CHAPTER 3

CHARACTERISATION OF SUBSTANCE P RELEASE FOLLOWING TRAUMATIC BRAIN INJURY
Chapter 3

3.1 Introduction

Traumatic brain injury (TBI) is the leading cause of disability in people under 40 of age, severely disabling 150-200 people per million population annually (Fleminger and Ponsford, 2005). The cost for rehabilitation and care of these individuals to the Australian community is in the order of two million dollars annually (Authority, 1993). Despite the enormity of this public health problem, no effective treatment currently exists. It is now accepted that brain injury results in the development of neurologic deficits through two main mechanisms. Firstly, the primary event, occurring at the time of the injury and including the mechanical processes such as shearing, laceration and stretching of nerve fibres (Graham et al., 1992; Graham et al., 1996). Besides using preventive measures such as helmets, airbags and seatbelts, little can be done for this initial event. In contrast, secondary injury is made up of biochemical and physiological factors that are initiated by the primary event and manifest over time (McIntosh et al., 1996). It has been previously demonstrated that much of the morbidity following brain injury is associated with the development of the secondary injury cascade (McIntosh et al., 1996). This cascade occurs over a period of time ranging from minutes and even months after the initial event, and as such, there are opportunities for interventional pharmacology to prevent further injury and improve the resultant outcome. A number of secondary injury factors have been identified including blood brain barrier (BBB) opening, oedema formation, release of excitatory amino acids (EAAs), ion changes, oxidative stress, and bioenergetic failure, amongst others. The goal for researchers has been to identify the secondary injury factors and develop interventional therapies that attenuate, or even prevent, their occurrence.
Of all the secondary injury factors that are involved with the development of neuronal deficits, the formation of oedema has been long known to be critical to outcome following injury (Lobato et al., 1988; Sarabia et al., 1988). This is particularly the case in younger victims of TBI where formation of oedema within the brain has been found to be responsible for 50% of all death and disability (Feickert et al., 1999). Within the brain the mechanisms associated with the formation of oedema are still unclear. However, research of oedema formation in peripheral tissues (Woie et al., 1993) has demonstrated an association between neuropeptides and the development of increased vascular permeability and oedema; this event has been termed neurogenic inflammation.

Neurogenic inflammation involves the release of sensory neuropeptides, including substance P (SP), calcitonin gene related peptide (CGRP) and neurokinin A (NKA), which produces vasodilation, oedema and tissue swelling. Studies of the peripheral inflammation have shown that such neurogenic inflammation is caused by the stimulation of the slow velocity C-fibres (nociceptors), initiating the release of these sensory neuropeptides that belong to the tachykinin family (Woie et al., 1993). However, it is the SP neuropeptide that has been generally accepted to be involved in increasing microvascular permeability and to oedema formation (Newbold and Brain, 1995). Oedema formation in the brain, when left uncontrolled, results in an increase in intracranial pressure (ICP) that may lead to a decrease in brain tissue perfusion, localised hypoxia and ischemia, and even tissue herniation and death. It is therefore of particular interest to attenuate or inhibit inflammation after TBI. While a number of studies have investigated the role of classical inflammation in oedema formation following TBI (Stahel et al., 1998; Lenzlinger et al., 2001; Stein and Hoffman, 2003b;
Besson et al., 2005), only one study has examined the role of neurogenic inflammation in this process. Nimmo et al (2004) demonstrated that capsaicin administered prior to TBI significantly attenuated BBB opening, oedema formation and the development of both motor and cognitive deficits. Capsaicin is a vanilloid receptor agonist that activates neuropeptide containing sensory nerves leading to the depletion of SP, NKA and CGRP stores. Induction of TBI during the neuropeptide-depleted state clearly prevented the development of neurogenic inflammation. Although, this study demonstrated a role for neurogenic inflammation in TBI, the identification of which neuropeptide is primarily involved in the formation of increased BBB permeability and oedema formation was not established.

Previous studies of peripheral oedema have shown that SP is the neuropeptide most closely associated with capsaicin sensitivity (Saria, 1984; Yonehara et al., 1987; De and Ghosh, 1990; Laird et al., 2000). It is also well known that SP is the neuropeptide responsible for increased vascular permeability whereas CGRP is primarily associated with vasodilation. Finally, Kramer et al (2003) have demonstrated in studies of cardiac ischaemia that SP release is increased with magnesium depletion; declines in magnesium concentration have been widely described following TBI (Vink et al., 1987; Vink et al., 1988). Accordingly, the present study examines whether SP may be upregulated following TBI. This was achieved using SP immunohistochemistry on injured rat brains at 5h, 1d, 3d or 7d following injury. Injury was induced using three different models of injury to establish whether SP upregulation was a ubiquitous feature of TBI or just specific to a certain model or severity of injury. The use of confocal microscopy was used to distinguish whether SP was co-localised with a
marker for BBB permeability (Evans blue). Finally, SP release into the blood after TBI was quantitated using an Elisa assay.

3.2 Methods

3.2.1 Induction of traumatic brain injury

Injury was induced using three separate models of rodent TBI as described in detail in Chapter 2. Moderate to severe diffuse injury was induced using the impact acceleration model (Foda and Marmarou, 1994; Marmarou et al., 1994), moderate to severe focal injury was induced using the lateral fluid percussion injury (FPI) model (McIntosh et al., 1987), while mild diffuse injury was produced using the recently developed hydraulic piston model of injury (Cernak et al., 2004). Briefly, diffuse impact-acceleration injury was induced in halothane anaesthetised male Sprague-Dawley rats (400 ± 20 g) by dropping a 450 g brass weight from a height of 2m onto a 10 mm diameter x 3mm thick stainless steel disc fixed centrally to the exposed skull with polyacrylamide adhesive. The head of the animal was supported by a 10 cm foam bed, which acted to decelerate the brass weight after impact. FPI was induced in halothane anaesthetised male Sprague-Dawley rats (400 ± 20 g) by rapidly injecting a small pulse of saline into the closed cranium of the rat via a craniotomy fitted with a Leur-loc fitting. The dura was intact at the opening and the pressure of the saline pulse was 2.5 ± 0.2 atm. Finally, mild, diffuse injury was induced in halothane anaesthetised male Sprague-Dawley rats (400 ± 20 g) using the hydraulic piston model (Cernak et al., 2004). In this model, a high-velocity air pressurised piston is targeted to contact a 10 mm diameter x 3 mm thick stainless steel disc fixed centrally with polyacrylamide adhesive to the exposed skull. The rat’s head was supported using a gel cushion and displacement after contact was set at 15 mm.
All animals were fed and watered ad libitum prior to induction of injury. During surgery, induction of injury, and during the immediate recovery phase, rat rectal temperature was maintained at 37° C using a thermostatically heated warming blanket. Immediately after injury in both the impact acceleration and FP injuries, animals were manually ventilated until stable respiration was restored, usually in less than 5 minutes. There was no requirement for assisted ventilation following injury induced with the hydraulic piston model. After injury, all wounds were sutured, animals withdrawn from anaesthia and returned to their cages following recovery.

### 3.2.2 Histology and Immunohistochemistry

For SP immunohistochemistry, 3 animals per group were injured and perfusion fixed at preselected time points. For the initial characterisation of SP immunoreactivity in the impact acceleration and FP, rats were perfusion fixed using 4% paraformaldehyde at 5h, 24h, 3d and 7d. An additional 3 animals per model were surgically prepared but not injured (shams). Having established the points of maximum SP immunoreactivity in both diffuse and focal injury, only two time points (24 h and 3d; n=3 per time point) were examined following induction of mild diffuse injury using the hydraulic piston model. All brains were perfusion fixed using paraformaldehyde and stored in 10% formalin. The brains were subsequently stained using SP antibody (Santa Cruz; 1:2000 dilution) using the antigen retrieval method as described in detail in Chapter 2.

### 3.2.3 Determination of blood brain barrier permeability

To examine the relationship between SP and BBB opening, a further 3 animals were injured using the impact acceleration model and injected with Evans blue (Sigma) (EB). EB binds to the plasma protein albumin and its appearance outside of the
vasculature indicates an increase in BBB permeability. EB (2ml/kg of 4%) was administered iv. to the animals 4.5 h after injury and left to equilibrate for a period of 30 min. This time point was chosen on the basis of previous studies that have demonstrated that the BBB permeability is maximal at 5 h after impact acceleration TBI (O'Connor et al., 2003a). At 5 h after TBI, animals were transcardially perfused with saline, their brains removed and stored in paraformaldehyde overnight. Consecutive 30μm coronal sections were cut using the vibratome and stained using the SP antibody (Santa Cruz; 1:200 dilution). Sections were examined using a confocal fluorescence microscope (BioRad Radiance 2100). As previously described by (Maier et al., 2004).

3.2.4 ELISA assay

Quantitative assessment of SP in blood plasma was assessed using a SP Elisa assay (R&D Systems, Cat no. DE1400), as described in detail in chapter 2. Male, Sprague-Dawley rats (400 ± 20 g; n=6) were injured using the impact acceleration model of TBI as described above. At 30 min and 5 hrs after injury, 1 ml of blood was collected from the tail vein. A further 6 animals served as uninjured sham controls. All blood samples were placed in ice and spun down within 5 minutes after injury. After collection, plasma samples were stored at –80°C until use.

3.2.4 Statistical Analysis

Data was analysed for statistical significance using one-way analysis of variance (ANOVA) followed by Student Neuman-Keuls tests (GraphPad Prism Software).
3.3 Results

3.3.1 Impact-Acceleration Injury (Weight-drop)

The cortex of sham animals (Fig 3.1) shows light SP immunoreactivity perivascularly and also within the parenchyma. Within the parenchyma, slight immunoreactivity can be observed on neuronal processes. At 5 h after injury (Fig 3.2A), there is an unequivocal upregulation of SP immunoreactivity with dense granulation noted along perivascular fibres, as well as along neuronal processes within the parenchyma. This time point has been previously shown to correlate with maximal formation of vasogenic oedema and opening of the BBB (O'Connor et al., 2003a). This pattern of increased SP immunoreactivity was still evident at the 24 h time point (Fig 3.2B), with perivascular and parenchymal neuronal fibres demonstrating strong evidence of immunoreactivity. At 3 days (Fig 3.2C), there was a qualitative decrease in immunoreactivity of SP in all areas, which by 7 days post-TBI (Fig 3.2D), had returned to levels similar to that observed in sham animals.

Since previous studies have shown that maximal opening of the BBB occurs at 5 h after impact acceleration injury (O'Connor et al., 2003a), and our immunohistochemistry shows clear perivascular SP upregulation at 5 h, we examined the association between Evans blue (EB) extravasation and SP immunoreactivity at this time point using confocal microscopy. At 4.5 h after injury, animals were administered an iv bolus of EB which was allowed to equilibrate for just 30 min before animals were decapitated and their brains removed for analysis. Confocal images of sham animals (Fig 3.3A) showed that there was slight SP immunoreactivity, consistent with the light
**Figure 3.1** SP stained section from male Sprague-Dawley rats after sham surgery. Sham treatment resulted in minimal SP immunoreactivity perivascularly and within the peranchyma.
**Figure 3.2** SP stained section from male Sprague-Dawley rats following the weight-drop model of diffuse TBI. (A) An unequivocal upregulation of SP immunoreactivity along the vessel (centre) and along the processes and within the parenchyma 5h after injury (B) Staining for SP was strong within the endothelial cells of the vessel and along some processes 24h after injury (C) Marked decrease in SP intensity at 3 days after injury (D) Still marked decrease in intensity of SP when compared with the earlier timepoints at 7 days after injury.
immunohistochemistry described above (Fig 3.1). Confocal images of EB in sham animals showed almost no fluorescence (Fig 3.3B), consistent with an intact BBB before injury. At 5h after injury, there was clear positive fluorescence for SP (Fig 3.3C), with granular deposits clearly defined along the vessel walls, and delineating the vessel, as well as in the parenchyma. This is consistent with that observed above with light immunohistochemistry. EB reactivity in the same slice of the 5h-injured brain (Fig 3.3D) also shows a marked increase in fluorescence along the vessel walls, again clearly delineating the vessel. Superimposing both SP and EB images (Fig 3.4) shows clear co-localization (purple) of fluorescence, implying that SP may play a role in the opening of the BBB following injury.

Given the high level of perivascular SP immunofluorescence at 5 h after TBI, it was of interest to establish whether SP is released into the bloodstream after TBI. Accordingly, we used a competitive Elisa assay, based on the competitive binding technique in which SP present in plasma samples competes with a fixed amount of alkaline phosphatase-labelled SP for sites on a rabbit polyclonal antibody at 30 min and 5 h after injury. At 30 min after injury, there was a significant increase (p < 0.01) in SP concentration to almost double the basal level observed in sham animals (Fig 3.5). These elevated levels of SP in the blood declined to sham levels by 5h after injury, suggesting that SP is released into the blood in large quantities immediately after injury and that this release may be associated with the early, but transient (<6 h), opening of the BBB in this model of TBI.
**Figure 3.3** Confocal imaging from the rat cortex (A) SP staining and (B) EB staining from male, sham treated Sprague-Dawley rats. Sham treatment resulted in minimal fluorescence (C) SP staining 5h after moderate/severe TBI and (D) EB staining from male Sprague-Dawley rats 5h after moderate/severe diffuse TBI. Injury resulted in distinct delineation of a cortical vessel as shown from the red fluorescence (SP = 488nm) and blue fluorescence (EB = 650nm). (Bar = 60 µm)
Figure 3.4 Confocal imaging from a male Sprague-Dawley rat 5h after injury. Injury results in co-localisation of both SP and EB at a cortical vessel. (Bar = 60 µm).
**Figure 3.5** Plasma concentration levels of SP in male Sprague-Dawley rats following diffuse TBI. 30 min after injury resulted in a significant increase in SP levels relative to sham treated animals as assessed by ELISA assay (n = 6 per group). **p < 0.001** relative to sham animals.
3.3.2 Lateral Fluid percussion injury

The cortex of sham animals (Fig 3.6) shows light SP immunoreactivity perivascularly and also within the parenchyma. Within the parenchyma, faint immunoreactivity can be observed on neuronal processes. At 5 h after injury (Fig 3.7A), there is an unequivocal upregulation of SP immunoreactivity with intense granulation noted along perivascular fibres. Prominent SP staining of cortical pyramidal neurons as well as along neuronal processes within the parenchyma is observed at the lesion site. These results are similar to those described above for impact-acceleration injury (section 3.3.1). The pattern of increased SP immunoreactivity was still evident at the 24 h time point (Fig 3.7B), with parenchymal neuronal fibres demonstrating strong evidence of immunoreactivity. At 3 d (Fig 3.7C) post-TBI, SP immunoreactivity had decreased, and unlike in impact acceleration injury, gliosis and possible microglial/macrophage accumulation was observed. The gliosis was increased considerably by 7 d post-trauma (Fig. 3.7D), while the SP immunoreactivity continued to decrease.

3.3.3 Impact-acceleration Injury (Hydraulic Piston)

As seen in all of the other sham animals, the cortex of sham animals for the hydraulic piston model of injury (Fig 3.8A) shows light SP immunoreactivity perivascularly and also within the parenchyma. At 24 h after injury (Fig 3.8B), there was still positive immunoreactivity in the perivascular and the parenchyma of 24h injured brains despite the induction of a much milder injury. After 3 d (Fig 3.8C) a similar pattern of immunoreactivity was still observed, although to a lesser degree then previously shown in the impact-acceleration injury (section 3.3.1), suggesting that there may be a graded level of SP release dependant upon severity of the insult. This is consistent with
Figure 3.6 SP stained section from male Sprague-Dawley rats after sham surgery (i.e. craniotomy performed but no injury given) Sham treatment resulted in minimal SP immunoreactivity perivascularly and within the parenchyma. (Bar = 100 µm)
**Figure 3.7** SP stained section from male Sprague-Dawley rats after moderate/severe lateral fluid percussion TBI. (A) An unequivocal upregulation of SP immunoreactivity perivascularly and along certain processes. Also, within the parenchyma 5h after injury (B) Staining for SP was evident around pyramidal neurons and processes 24h after injury. (C) Marked decrease in SP intensity at 3 days after injury with an increased proliferation of glia cells directly below the injury site (D) Marked decrease in SP immunoreactivity at 7 days post-injury with gliosis and microglial/macrophage accumulation prominent.
**Figure 3.8** SP stained section from male Sprague-Dawley rats after mild diffuse TBI. (A) Sham treatment resulted in minimal SP immunoreactivity perivascularly and within the peranchyma. (B) Upregulation of SP evident perivascularly and perineuronal at 24hr. (C) Evidence of SP immunoreactivity at 3days although a decrease compared to 24hr after injury.
previous studies that have shown that SP release is proportional to the intensity and frequency of stressful stimuli (Mantyh, 2002).

3.4 Discussion

In this study we have shown that the neuropeptide SP is upregulated following TBI, irrespective of whether TBI was induced using a diffuse or focal model of injury, or whether the injury severity was mild, moderate or severe. We are left to conclude that increased SP immunoreactivity is a ubiquitous feature of TBI. SP was particularly apparent in the perivascular nerve fibres, cortical neurons, neuronal processes and within the parenchyma of injured animals. The upregulation of the perivascular SP positive fibres implies a potential role in the breakdown of the cerebral cortical vasculature that follows TBI. This has been supported by recent work that suggests an important role for tachykinins in BBB permeability following ischaemic and posttraumatic cerebral injury (Bruno et al., 2003; Nimmo et al., 2004). The perivascular nerve fibres may be the source of SP release following TBI, similar to that which has been shown to occur following clinical cerebral ischaemia (Bruno et al., 2003). In these studies, serum SP levels were significantly greater than controls, with higher levels being observed at 12 hr following the clinical onset of cerebral ischemia compared to 24 hr.

In our studies, maximal levels of SP immunoreactivity were observed for up to 24h, followed by a gradual decline over the following 6 days. These immunohistochemistry results are temporally correlated with oedema formation, which has been shown to occur in two phases. Initially there is a vasogenic phase at 4-5 h following TBI (Albensi et al., 2000; Beaumont et al., 2000) associated with increased BBB
permeability (Barzo et al., 1996; Vink et al., 2003a), followed by the gradual development of cytotoxic oedema, which can persist for a number of days after injury. Quantitatively, SP levels were measured from plasma at 30 min and 5 h after injury using a competitive Elisa assay. Results show a significant increase in SP levels shortly after (30 min) TBI relative to sham treated animals, followed by a return to pre-injury levels at 5 h after injury. This finding suggests that SP is released immediately after injury and may be associated with a resultant opening of the BBB. By 5 h, SP levels in the blood have returned to normal values although BBB permeability remains dysfunctional. The temporal association of early increases in blood SP, together with the increased brain SP immunoreactivity, with increased BBB permeability and vasogenic oedema formation implies that SP may have a potential role in these events.

Having characterised an increase in brain SP immunoreactivity, and release into the blood, following injury, the next step is to determine a way of inhibiting its action. SP is a neuropeptide preferentially binding to the neurokinin-1 (NK1) receptors. Recent work has shown NK1 receptors to be widespread in the cortex, hippocampus, amygdala and other areas (Tooney et al., 2000). There is therefore an opportunity to ascertain whether administration of a NK1 receptor antagonist decreases BBB permeability and oedema formation. This is the subject of the following chapter.
CHAPTER 4
CHARACTERISATION OF n-ACETYLM L-TRYPTOPHAN
IN TRAUMATIC BRAIN INJURY
4.1 Introduction

In the previous chapter we showed a significant upregulation of SP following TBI, particularly on perivascular fibres and within the parenchyma. The fact that SP immunoreactivity was present in these areas shortly after injury (5 - 24 h) suggests that the neuropeptide may have a role in increased BBB permeability and formation of oedema. This neurogenic inflammation, putatively mediated by the binding of SP to tachykinin receptors, has been previously shown to be associated with oedema formation and functional deficits following injury (Vink et al., 2003b; Nimmo et al., 2004).

The tachykinin receptors are a family of G-protein coupled receptors and include neurokinin-1 (NK₁), neurokinin-2 (NK₂) and neurokinin-3 (NK₃) (Buck et al., 1986; Advenier and Emonds-Alt, 1996). SP has the greatest affinity for the NK₁ receptor (Lew et al., 1990; Quartara and Maggi, 1997), which are widely expressed in the mammalian periphery and central nervous system (Quartara and Maggi, 1998). In the cardiovascular system, NK₁ receptors mediate endothelium dependent vasodilation and plasma protein extravasation, whereas in the respiratory system they mediate neurogenic inflammation upon exposure of the airways to irritants (Quartara and Maggi, 1998). Knockout of the NK₁ receptors in mice results in near complete protection from immune complex inflammation in the lung (Bozic et al., 1996; Yonehara and Yoshimura, 2000), suggesting that pharmacological antagonism of the receptors may similarly inhibit neurogenic inflammation.

A number of studies have shown that the use of a NK₁ receptor antagonist can be beneficial to outcome. Specifically, Pothoulakis et al (Pothoulakis et al., 1994)
demonstrated that pre-treatment with a NK₁ antagonist significantly inhibited the effects of an inflammatory toxin within the ilium of rats. Rupniak and Kramer (Rupniak and Kramer, 1999) have described the efficacy of NK₁ antagonists in the treatment of experimental depression and emesis, while Ranga and Krishnan (Ranga and Krishnan, 2002) first published the use of the NK₁ antagonist MK-0869 in the treatment of clinical depression and anxiety. A similar beneficial role for NK₁ antagonists has been suggested by Nimmo et al (2004) (Nimmo et al., 2004), who showed that neuropeptide-depletion prior to induction of TBI attenuated the development of neurogenic inflammation. Whether NK₁ antagonists can prevent the BBB opening and vasogenic oedema formation associated with TBI has not yet been demonstrated.

The current chapter will confirm the co-localisation of SP with NK₁ receptors, and then characterise the effects of the NK₁ antagonist, n-acetyl L-tryptophan (NAT) (MacLeod et al., 1993), on BBB permeability following TBI using the extravasation of Evans blue (EB) to determine the dose optimum (Mikawa et al., 1996; Kaya et al., 2001). Having established the optimum dose for NAT, we will then characterise the effects of NAT on brain oedema, brain free Mg²⁺ concentration, SP immunoreactivity, and aquaporin-4 (AQP-4) immunoreactivity.

4.2 Methods

4.2.1 Induction of traumatic brain injury

Animals were injured using either the weight drop or hydraulic piston models of diffuse brain injury as described in detail in Chapter 2. Both models of acceleration injury have been previously shown to produce similar degrees of oedema, BBB
opening, Mg$^{2+}$ decline and histological changes (Cernak et al., 2004).

Briefly, diffuse impact-acceleration injury was induced in halothane anaesthetised male Sprague-Dawley rats (400 ± 20 g) by dropping a 450 g brass weight from a height of 2m onto a 10 mm diameter x 3mm thick stainless steel disc fixed centrally to the exposed skull with polyacrylamide adhesive. The head of the animal was supported by a 10 cm foam bed, which acted to decelerate the brass weight after impact. For nuclear magnetic resonance studies, adult male Sprague-Dawley rats (n=12; 400 ± 20 g) were injured using the hydraulic piston impact acceleration model of diffuse TBI. Rats were anaesthetised with halothane and injury induced using a hydraulically controlled, high-velocity impactor to contact a 10mm diameter x 3mm thick steel disc fixed centrally between the bregma and lambda sutures using polyacrylamide adhesive. The head is decelerated after impact using a moulded, gel-filled base upon which the animals head is supported during injury. In the current experiments, the impactor travelled a distance of 18 mm after impact, which results in moderate to severe injury.

### 4.2.2 n-acetyl L-tryptophan dose – response

The optimal dose of the NK$_1$ receptor antagonist was determined from the level of BBB permeability using EB extravasation as the BBB marker, as previously described in detail elsewhere (Mikawa et al., 1996; Kaya et al., 2001). Briefly, EB dye (Sigma cat no; E-2129; 2mL/kg of 4%) was administered intravenously at 4.5 h after impact acceleration induced TBI and left to equilibrate for 30 min. At 5 h after TBI, animals were transcardially perfused with saline to remove intravascular EB dye and decapitated. After decapitation, the brain was removed and the cortex separated and weighed, and then homogenised. After the protein was precipitated with trichloroacetic
acid, samples were cooled and then centrifuged so that the supernatant could be assessed for EB concentration using a spectrophotometer.

The NAT was administered intravenously at 30 min after TBI at doses ranging between 0.0025 mg/kg to 25 mg/kg in 4 steps. This range was based upon the previously published work of Towler et al (1998) (Towler et al., 1998).

4.2.3 Oedema

The amount of brain water was calculated using the wet weight-dry weight method. Animals were separated into 3 groups either treated with a vehicle 30 min post-TBI, treated with NAT 30 min post-TBI or were treated as sham controls. They were then re-anaesthetised with 4% halothane at pre-selected timepoints and decapitated. The brain was removed rapidly from the skull, the olfactory bulbs and cerebellum discarded and the cortex and sub-cortex separated. The cortex and sub-cortex of each rat were placed separately into pre weighed and labelled glass vials with quick fit lids (to prevent evaporation) and weighed immediately for wet water content. The vials (glass lids removed) were then placed in an oven for at 100°C for 72 hrs. Vials and brain segments were then re-weighed to obtain dry weight content. Oedema in each brain sample was calculated using the wet/dry method formula:

\[
% \text{ Water} = \frac{(\text{Wet Weight} – \text{Dry Weight}) \times 100}{\text{Wet Weight}} \tag{1}
\]
4.2.4 Nuclear magnetic resonance studies

*Magnetic resonance imaging (MRI)*

At 5h and 24 h, after TBI, animals (n=6/group) were re-anæsthetised with halothane and subject to magnetic resonance imaging examination as previously described (Cernak et al., 2004). Briefly, animals were placed in the heated plexiglas holder and a respiratory motion detector positioned over the thorax to facilitate respiratory gating. The plexiglas holder was then positioned in the centre of the magnet bore where a 72 mm proton tuned birdcage coil had been positioned. Field homogeneity across the brain was then optimised, and sagittal and coronal high resolution images were acquired with a multislice-multiecho pulse sequence or a rapid acquisition relaxation enhancement pulse sequence; TR 2 s, TE 30/60 ms, field of view of 4 cm, a matrix of 512 x 512, and a slice thickness of 1 mm. Five to seven coronal slice were obtained per animal, which were positioned precisely using a sagittal pilot scan and by measuring a known distance from the anterior pole of the frontal lobe to an index slice. Multi-slice, multi-echo T2-weighted images were acquired to obtain eight contiguous slices from the dorsal end of the olfactory bulb to rostral margin of cerebellum: FOV = 4 cm x 4 cm, slice thickness = 2 mm, resolution 256 x 256, TR/TE = 2000/20 ms, 4 echo images, and 2 averages. Diffusion weighted images (DWI) were then acquired with a spin echo pulse sequence that had diffusion gradients added before and after the refocusing pulse. Gradient strength was varied in six steps using sensitization values ranging from 20 to 1000 s/mm². A 256 x 256 matrix was used with a 3 cm field of view, TR 2.0 s, TE 502 msec, slice thickness of 2 mm and 4 echoes. Diffusion maps were generated by applying the Stejskal-Tanner equation in association with a Marquart algorithm using a commercially available Paravision software (Bruker, Billerica, MA, USA). Apparent diffusion coefficients (ADCs) were calculated for four
regions: left cortex, right cortex, left subcortex and right subcortex. ADC were expressed as $10^{-5}$ mm$^2$/sec ± SEM.

**Phosphorus magnetic resonance spectroscopy**

Animals used in MRI experiments were also subject to phosphorus magnetic resonance spectroscopy at 4 h post-trauma as previously described in detail elsewhere (Heath and Vink, 1999). Briefly, animals were placed in a specially constructed, temperature controlled plexiglas holder and a 5 mm x 9 mm surface coil was placed centrally over the exposed skull. Skin and muscle were retracted well clear of the coil to prevent contributions from these tissues. The animals was then inserted into the centre of a 7.0 Tesla magnet interfaced with a Bruker spectrometer and field homogeneity optimized on the water signal prior to acquisition of phosphorus spectra. Phosphorus spectra were obtained in 20 min blocks using a 90° pulse calibrated for a 2 mm cortical depth, a 700 msec delay time, and a 5,000 Hz spectral width containing 2048 data points. Rectal temperature and respiration was monitored at all times. The anaesthesia was maintained using halothane.

Phosphorus magnetic resonance spectra were analyzed using the resident Bruker computer software program. Following convolution difference (400/20 Hz), chemical shifts and integrals of the individual peaks were determined following line fitting. Intracellular pH and brain free Mg$^{2+}$ concentrations were then determined as described in detail elsewhere (Heath and Vink, 1999). Briefly, intracellular pH was determined from the chemical shift of the inorganic phosphate peak ($\delta$Pi) relative to phosphocreatine (PCr) in the magnetic resonance spectra using the equation
\[ pH = 6.77 + \log \left( \frac{\delta P_1 - 3.29}{5.68 - \delta P_1} \right) \]  

Similarly, free Mg\(^{2+}\) concentration was determined from the chemical shift difference between the \(\alpha\) and \(\beta\) peaks of ATP using the equation

\[ [Mg^{2+}] = K_d \left( \frac{10.82 - \delta_{\alpha-\beta}}{\delta_{\alpha-\beta} - 8.35} \right) \]

where \(\delta_{\alpha-\beta}\) is the chemical shift difference between the \(\alpha\) and \(\beta\) peaks of ATP. The \(K_d\) for MgATP was initially assumed to be 50 \(\mu\)M at pH 7.2 and 0.15 M ionic strength and were corrected for pH according to Bock et al (1987). (Bock et al., 1987).

### 4.2.4 Histology and Immunohistochemistry

**Confocal**

For confocal microscopy, 10\(\mu\)m thick paraffin sections from 5 h injured brains were examined using double immunofluorescent staining with the SP antibody (Santa Cruz; 1:200 dilution) and the NK\(_1\) receptor antibody (Advanced Targeting Systems; 1:150 dilution). Sections were incubated overnight in the primary antibodies, then the secondary antibodies for 1 hour. Secondary antibodies used were an Alexa fluor 488 donkey anti-goat (Molecular probes; 1:200 dilution) for SP and Alexa fluor 660 goat anti-rabbit (Molecular probes; 1:200 dilution). After washing in PBS, slides were mounted using an aqueous mounting solution with antifade and sealed using varnish. Positive and negative controls were routinely used for all antibodies. Sections were examined using a confocal fluorescence microscope (BioRad Radiance 2100).
Light immunohistochemistry

For SP immunohistochemistry, 3 animals per group were injured and perfusion fixed at preselected time points. Having established in chapter 3 the points of maximum SP immunoreactivity in the diffuse impact-acceleration injury, only three time points (5 h, 24 h and 3d; n=3 per time point) were examined following induction of moderate diffuse injury and treatment. All brains were perfusion fixed using paraformaldehyde and stored in 10% formalin. The brains were subsequently stained using SP antibody (Santa Cruz; 1:2000 dilution) and examined using a Leica light microscope.

Electron microscopy

For aquaporin studies, 3 injured animals were perfusion fixed at 5 h after TBI with 4% paraformaldehyde fixative in 0.1M-phosphate buffer, pH 7.4, and the brains collected. Vibratome sections were cut and immunolabelled for AQP-4 using a rabbit polyclonal antibody (Alpha Diagnostic International, Inc., San Antonio) at 1/1000 or 1/2000 dilutions in normal goat serum (NGS) overnight. Immunolabelling was completed using biotinylated secondary antibody (goat anti-rabbit IgG) and streptavidin-peroxidase complex (1:1000, Rockland, Gilbertsville, PA). The peroxidase reaction product was developed using liquid DAB and substrate-chromogen system (DakoCytomation, CA) for 10 min. Rectangles of tissue were cut from the parietal lobe and pons from immunolabelled vibratome sections, osmicated and processed in resin, and ultrathin sections examined with the electron microscope for the detection of peroxidase reaction product.
4.2.5 Statistical analysis

All data are expressed as mean ± SEM. Data was analysed using one way analysis of variance (ANOVA) followed by Student Neuman Keuls tests (Prism software; Graphpad). A p value of < 0.05 was considered significant.

4.3 Results

4.3.1 confocal microscopy

To establish that NK₁ receptors are present in rat brain, and that SP co-localises with these receptors, confocal microscopy was used to analyse SP and NK₁ fluorescence from the same section of injured cortex 5 h post-TBI. The SP antibody (Fig 4.1A) demonstrated fine and punctuate staining perivascularly, consistent with confocal and light microscopy images from the previous chapter. NK₁ receptor activity from the same slice of brain (Fig 4.1B) also shows clear fluorescence along the vessel wall with intense granular deposits. Superimposing both SP and NK₁ images (Fig 4.1C) shows clear co-localisation (orange) of fluorescence, implying an association of SP with the NK₁ receptor along the vasculature. The vascular co-localisation of SP and the NK₁ receptors supports a potential role in regulation of BBB function.

4.3.2 n-acetyl L-tryptophan optimal dosage

Previous studies have established that impact acceleration injury results in an increased permeability of the BBB (Vink et al., 2003b). Having characterised the vascular co-localisation of SP with the NK₁ receptors, and given their potential role in increasing vascular permeability (Nimmo et al., 2004), we examined the effects of different doses of NAT on BBB permeability following injury (Fig 4.2). EB extravasation into the brain tissue at 5 h following injury was significantly increased (0.0387±0.0005 µg/mg
Figure 4.1 Confocal imaging of cortex from male, Sprague-Dawley rats after diffuse TBI (A) Fine and punctuate SP staining perivascularly 5h after injury (B) NK₁ receptor staining evident perivascularly with granular fluorescence 5h after injury. (C) Injury results in co-localisation of both SP and the NK₁ receptor perivascularly (orange). (Bar = 100 µm)
Figure 4.2 EB brain tissue concentration in male Sprague-Dawley rats following diffuse TBI. Injury resulted in a significant increase of EB within the brain tissue 5hr after injury. A concentration of 2.5mg/kg was determined to be the optimum dosage for n-acetyl L-tryptophan. *** p < 0.001 compared with sham levels.
in brain tissue) relative to shams. Treatment with the NK$_1$ receptor antagonist significantly attenuated EB extravasation after injury with the optimal dosage at 30 minutes post-trauma being 2.5 mg/kg.

4.3.3 Nuclear magnetic resonance studies

Having established the dose optimum for NAT, its effects on oedema formation following TBI was assessed using MRI. Qualitatively, ADC maps derived from DWI show bright areas of hyperintensity throughout the brain of vehicle treated rats (Fig 4.3B) compared to that in naïve animals (not surgically prepared or injured; Fig 4.3A). This hyperintensity reflects less restriction for water diffusion, consistent with increased extracellular water. This hyperintensity is unlikely to be due to loss of diffusion barriers (membrane breakdown) given that the images were acquired at between 4 and 5 h after injury. Thus, the hyperintensity is thought to reflect vasogenic oedema formation, consistent with previous DWI results (Hanstock et al., 1994; Nimmo et al., 2004). In contrast, animals treated with the NK$_1$ receptor antagonist at 30 min after TBI did not exhibit any hyperintensity throughout the brain, suggesting an attenuation of oedema formation after injury compared to vehicle treated animals (Fig 4.3C).

Quantitatively, the ADC differences are shown in Fig 4.3D. Naïve and sham treated animals demonstrated similar mean ADC values in all regions of the brain (72.63±1.09 and 74.8±2.68 x 10$^{-5}$ mm$^2$/sec respectively), which is similar to previously published results (Hanstock et al., 1994; Albensi et al., 2000). After injury, there was a significant increase (p < 0.001) in ADC values in vehicle treated animals (88.5±2.67 x
Figure 4.3 (A) Apparent diffusion coefficients (ADC) maps derived from the diffusion weighted magnetic resonance images obtained from naïve male Sprague-Dawley rats. Naïve treatment resulted in negative water movement while (B) following diffuse TBI areas of subcortical hyperintensity indicates the presence of vasogenic oedema. (C) When treated with NAT this hyperintensity is decreased and oedema is resolved. (D) ADC obtained from the brain following naïve, sham, moderate TBI and moderate TBI + treatment in rats; *** p < 0.001 compared to sham control values.
10^{-5} \text{ mm}^2/\text{sec}) compared to sham treated animals, confirming an increased ADC for water, and consistent with vasogenic oedema formation. This increase was significantly inhibited (p < 0.001) in the NAT treated animals (69±6.7 \times 10^{-5} \text{ mm}^2/\text{sec}), which were not significantly different from sham animals.

4.3.4 Effects of NAT on Brain water content

The changes in oedema described using MRI were confirmed using wet weight/dry weight determinations of brain water content (Fig 4.4). The percentage of water content in sham animals (77.8±0.2\%) was consistent with those previously published (Kita and Marmarou, 1994; Bareyre et al., 1997; Nimmo et al., 2004). At 5 h after injury, vehicle treated animals had a significantly increased level of brain water content (80±0.4\%; p < 0.001) indicating the development of oedema following diffuse TBI. Treatment with NAT 30 min post-TBI led to a reduction of oedema at 5 h following injury (78.5±0.2\% p < 0.001) compared to vehicle controls. This water content after NAT treatment was not significantly different from sham values.

4.3.5 Effects of NAT on blood brain barrier permeability

The observed reduction in oedema was associated with a significant reduction in BBB permeability as shown by the penetration of EB dye 5 h following injury (Fig 4.5). In sham animals, the negligible penetration of the EB dye (0.0028 EB/mg) confirmed that the BBB was intact despite induction of anaesthesia and surgery. At 5 h after injury, a significant increase (p < 0.001) in EB dye (0.0387 EB/mg) was detected indicating increased BBB permeability. In contrast, NAT treatment resulted in a profound reduction in EB penetration (0.0058 EB/mg) compared to vehicle controls (p < 0.001). Since the increase in BBB permeability was associated with the increased brain water
Figure 4.4 Percentage cortical water content in male rats 5h following moderate/severe TBI and treated with NAT. Data are the means ± SEM (n=6 per group) (** p < 0.01 compared to sham levels).
Figure 4.5 The level of BBB permeability in male Sprague-Dawley rats as determined via EB penetration into the brain tissue. Moderate diffuse TBI resulted in a significant increase in EB concentration within the brain tissue, while following treatment the level of EB penetration returns back to pre-injury levels; ** p < 0.01 compared to sham levels.
content shortly after injury (4-5 h), the oedema is thought to represent vasogenic oedema (Beaumont et al., 2000). These results are consistent with the DWI results shown above. Finally, the impact of treatment with the NK₁ receptor antagonist following injury implies that release of neuropeptides, and formation of neurogenic inflammation may play a significant role in these events.

4.3.6 Effects of NAT on Brain Free Mg²⁺

Effects of the NK₁ receptor antagonist on brain free Mg²⁺ concentration are shown in Fig. 4.6. From the phosphorus MR spectrum (Fig 4.6A), brain pH in all animals prior to injury was 7.13 ± 0.04 while brain intracellular free Mg²⁺ was 0.51 ± 0.05 mM. After injury, brain ATP concentration and pH did not change significantly in either vehicle treated animals, or animals treated with the NK₁ receptor antagonist. In contrast, there was a significant decline (p < 0.05) in brain intracellular free Mg²⁺ concentration to 0.27 ± 0.02 mM in vehicle treated control animals by 4 h post-TBI (Fig 4.6B). These values are consistent with previous results in rodent TBI (Heath and Vink, 1999; Vink and Cernak, 2000; Cernak et al., 2004). Animals administered NAT at 30 min post-TBI demonstrated a brain intracellular free Mg²⁺ concentration of 0.47 ± 0.06 at 4 h after injury which was significantly greater (p < 0.05) that that recorded in saline treated control animals, but not significantly different from the pre-injury brain free Mg²⁺ concentration.

4.3.7 Immunohistochemistry

Immunohistochemical analysis of tissue following administration of NAT is shown in Fig. 4.7-4.9. At 5 h after injury, the cortex from sham operated animals (Fig 4.7A)
Figure 4.6A Phosphorus MR spectrum displaying the effects of NAT on brain free magnesium concentration. Brain pH in all animals prior to injury was 7.13 ± 0.04 while brain intracellular free magnesium was 0.51 ± 0.05 mM. After injury, brain ATP concentration and pH did not change significantly in either vehicle treated animals, or animals treated with the NAT.
Figure 4.6B Brain intracellular free magnesium concentration following diffuse TBI in male Sprague-Dawley rats. Injury resulted in a significant decline ($p < 0.05$) in brain intracellular free magnesium concentration to $0.27 \pm 0.02$ mM in vehicle treated control animals by 4 h post-TBI. Following treatment with NAT, brain intracellular free magnesium concentration was recorded as $0.47 \pm 0.06$ at 4 h after injury which was significantly greater saline treated control animals; * $p < 0.05$ compared to sham levels.
Figure 4.7 SP stained section from male Sprague-Dawley rats after moderate/severe diffuse TBI. (A) Sham treatment resulted in minimal SP immunoreactivity perivascularly and within the perichyma. (B) An unequivocal upregulation of SP immunoreactivity along the vessel (centre) and along the processes and within the parenchyma 5h after injury (C) treatment with NAT 30 min post-TBI resulted in a marked decrease in SP immunoreactivity. (Bar = 100 µm)
show light SP immunoreactivity perivascularly and also within the parenchyma. Within the parenchyma, slight immunoreactivity can be observed on neuronal processes. At 5 h after injury (Fig 4.7B), there is an unequivocal upregulation of SP immunoreactivity with dense granulation noted along perivascular fibres, as well as along neuronal processes within the parenchyma. This time point has been previously shown to correlate with maximal formation of vasogenic oedema and opening of the BBB (O'Connor et al., 2003a). Following the administration of NAT (Fig 4.7C), there was a marked reduction in SP immunoreactivity, perivascularly and in the parenchyma, which had levels of SP immunoreactivity similar to that observed in sham animals.

The pattern of increased SP immunoreactivity was still evident at the 24 h time point (Fig 4.8B), with perivascular and parenchymal neuronal fibres demonstrating strong evidence of immunoreactivity, which with the administration of the NK₁ receptor antagonist showed areas of reduction (Fig 4.8C).

At 3 days (Fig 4.9B), there was a qualitative decrease in immunoreactivity of SP in all areas, although treatment at this time point post-TBI demonstrated the levels of SP immunoreactivity had returned to that seen in vehicle controls (Fig. 4.9B), implying that the affects of the bolus administration of NAT at 30 min post-TBI had decreased by that time point.

Aquaporin channels have been implicated in oedema formation, particularly AQP-4 in the development and resolution of vasogenic brain oedema (Papadopoulos et al., 2004). To determine the effect of the NK₁ receptor antagonist on aquaporin channels, electron
Figure 4.8 SP stained sections from male Sprague-Dawley rats after moderate/severe diffuse TBI. (A) Sham treatment resulted in minimal SP immunoreactivity perivascularly and within the parenchyma. (B) Staining for SP was strong within the endothelial cells of the vessel and along some processes 24h after injury (C) Treatment with NAT 30 min post-TBI resulted in a marked decrease in SP immunoreactivity. (Bar = 100 µm)
Figure 4.9 SP stained sections from male Sprague-Dawley rats after moderate/severe diffuse TBI. (A) Sham treatment resulted in minimal SP immunoreactivity perivascularly and within the parenchyma. (B) Staining for SP was qualitatively decreased 3days after injury (C) Treatment with NAT 30 min post-TBI resulted in a clear increase in SP immunoreactivity. (Bar = 100 µm)
**Figure 4.10** AQP-4 staining within the perivascular astrocyte processes at 5hr following diffuse TBI. (A) Sham operation showed an upregulation within the astrocyte end-feet process surrounding the capillary vessel (B) Injury results in a qualitative decrease in AQP-4 staining while (C) NAT-treated animals showed an intense staining within the astrocyte end foot processes surrounding the capillary.
microscopy was utilised to examine perivascular AQP-4 immunoreactivity. In sham animals, electron dense reaction product was present in astrocytic processes surrounding brain microvessels at the astrocyte-endothelial cell interface indicative of AQP-4 immunolabelling (Fig. 4.10A). After injury, there was a profound reduction or loss of perivascular immunoreactivity (Fig. 4.10B), indicating a loss of AQP-4 water channels from the membrane surface. In contrast, treatment with the NK₁ receptor antagonist resulted in an increase or preservation of perivascular labelling (Fig. 4.10C).

4.4 Discussion

The present study demonstrates that administration of the NK₁ receptor antagonist NAT following TBI results in the attenuation of post-traumatic BBB permeability, oedema formation. It leads to improved brain free Mg²⁺ concentration and a down regulation of SP and upregulation of AQP-4 expression.

Prior to the present results, only one study had previously implicated a role for sensory neuropeptides, and in particular SP, in neurogenic inflammation following TBI. Nimmo et al (2004) (Nimmo et al., 2004) showed that prior depletion of neuropeptides from sensory nerves led to an inhibition of BBB opening, oedema formation, free Mg²⁺ decline and development of motor and cognitive deficits after TBI. Prior to these findings, very few reports describing a role of neuropeptides in nervous system injury had been published. Indeed, only isolated reports in peripheral nerve injury (Malcangio et al., 2000), traumatic spinal cord injury (Sharma et al., 1993) and brain ischemia (Stumm et al., 2001) had described inflammatory release of sensory neuropeptides. Of these, only the studies of brain ischaemia showed an activation of neuropeptide receptors in the endothelium related to oedema formation (Stumm et al.,
2001), similar to what has been well characterised in peripheral tissue injury (Woie et al., 1993). This observation is consistent with our current results, where TBI leads to the development of oedema shortly after injury along with increased extravasation of EB across the BBB.

The early increase in BBB permeability supports previous findings showing that TBI induces a maximal increase in permeability within the first 3-6 h following injury, along with early oedema formation (Vink et al., 2003b). This same study described that the oedema formation following injury occurred in two phases with an early vasogenic form directly related to BBB permeability and a second cytotoxic form occurring over the subsequent days independent of increased BBB permeability. Administration of the NK₁ receptor antagonist 30 min post-TBI attenuated both BBB opening and early vasogenic oedema formation, and supports a direct role for SP in these events.

The role of SP in vasogenic oedema was confirmed using MRI diffusion-weighted imaging, where bright areas of hyperintensity throughout the ADC maps of the brain occurred 4-5 h after injury with a marked reduction following treatment with the NK₁ receptor antagonist. This early formation of vasogenic oedema was consistent with previously published results, including those of Hanstock et al (1994) (Hanstock et al., 1994) who used the lateral fluid percussion (FP) device to show that TBI results in an increased water diffusion distance with the directionality indicative of bulk flow of extracellular fluid toward the lateral ventricles (vasogenic edema). Albensi et al (2000) (Albensi et al., 2000) also showed that the FP model resulted in regions of increased ADC early after TBI that may be associated with vasogenic oedema formation. Beaumont et al (2000) (Beaumont et al., 2000) demonstrated that diffuse impact
acceleration induced injury resulted in vasogenic oedema despite only a very brief BBB opening (Beaumont et al., 2000).

The present study has also shown that administration of the NK₁ receptor antagonist results in a profound attenuation of Mg²⁺ decline in the first 4-5 h after TBI. Decline in brain free Mg²⁺ concentration after TBI has been associated with the development of neurological deficits, and has been extensively demonstrated using MRS in a number of experimental injury models, including the impact acceleration model used in this current study (Vink and Cernak, 2000). Furthermore, studies have shown that pharmacological interventions that improve functional outcome after TBI generally all improve brain free Mg²⁺ concentration (Vink, 1993). While the improvement in brain free Mg²⁺ concentration may simply be a reflection of reduced neuronal injury, the fact that administration of Mg²⁺ alone also improves functional outcome (Vink and Cernak, 2000) suggests that restored brain Mg²⁺ homeostasis may be a mechanism of action by which neuroprotective effects are mediated. Since the NK₁ receptor antagonist restores brain free Mg²⁺ concentration after TBI, it may be that some of its neuroprotective actions may also be mediated by the Mg²⁺ ion. Magnesium is involved in many cellular processes including inhibiting excitotoxicity and apoptosis, and improving the brain bioenergetic state (Van Den Heuvel et al., 2004). Magnesium has also been shown to have direct effects on BBB permeability and vasogenic oedema formation (Esen et al., 2003), and learning and memory (Smith et al., 1993). Also, SP has a number of additional central nervous system effects that are independent of Mg²⁺, including effects on depression, anxiety, emesis, motor control and cognition (Hasenohrl et al., 2000; Hokfelt et al., 2001). Accordingly, NK₁ antagonists may be a novel
multifactorial drug intervention for TBI that incorporates Mg$^{2+}$-dependent and Mg$^{2+}$-independent neuroprotective actions.

Previously we proposed that an increase in SP immunoreactivity is a ubiquitous feature of TBI. In this chapter we show that administration of the NK$_i$ receptor antagonist at pre-selected timepoints following injury results in a marked reduction in SP immunoreactivity. This decrease is particularly evident at 5 h and 24 h following injury, which coincides with the timeframe of vasogenic oedema. By 3 d after injury there is qualitative evidence of an increase in SP immunoreactivity perivascularly and within the parenchyma suggesting a decline in the antagonist’s effects, which may be a reflection of its half-life.

The relationship between AQP-4 expression and brain injury is unclear, despite a number of reports implicating aquaporins in oedema formation. An in vitro study reported that hypoxia and reoxygenation leads to an upregulation in AQP-4 in astrocytes (Yamamoto et al., 2001). In human oedematous brain tumours there is an upregulation of AQP-4 expression (Saadoun et al., 2002). AQP-4 knockout mice show less brain oedema formation after water intoxication and cerebral ischaemia (Manley et al., 2000). The conclusion from the above studies is that brain oedema is associated with an upregulation of AQP-4. In contrast, a recent study of cultured astrocytes reported a decrease in AQP-4 levels in co-culture with glioma cells, and the authors suggested that a decreased expression of AQP-4 induced by glioma cells is a mechanism for brain oedema in human glioma (Chen et al., 2003b). Another recent study reported a reduction in AQP-4 immunoreactivity at 1h following cerebral hypoxia-ischaemia, although a significant difference using western blotting was
detected only at 24h (Meng et al., 2004). A down regulation of AQP-4 mRNA expression was suggested as a mechanism for water imbalance in a model of TBI with hyponatraemia (Ke et al., 2002), while a global reduction in brain AQP-4 expression coincides with the development of oedema in focal cortical impact injury (Kiening et al., 2002). In the current study of impact acceleration diffuse head trauma we have shown a general decreased expression of perivascular AQP-4 at 5h post-injury. This decrease in expression would presumably lead to reduced movement of water from the brain parenchyma and accordingly increased oedema. It is unknown whether the rapid decline in AQP-4 is the result of a redistribution of the protein between different domains of astrocytes. In contrast, the administration of NAT at 30 min after trauma increased AQP-4 immunoreactivity relative to untreated animals, suggesting that the NK1 receptor antagonist may reduce brain water content by increasing the expression of AQP-4 channels in astrocytes and allowing parenchymal water to enter the vasculature.

In conclusion, we have demonstrated that inhibition of neurogenic inflammation by post-traumatic administration of the NK1 receptor antagonist NAT inhibits BBB permeability, vasogenic oedema formation, free Mg2+ decline, down regulates SP and upregulates perivascular AQP-4 immunoreactivity. These results show sensory neuropeptides, and in particular SP, may play a significant role in the post-traumatic, secondary injury processes and may offer a novel target for the development of interventional pharmacological strategies. Whether these interventional strategies improve functional outcome following moderate to severe TBI is the subject of the next chapter.