischaemia is a likely cause of this loss of consciousness (due to extremely high ICP during the initial bleed), the association may point towards cytotoxic mechanisms of oedema formation. Interventions targeting cytotoxic oedema may therefore show more promise in the management of SAH for some patients. In the clinical setting, multiple mechanisms of oedema formation are likely to be involved, and cytotoxic and vasogenic mechanisms of oedema formation may potentiate each other. For example, the NMDA receptor antagonist felbamate, which may be expected to ameliorate cytotoxic oedema, also ameliorates blood brain barrier disruption (assessed by evens blue extravasation) following experimental SAH (Germano *et al* 2007).

There are now several studies that have demonstrated an association between brain oedema and outcome following SAH. Global cerebral oedema is an independent predictor for death and disability following clinical SAH (Claassen *et al* 2002) and the correlation between increased brain water content and outcome that was observed in this study is consistent with that reported by others (Thal *et al* 2009a). Blockade of bradykinin B2 receptors prior to induction of rat SAH reduces brain oedema formation and improves functional outcome, possibly supporting a cause and effect relationship between oedema and outcome. Nonetheless, clinical application of this finding remains limited as animals treated post-SAH demonstrate no benefit (Thal *et al* 2009b).

In the present studies, it is uncertain if brain oedema itself was the cause of poor outcome, or if an event such as cerebral ischaemia was responsible for both oedema formation and poor outcome via separate mechanisms. For example, by acting on B2 receptors, bradykinin is known to cause smooth muscle constriction as well as blood-brain barrier opening. So B2 receptor blockade prior to SAH may have ameliorated cerebral vasospasm (thus preserving regional cerebral blood flow) in addition to preserving blood-brain barrier integrity. There are, therefore multiple mechanisms that may account for the improved outcome observed by Thal and colleagues (Thal *et al* 2009b). Similarly, the NMDA receptor antagonist felbamate, which has been shown to reduce blood brain barrier permeability and improve outcome in experimental SAH (Germano *et al* 2007), may also elicit neuroprotective effects through other mechanisms. Felbamate is an anti-epileptic drug, and so may ameliorate cortical spreading depression (CSD), as described in Chapter 1. CSD has been shown to be another important mechanism of poor outcome following SAH (Macdonald *et al* 2007).

Further studies will be useful to clarify the mechanism of oedema formation in experimental SAH and also the mechanisms underlying the secondary ICP increase. Whilst SP mediated vasogenic oedema did not appear to contribute to ICP following SAH in this study, the secondary ICP increase may have been caused by oedema formed by another mechanism, hydrocephalus or increased volume of circulating intracranial blood. As our results only describe ICP changes within 5 hours of experimental SAH, investigation of ICP over longer monitoring periods may also be useful, as different mechanisms of oedema formation are likely to be more important at different time points after the ictus.

Studies on the impact of cerebral oedema on ICP in these models may be worthwhile, using pre-treatment with a B2 receptor antagonist (known to ameliorate vasogenic oedema) (Thal *et al* 2009b), or using aquaporin-4 inhibitors (which may reduce cytotoxic cerebral oedema) (Bloch and Manley 2007). The impact of reducing CSF production on ICP in these models (eg using aquaporin-1 inhibitors or acetazolamide) may also be useful. Methods of monitoring cerebral autoregulation, by monitoring slow wave changes in ICP and ABP (Brady *et al* 2008; Jaeger *et al* 2007; Zweifel *et al* 2008) may indicate if cerebral vasodilation following the ictus contributed to the secondary ICP increase in these studies, pointing to interventions such as transient hyperventilation to maintain brain tissue oxygenation in the presence of elevated ICP (Newell *et al* 1996).

7.1.3 Implications for clinical management

The amount of oedema produced in experimental SAH is relatively modest, even in animals with profound functional deficits, suggesting that anti-oedema interventions may have limited potential to be efficacious in the management of SAH than some other acute brain injuries. In the studies that formed part of this thesis, the ICP-guided filament SAH group demonstrated the most severe functional deficits and had an increase in whole brain water content of 0.87%. Far greater increases in brain water content have been reported following experimental filament occlusive stroke (3.6%) (Turner *et al* 2006), intracerebral heamorrhage (3.2%) (Li *et al* 2009), endothelin stroke (1.3%) (Huang *et al* 2007) and brain trauma (2.2%) (Nimmo *et al* 2004). These models produced 2 to 5 times more oedema but have substantially lower mortality rates than filament SAH. However, a key feature of experimental SAH is the rapid development of intracranial hypertension, such that compensatory mechanisms, that may offset the effect of brain oedema on ICP in other

models of brain injury, are rapidly exhausted in experimental SAH. If follows that a relatively small amount of brain oedema in experimental SAH may have a substantial impact on ICP and outcome.

An important finding of the ICP studies in this thesis, is that following the peak ICP of the ictus, ICP remained elevated and increased again within the following 2 hours. These primary and secondary ICP increases were observed in every animal that was subject to SAH including injection SAH animals that demonstrated no functional deficits. The clinical implication is that all SAH patients may have elevated ICP and reduced brain compliance, including those who do not demonstrate neurological signs. SAH patients may be vulnerable to a secondary ICP increase early after the ictus and this may be a mechanism of secondary injury that is potentially preventable.

Clinical deterioration early after the ictus, before neurosurgical and neurological management has been commenced, is very common and has been attributed to aneurysmal rebleeding (Ohkuma *et al* 2001). However, the secondary ICP increase occurring in this period and is another potential source of secondary injury, as is plateau wave development. Identifying patients who have reduced brain compliance, or who are at risk of developing intracranial hypertension, is a clinical challenge that may be more readily addressed as new technologies are developed which enable non-invasive methods of monitoring ICP (Schmidt *et al* 2002).

7.2 Limitations

A limitation of the filament SAH studies detailed in this thesis is the variability of haemorrhage produced by this model. Whilst filament SAH reproduces features of human SAH, as detailed in Chapter 2, we also noted that functional deficits produced by filament SAH correlated with haemorrhage volume. The prechiasmatic cistern injection SAH model produces a similar distribution of subarachnoid blood to that produced by the filament SAH model, and injection SAH may produce functional deficits if a larger blood volume is injected. Thus, injection SAH may provide a highly reproducible model of SAH that may provide advantages for investigating the impact of interventions on functional outcome as well as ICP following SAH.

Another important limitation of this series of studies is that a single dosage regime for NAT treatment was used. The final study, detailed in Chapter 6, found that SP blockade may potentially dampen a pressor response to SAH, and this pressor response may be beneficial by elevating CPP in the early hours after the ictus. This suggests that any potential benefit that NAT treatment may exert by reducing brain oedema would be offset by a deleterious effect on CPP. Therefore, NAT administration at a later time point may have been more appropriate. Further studies are needed to clarify if SP is involved in a protective, early pressor response to SAH. However, it is interesting to note that studies of intracerebral haemorrhage (ICH) in this laboratory found that NAT demonstrated more benefit in an ICH model in which ICP possibly rose more gradually (collagenase induced ICP) but less benefit in a model in which ICP probably rose more rapidly (injection ICH) (Kleinig 2010).

7.3 Conclusions

Both experimental models of SAH used in these studies produced profound, acute increase in ICP followed by a secondary ICP elevation within 2 hours of SAH. The impact of oedema on ICP and functional outcome in these studies is unclear, although it can be concluded that oedema was not the cause of the primary increase in ICP. Vasogenic oedema was most likely present, as evidenced by an open BBB and brain water accumulation, and increased substance P immunoreactivity indicates that neurogenic inflammation may have contributed to the blood-brain barrier breakdown. However, other mechanisms of blood-brain barrier opening may have played a more important role given that treatment with the SP antagonist, NAT, did not reduce brain oedema or ICP. Whilst the amount of oedema produced in these models may have been too small for NAT to demonstrate any significant benefit, our findings that there was also no improvement in survival or functional outcome in these treated animals suggest that SP-mediated neurogenic oedema may not be an important mechanism of secondary injury in SAH. We conclude that SP does not play a critical role in the development of functional deficits following SAH and that therapies are better directed toward alternative targets

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