

**CHARACTERISING THE ROLE OF SUBSTANCE P
IN ACUTE ISCHAEMIC STROKE**

RENÉE JADE TURNER

B.Sc (Hons)

Discipline of Pathology, School of Medical Sciences

The University of Adelaide

November 2007

A thesis submitted to the University of Adelaide in fulfilment of the requirements
for the degree of Doctor of Philosophy

CHAPTER 8:
CHARACTERISATION OF THE EFFECTS OF
NEUROPEPTIDE DEPLETION WITH CAPSAICIN
FOLLOWING ACUTE ISCHAEMIC STROKE

8.1 Introduction

Chapter 3 demonstrated that there was an increase in the neuropeptide SP following stroke, and that this was associated with neurogenic inflammation and subsequent BBB dysfunction, cerebral oedema and functional deficits. Chapter 4 subsequently demonstrated that blocking the action of SP with a NK₁ receptor antagonist was highly efficacious in improving post-stroke outcome through reduction of BBB permeability, cerebral oedema and functional deficits. These studies specifically focused on the SP pathway following stroke and it is therefore unclear what role other neuropeptides may play following stroke. The current study will examine the role of total neuropeptide depletion, using capsaicin, on outcome following stroke.

Capsaicin, an agent isolated from chilli peppers, is able to stimulate the release of sensory neuropeptides, including SP and CGRP, to the point of depletion (Wimalawansa, 1996; Kashiba et al., 1997). In neonatal animals, capsaicin pre-treatment produces permanent sensory neuropeptide depletion, whereas in adults it produces transient sensory neuropeptide depletion, reported to last for at least 4 weeks. Thus, capsaicin treatment is an extremely useful experimental tool enabling researchers to study the functions of various neuropeptides. Previous studies from our laboratory (Nimmo et al., 2004; Vink et al., 2003) have shown that pre-treatment with capsaicin markedly improved outcome following diffuse traumatic brain injury by reducing BBB dysfunction, cerebral oedema and functional deficits. The fact that the protection conferred by capsaicin was almost identical to that conferred by NK₁ receptor antagonists confirmed a dominant role for SP following injury to the brain. As such, the aim of the present study was to determine whether

the depletion of all neuropeptides by capsaicin similarly conveys any protection from the development of functional deficits following ischaemic stroke.

8.2 Study Design

Animals (n=14) were randomly assigned to treatment and control groups. Capsaicin (Sigma) was dissolved in 20% Tween 80, 20% ethanol and 60% saline. Capsaicin, or equal volume of vehicle, was then administered subcutaneously over a 3 d period at a dose of 125 mg/kg (50 mg/kg on day 1, 50 mg/kg on day 2 and 25 mg/kg on day 3). From our previous trauma studies this regime was found to be effective in ablating neuropeptides (Nimmo et al., 2004; Vink et al., 2003). Previous studies have reported that the level of SP in sensory nerves remains depleted for more than four weeks using this protocol. 14 d after capsaicin pre-treatment animals were subject to 2 h MCAO followed by reperfusion (as described in Chapter 2.2.2). Following surgery, animals were assessed for functional and histological outcome (as described in Chapters 2.4-2.5). Results were compared with those obtained for the NAT treatment at 4 h after stroke study in Chapter 4.

8.2.1 Functional Outcome

Commencing at 24 h after surgery, functional outcome testing was carried out daily for a 7 d period. Functional outcome was assessed using the rotarod, bilateral asymmetry test, neuroscore, open field and angleboard tests, as previously described (Chapter 2.4).

8.2.2 Histological Outcome

At 7 d following stroke, animals were perfused with 10% formalin under isoflurane anaesthesia, as described in detail in chapter 2. Brains were then removed and processed for immunohistochemistry. Slides were stained for H&E, SP, APP, FJC, GFAP and ED-1. Sections were then assessed using light microscopy or fluorescence microscopy as appropriate.

8.2.3 Statistical Analysis

All parametric data was analysed using analysis of variance followed by Bonferroni post-tests. The neuroscore data was analysed using the Kruskal Wallis ANOVA followed by Dunn's multiple comparisons test. All parametric data are expressed as mean +/- SEM, while the neuroscore data is expressed as the median.

8.3 Results

8.3.1 Functional Outcome

There was no significant difference observed between the vehicle pre-treatment or post-treatment groups ($p > 0.05$; results not shown), demonstrating that untreated animals perform similarly, regardless of the time of vehicle administration. As such, the data for these groups was combined and they are represented as the "vehicle" group on all of the functional outcome measures.

Motor Function - Rotarod

Sham animals recorded no motor deficits for the 7-day assessment period, indicating that the MCAO surgical procedure without the arterial occlusion had no effect on motor function. Following stroke, the vehicle group showed marked

functional deficits (Figure 8.1), scoring an average of 31 s on day 1, and only improving to 57 s by day 7. The performance of the vehicle-treated group was significantly worse than shams on all days post-stroke ($p < 0.001$). Capsaicin-treated animals scored 51 s on day one, they gradually improved over the assessment period, such that by day 7 their performance was comparable to shams ($p > 0.05$). Nonetheless, significant motor deficits compared to shams ($p < 0.001$) were observed on days 1-6 post-stroke. NAT-treated animals demonstrated a more rapid recovery in motor function than capsaicin-treated animals. Animals in the NAT group recorded a rotarod score of 69 s on day 1 post-stroke and rapidly improved to normal functional levels by day 3 post-stroke. The rotarod performance of this group was significantly better ($p < 0.001$) than vehicles on days 2-7 post-stroke. Although demonstrating the same pattern of recovery, capsaicin animals scored consistently lower ($p < 0.001$) on the rotarod compared to NAT-treated animals on days 2-6 post-stroke, and recovery of motor function was accelerated in the NAT-treated group compared to the capsaicin-treated group.

Sensory Function: Bilateral Asymmetry Test

Sham animals showed no sensory deficits, rapidly removing the tape on their forepaws on each of the assessment days. Following stroke, vehicle animals displayed profound sensory deficits, as demonstrated by their difficulty in removing the tape on any assessment day. As such, time to removal was significantly greater ($p < 0.001$) than shams on all days post-stroke. Conversely, both the capsaicin-treated group and the NAT-treated group demonstrated a recovery of sensory function over the assessment period. Specifically, the capsaicin animals recorded improved latencies over time, performing significantly better than vehicles on days

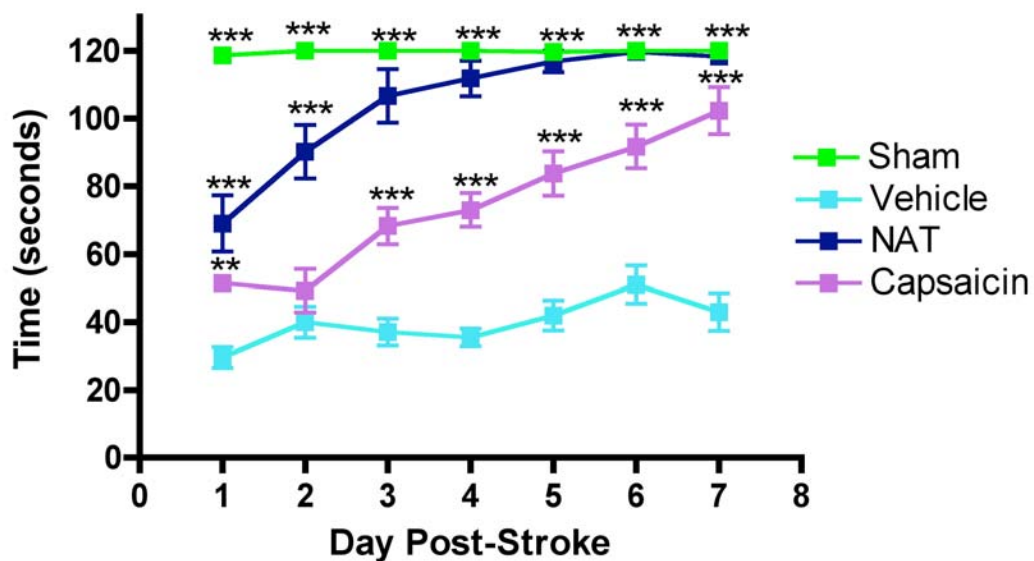


Figure 8.1 Capsaicin pre-treatment – Motor function as assessed by the rotarod.

Sham animals had no motor deficits (green). Following stroke, vehicle animals (aqua) demonstrated profound motor deficits that persisted for the 7 d assessment period. In contrast, NAT-treated animals (navy) showed a steady improvement in motor function, such that by day 3 post-stroke they were performing at normal levels. Similarly, capsaicin-treated animals (purple) showed improved motor function over time, performing at normal levels by day 7 post-stroke (** denotes $p < 0.01$ versus vehicle; *** denotes $p < 0.001$ versus vehicle) (sham $n = 6$; vehicle $n = 12$; NAT $n = 6$; capsaicin $n = 8$).

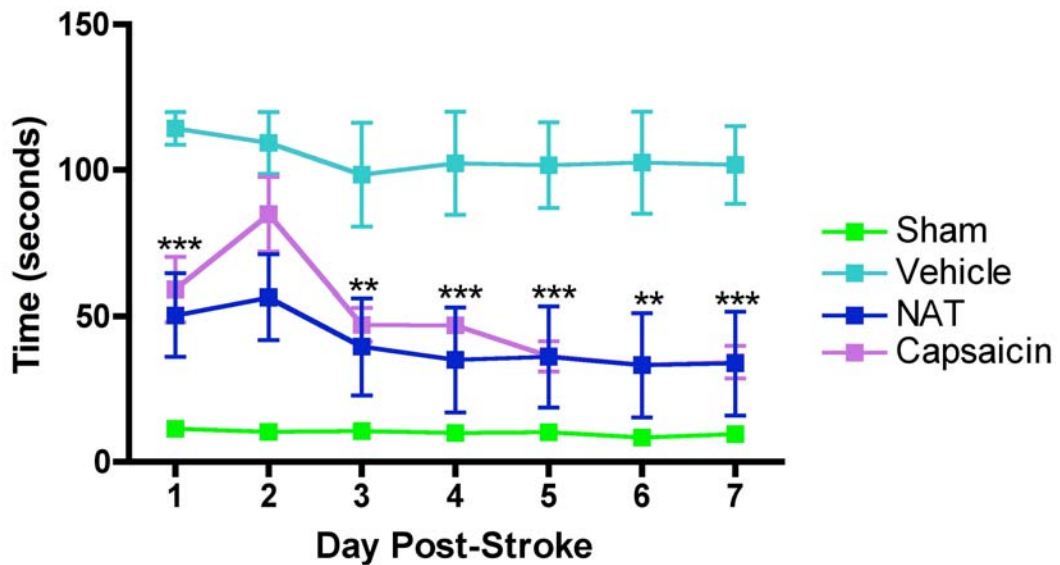


Figure 8.2 Capsaicin pre-treatment – Sensory function as assessed by the bilateral asymmetry test.

No sensory deficits were observed in sham animals (green). Following stroke, vehicle animals (aqua) demonstrated profound sensory deficits that persisted for the entire assessment period. In contrast, NAT-treated animals (navy) showed a recovery of sensory function over time, recording latencies significantly better than vehicles on days 1 and 3-6 post-stroke. Capsaicin-treated animals (purple) showed a similar pattern of recovery in sensory function, scoring significantly better than vehicles on days 1 and 3-6 post-stroke (** denotes $p < 0.01$ versus vehicle; *** denotes $p < 0.001$ versus vehicle) (sham $n = 6$; vehicle $n = 12$; NAT $n = 6$; capsaicin $n = 8$).

1 and 3-7 post-stroke ($0.001 < p < 0.01$), reaching normal functional levels by day 3 post-stroke. Similarly, NAT-treated animals showed improved sensory function over the 7 d assessment period. Animals in this group had reached normal functional levels by day 3 post-stroke, recording latencies significantly better than vehicles on days 3-7 post-stroke. NAT-treated animals also demonstrated a more rapid recovery in sensory function, as compared to the capsaicin-treated animals, although this trend was not statistically significant.

Spontaneous Exploratory Behaviour: Open Field

Sham animals demonstrated normal activity levels in the open field. However, a decline in spontaneous exploratory behaviour was noted over time, and this is most likely to be due to habituation, a well-described event in uninjured animals (McIlwain et al., 2001; Paylor et al., 2006; Stohr et al., 1998). Following stroke, vehicle animals consistently travelled through less than 50 squares on all assessment days (Figure 8.3), which was significantly reduced compared to shams on days 1 and 3 ($0.01 < p < 0.05$) post-stroke. No improvement in spontaneous exploratory behaviour was observed in this group. The capsaicin treated group was significantly worse than shams on day 1 post-stroke ($p < 0.05$) and then demonstrated an increase in open field activity as reflected by an improved score that was not significantly different from shams (days 3-7; $p > 0.05$). However, despite a trend towards increased exploratory behaviour compared to vehicles, these differences were not significant. In contrast, the level of spontaneous exploratory behaviour in the NAT-treated group was not significantly different that shams on any day post-stroke. As such, NAT-treated animals consistently demonstrated spontaneous exploratory behaviour levels in the open field significantly higher than vehicle-treated animals

on all assessment days ($0.001 < p < 0.05$). This difference was significantly ($p < 0.05$) greater than capsaicin pre-treated animals only on day 3 post-stroke. On all other days there was no significant ($p > 0.05$) difference between the activity level of the two groups despite a trend towards an increased number of squares by the NAT treated group.

Neurological Function: modified Neuroseverity Score

Sham animals displayed no neurological deficits on any of the assessment days, confirming that the surgical procedure had no effect on neurological function. Following stroke, vehicle animals consistently recorded an mNSS ranking of 6 or more, indicative of moderate injury (Figure 8.4). No improvement in neurological function was observed in this group following stroke and as such, their neurological function was significantly ($p < 0.001$) worse than shams at all time-points. In comparison, both the NAT and capsaicin-treated groups demonstrated a recovery of neurological function over the 7 d assessment period, recording a neurological score not significantly different from shams ($p > 0.05$). The NAT performed at levels significantly better than vehicle treated animals ($p < 0.05$), while the capsaicin treated group did not perform significantly better than vehicle treated animals ($p > 0.05$). Despite this, there was no significant difference between the NAT- and capsaicin-treated groups, largely because of the within group variation.

Hemiparesis: Angleboard

No hemiparesis was observed in sham animals, indicating that the surgical procedure had no effect on balance and muscle strength. Following stroke, vehicle animals demonstrated profound hemiparesis that persisted for the 7 d assessment

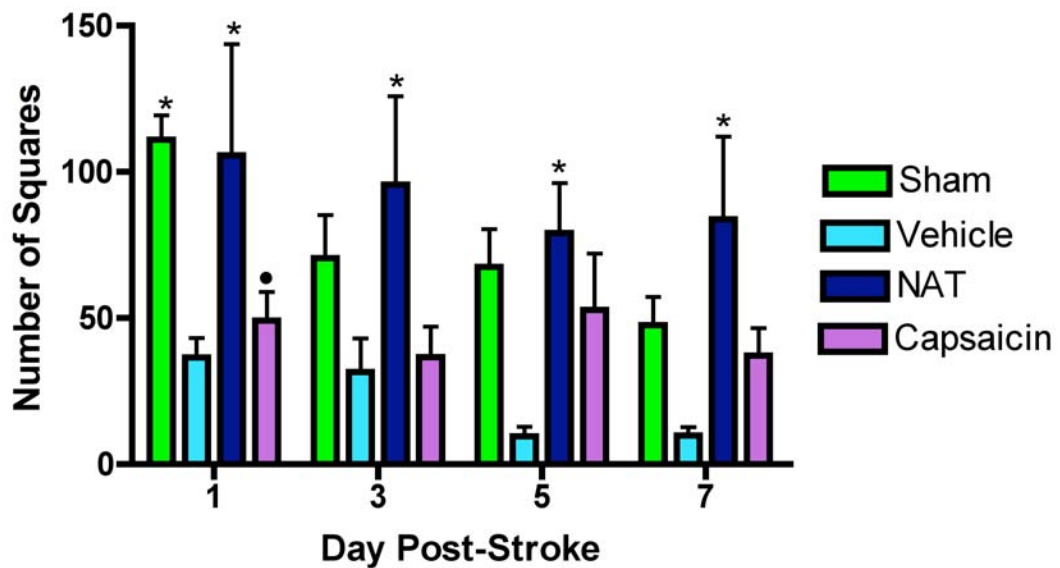


Figure 8.3 Capsaicin pre-treatment – Spontaneous exploratory behaviour, as assessed by the open field test.

Normal levels of spontaneous exploratory behaviour were observed in sham animals (green). Following stroke, vehicle animals (aqua) demonstrated a significant decline in spontaneous exploratory behaviour. In contrast, NAT-treated animals (navy) showed a recovery in spontaneous exploratory behaviour with an increase ($p < 0.05$) in the number of squares transversed compared to vehicles. Capsaicin-treated animals did not record open field scores significantly different from vehicles on any assessment day, despite a trend towards increased spontaneous exploratory behaviour (* denotes $p < 0.05$ versus vehicle; • denotes $p < 0.05$ versus sham) (sham $n = 6$; vehicle $n = 12$; NAT $n = 6$; capsaicin $n = 8$).

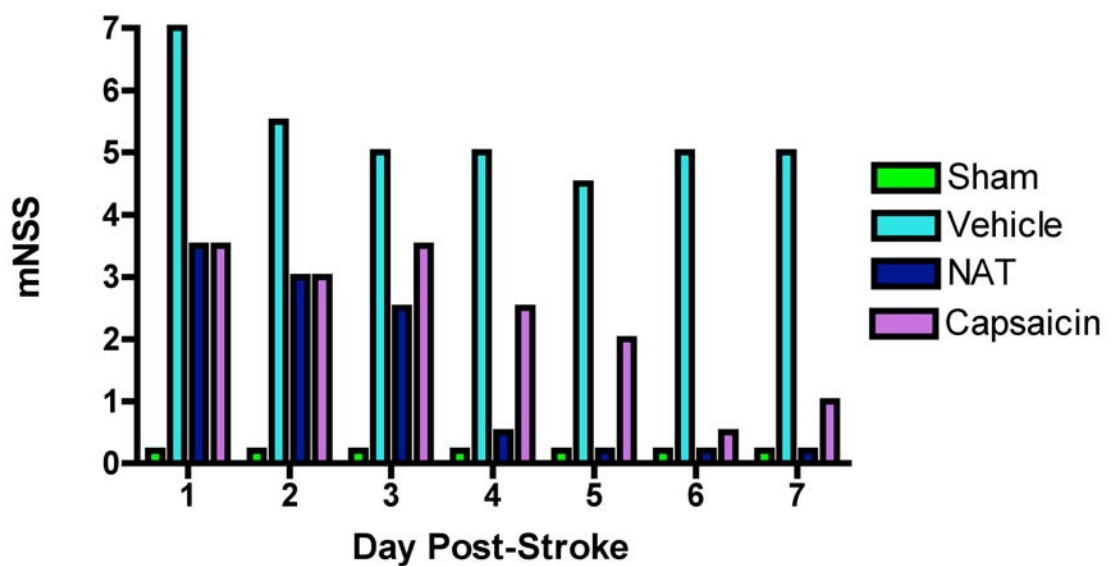


Figure 8.4 Capsaicin pre-treatment – Neurological function, as assessed by the modified neuroseverity score.

No neurological deficits were observed in sham animals (green). Following stroke, vehicle animals (aqua) showed profound neurological deficits ($p < 0.001$ versus shams), which persisted for the 7 d assessment period. In contrast, NAT-treated animals (navy) recovered from mild neurological deficits to no observable deficit by day 5 post-stroke ($p < 0.05$ versus vehicles). Capsaicin-treated animals (purple) also showed a recovery of neurological function over the assessment period, but were still ranked as having a mild injury by day 7 post-stroke (sham $n = 6$; vehicle $n = 12$; NAT $n = 6$; capsaicin $n = 8$).

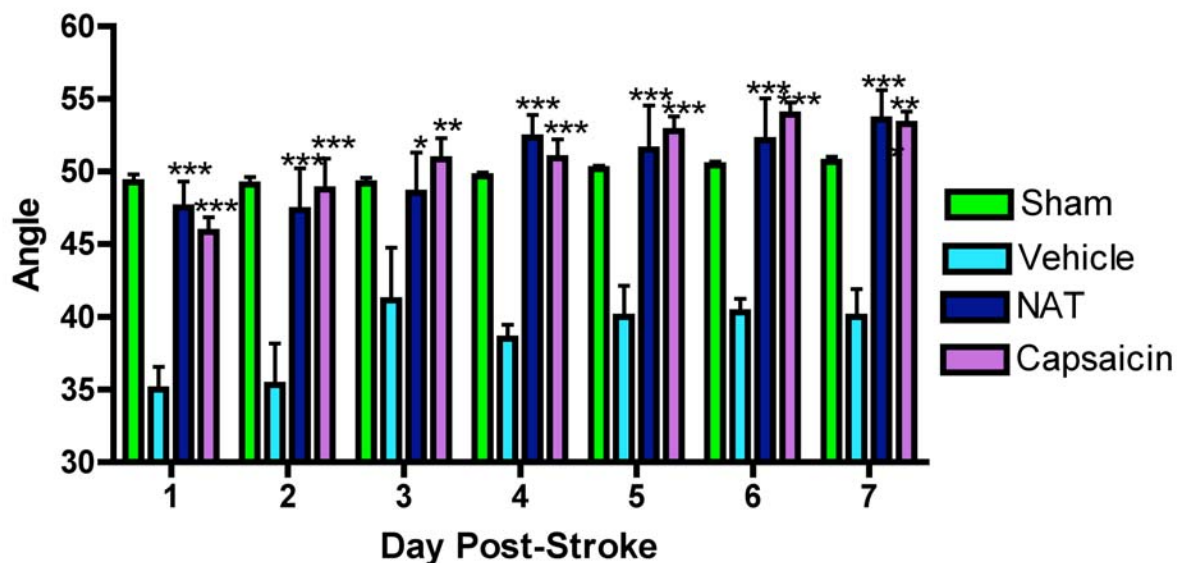


Figure 8.5 Capsaicin pre-treatment – Hemiparesis, as assessed by the angleboard.

Sham animals showed no signs of hemiparesis (green). Following stroke, vehicle animals (aqua) showed profound hemiparesis that persisted for the entire assessment period ($p < 0.001$ versus shams). In contrast, NAT-treated animals (navy) showed a recovery in hemiparesis over the assessment period, recording angleboard scores significantly better ($0.05 < p < 0.001$) than vehicles on all days post-stroke. Similarly, capsaicin-treated animals (purple) demonstrated improved angleboard function over time and scored significantly better ($0.01 < p < 0.001$) than vehicles on all assessment days (* denotes $p < 0.05$ versus vehicle; ** denotes $p < 0.01$ versus vehicles; *** denotes $p < 0.001$ versus vehicles) (sham $n = 6$; vehicle $n = 12$; NAT $n = 6$; capsaicin $n = 8$).

period (Figure 8.5). No improvement in angleboard performance was observed over time, with scores significantly worse ($p < 0.001$) than shams on all assessment days. In contrast, a reduction in hemiparesis was observed over time in the NAT group, as evidenced by improved angleboard scores. This group performed significantly better ($0.001 < p < 0.05$) than vehicles on all assessment days, reaching normal functional levels. Similarly, the capsaicin-treated group showed improved angleboard performance over time, scoring significantly better than vehicles and comparable to shams on all assessment days. There was no significant difference between the capsaicin and NAT-treated groups.

8.3.2 Histological outcome

General Pathology - H&E

As reported in previous chapters, no abnormalities were observed in sham tissue. At 7 d following stroke, extensive reactive gliosis occupied the cortex (Figure 8.6), accompanied by a complete loss of normal cortical architecture. However, treatment with either NAT or capsaicin was able to reduce the extent of the reactive gliosis, such that regions of tissue preservation were observed. Specifically, there was a modest reduction in reactive gliosis with both NAT and capsaicin treatment. Within the white matter of vehicle animals, extensive tissue destruction was observed (Figure 8.7), characterised by tissue vacuolation and the influx of inflammatory cells. NAT or capsaicin treatment did not affect the extent of tissue damage within the white matter.

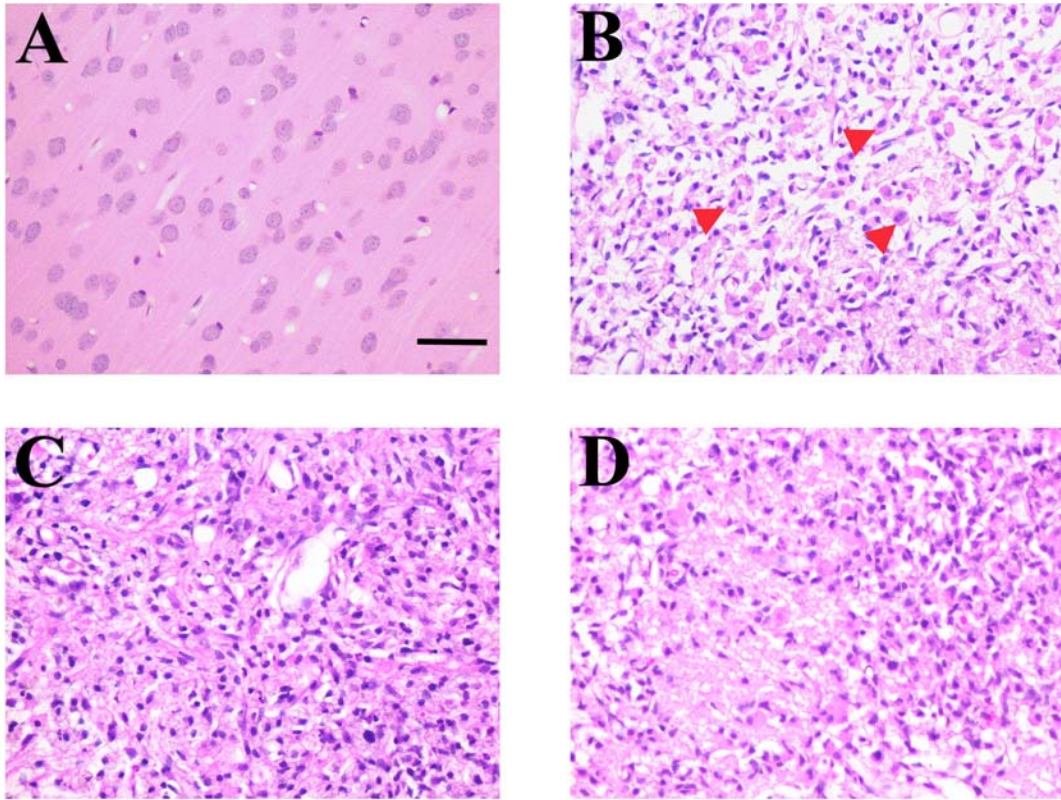


Figure 8.6 Capsaicin pre-treatment –Cortex at 7 d following stroke. H&E stained sections (Bar = 100 μ m).

No abnormalities were observed in sham tissue (A). Following stroke (B), extensive reactive gliosis was observed to occupy the cortex, along with the influx of macrophages (red arrowheads). Treatment with NAT (C) or capsaicin (D) resulted in a modest reduction in reactive gliosis and a degree of tissue preservation was observed.

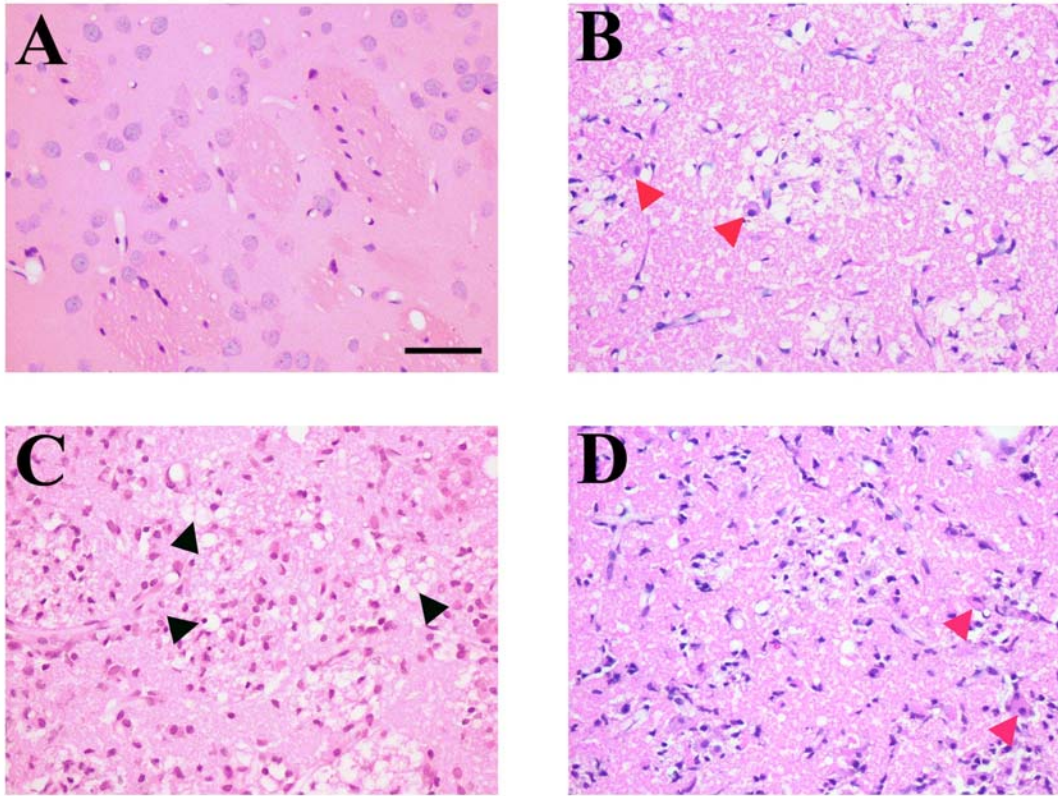


Figure 8.7 Capsaicin pre-treatment –White matter at 7 d following stroke. H&E stained sections (Bar = 100 μ m).

No abnormalities were observed in sham tissue (A). Following stroke (B), extensive tissue destruction was observed within the white matter, along with the influx of macrophages (red arrowheads) and this was largely unaffected by NAT (C) or capsaicin (D) treatment.

SP response: SP Immunohistochemistry

Light SP immunoreactivity was observed within the perivascular tissue of sham animals. At 7 d following stroke in vehicle animals, little perivascular SP immunoreactivity was observed, due to the extensive reactive gliosis that occupied the infarct (Figure 8.8). Treatment with either NAT or capsaicin did not affect the perivascular SP response, as observed at 7 d following stroke. Light SP immunoreactivity was observed within the cortex (Figure 8.9), consistent with previous reports. Following stroke, extensive reactive gliosis occurred by day 7 within cortical tissue and as a result, little cortical SP immunoreactivity was evident. Treatment with either NAT or capsaicin did not appear to affect the level of cortical SP immunoreactivity observed at 7 d post-stroke.

Axonal Injury: APP Immunohistochemistry

No axonal injury was observed within the white matter of sham animals, indicating that the surgical procedure had no effect of white matter tissue integrity. At 7 d following stroke, florid axonal injury was observed in vehicle tissue (Figure 8.10), with large retraction balls observed throughout the white matter. NAT treatment resulted in a reduction in axonal injury, with the appearance of fewer retraction balls. Capsaicin treatment also reduced the degree of white matter axonal injury, with fewer and smaller retraction balls observed. Faint APP immunoreactivity was observed within the cortex of sham animals (Figure 8.11). Following stroke, an increase in cortical APP immunoreactivity was observed in vehicle animals. Treatment with NAT or capsaicin did not appear to affect the degree of cortical APP immunoreactivity.

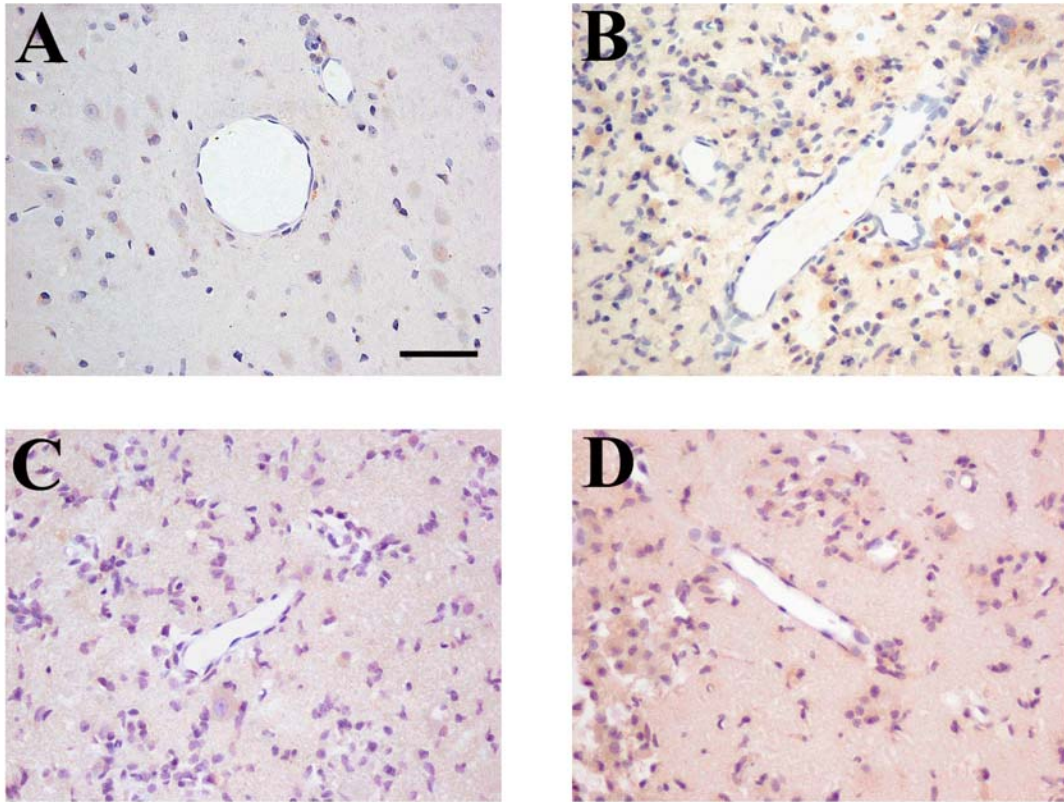


Figure 8.8 Capsaicin pre-treatment –Perivascular SP immunoreactivity at 7 d post-stroke. SP stained sections (Bar = 100 μ m).

Faint SP immunoreactivity was observed in the perivascular tissue of sham animals (A). At 7 d following stroke, reactive gliosis occupied the infarct and as a result little perivascular SP immunoreactivity was observed in vehicle (B), NAT (C) or capsaicin-treated (D) tissue.

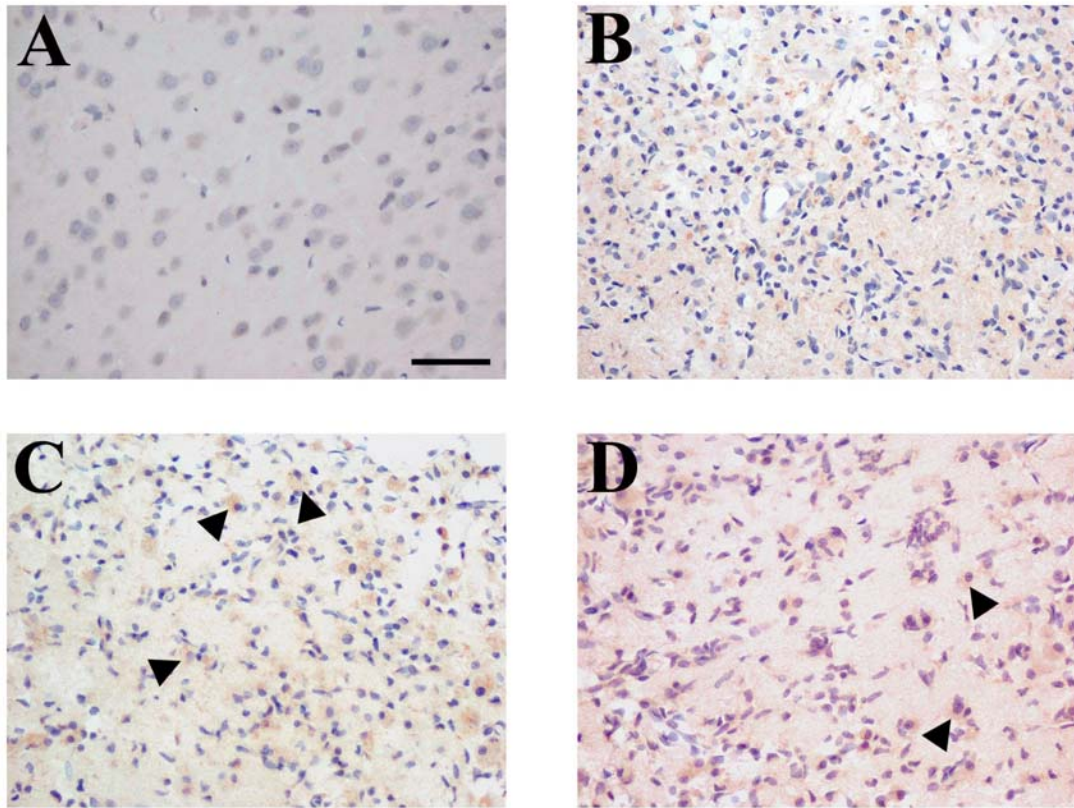


Figure 8.9 Capsaicin pre-treatment – Cortical SP immunoreactivity at 7 d following stroke. SP stained sections (Bar = 100 μ m).

Faint SP immunoreactivity was observed in sham tissue (A). At 7 d post-reperfusion (B), reactive gliosis occupied the cortex and little SP immunoreactivity (arrowheads) was observed. This was largely unaffected by NAT (C) or capsaicin (D) treatment.

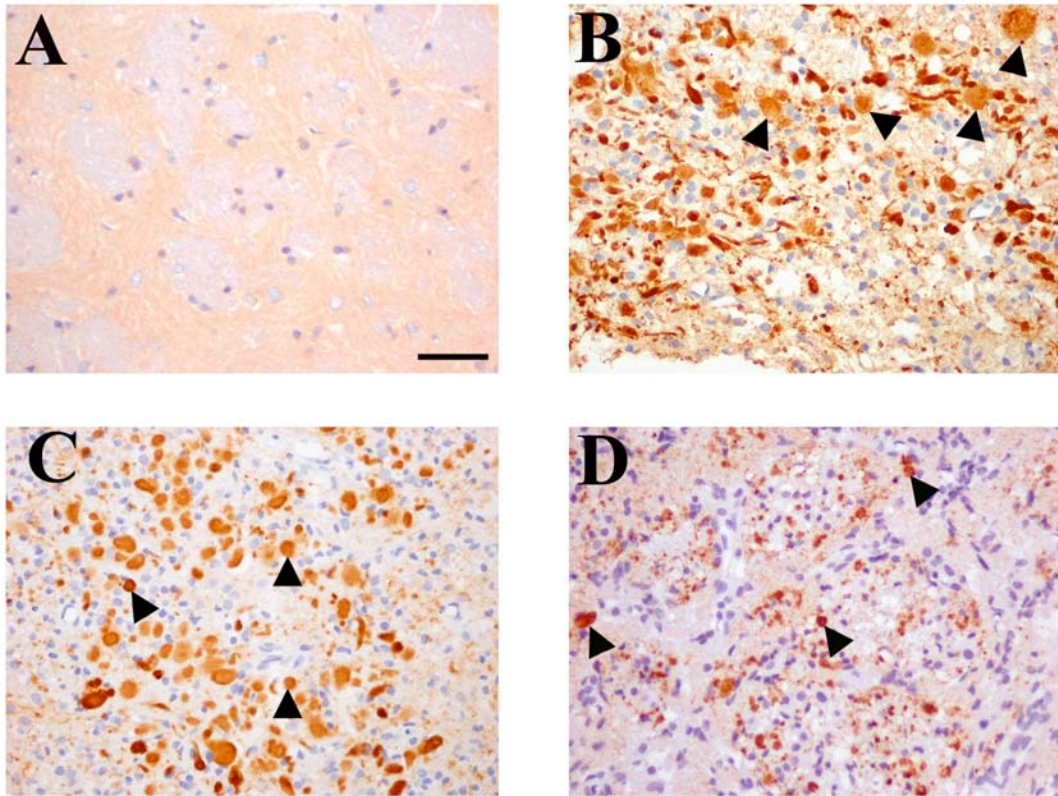


Figure 8.10 Capsaicin pre-treatment – Axonal injury within the white matter at 7 d following stroke. APP stained sections (Bar = 100 μ m).

No axonal injury was observed in sham tissue (A). At 7 d post-reperfusion (B), florid axonal injury (arrowheads) was observed. Treatment with NAT (C) or capsaicin (D) reduced the axonal injury observed within the white matter at 7 d post-stroke, with fewer and smaller retraction balls seen.

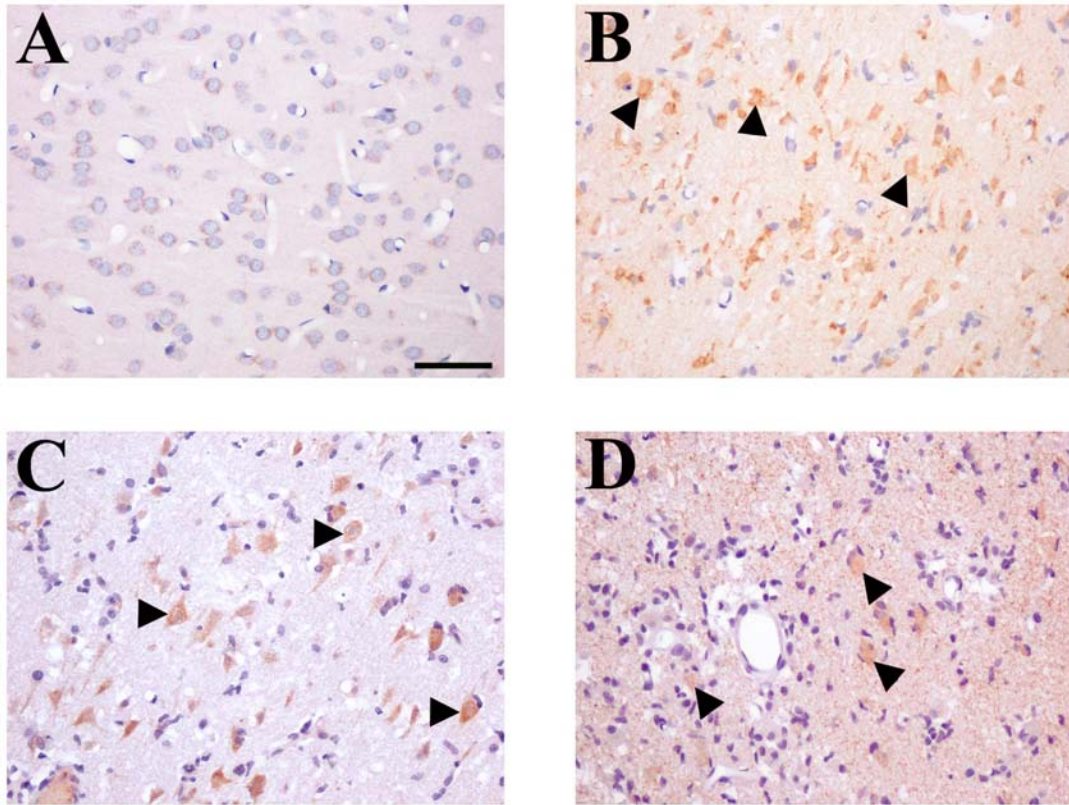


Figure 8.11 Capsaicin pre-treatment – Cortical APP immunoreactivity at 7 d following stroke. APP stained sections (Bar = 100 μ m).

Faint APP immunoreactivity was observed in sham tissue (A). At 7 d post-reperfusion (B), increased neuronal APP immunoreactivity (arrowheads) was observed. Treatment with NAT (C) or capsaicin (D) did not affect neuronal APP immunoreactivity.

Degenerating Neurons: FJC

No degenerating neurons were observed in sham tissue, confirming that the surgical procedure had no significant effect on neuronal survival. At 7 d following stroke, extensive reactive gliosis occupied the cortex (Figure 8.12) and as such, no degenerating neurons could be observed. However, a modest reduction in reactive gliosis was observed in NAT- and capsaicin-treated animals, such that some degenerating neurons were observed. Within the white matter of vehicle animals, a marked loss of normal tissue architecture was observed (Figure 8.13), characterised by reactive gliosis and tissue vacuolation. As a result, few degenerating neurons were observed. Treatment with either NAT or capsaicin produced a modest reduction in the reactive gliosis within the white matter.

Astrocyte Response: GFAP Immunohistochemistry

Faint GFAP immunoreactivity was observed within sham tissue (Figure 8.14). At 7 d following reperfusion, an increase in GFAP immunoreactivity was observed within the infarct border zone of vehicle animals. This response to ischaemia was further increased with NAT treatment, as evidenced by marked astrocyte hypertrophy and hyperplasia. In contrast, the GFAP response within the infarct boundary zone of capsaicin-treated animals was comparable to that of vehicle animals. Within perivascular tissue, an increase in GFAP immunoreactivity was also observed following stroke (Figure 8.15) in vehicle animals. Once again, NAT treatment was observed to further increase this response to ischaemia, with profound GFAP immunoreactivity observed in perivascular tissue. Capsaicin treatment produced a pattern of perivascular GFAP immunoreactivity that was comparable to that observed in vehicle-treated animals.

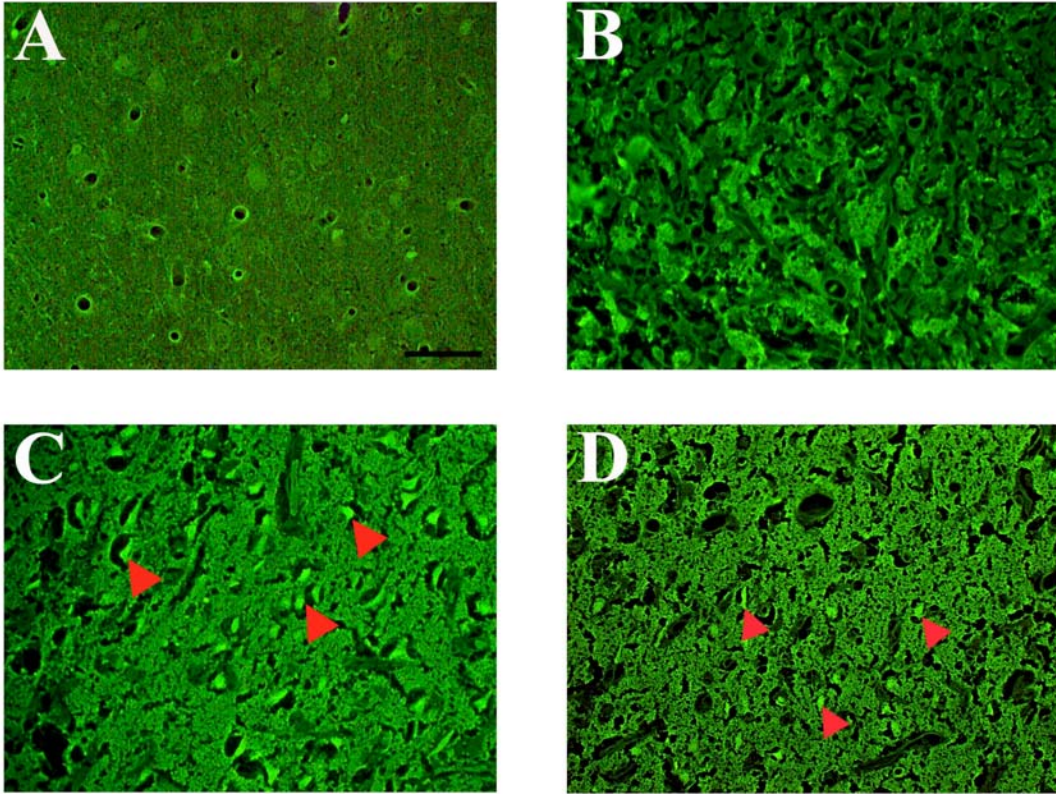


Figure 8.12 Capsaicin pre-treatment – Degenerating neurons within the cortex at 7 d following stroke. FJC stained sections (Bar = 100 μ m).

No degenerating neurons were observed in sham tissue (A). At 7 d post-reperfusion (B), extensive reactive gliosis occupied the cortex and no degenerating neurons (arrowheads) were observed. Treatment with NAT (C) or capsaicin (D) resulted in a modest reduction in the degree of reactive gliosis and some degenerating neurons were observed.

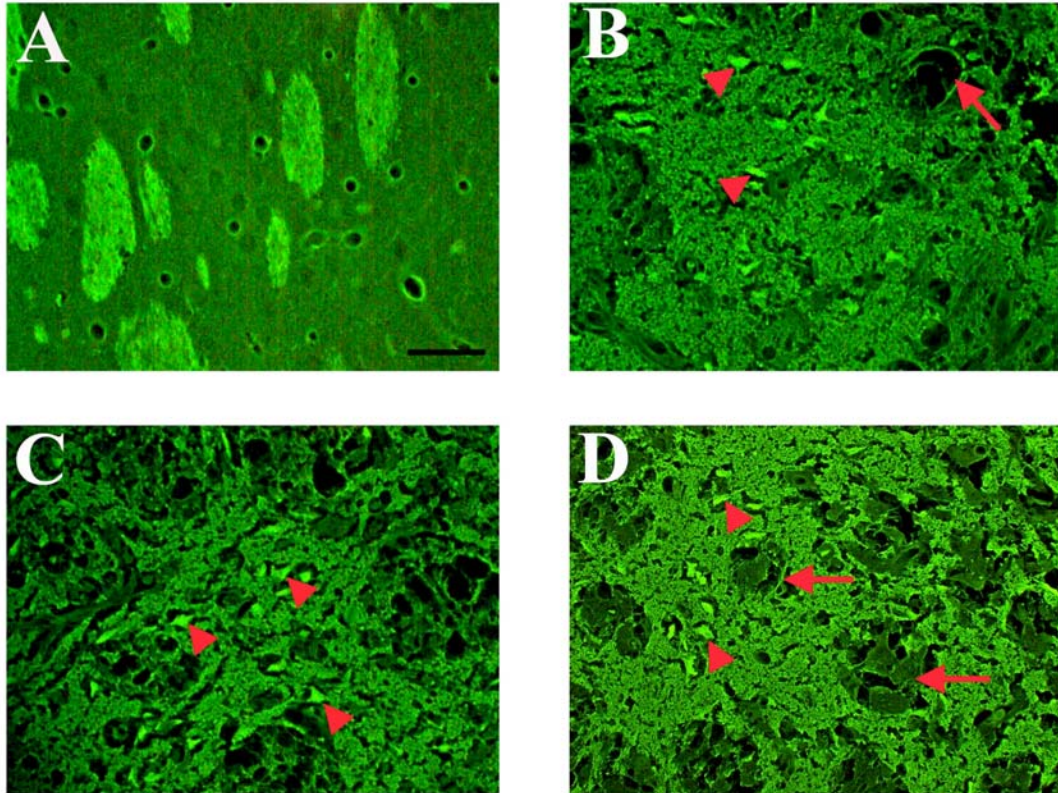


Figure 8.13 Capsaicin pre-treatment – Degenerating neurons within the white matter at 7 d following stroke. FJC stained sections (Bar = 100 μ m).

No degenerating neurons were observed in sham tissue (A). At 7 d following reperfusion (B), marked loss of white matter architecture was observed with reactive gliosis occupying the tissue, few degenerating neurons (arrowheads) were seen. Treatment with NAT (C) or capsaicin (D) treatment resulted in a modest reduction in reactive gliosis and some degenerating neurons were observed.

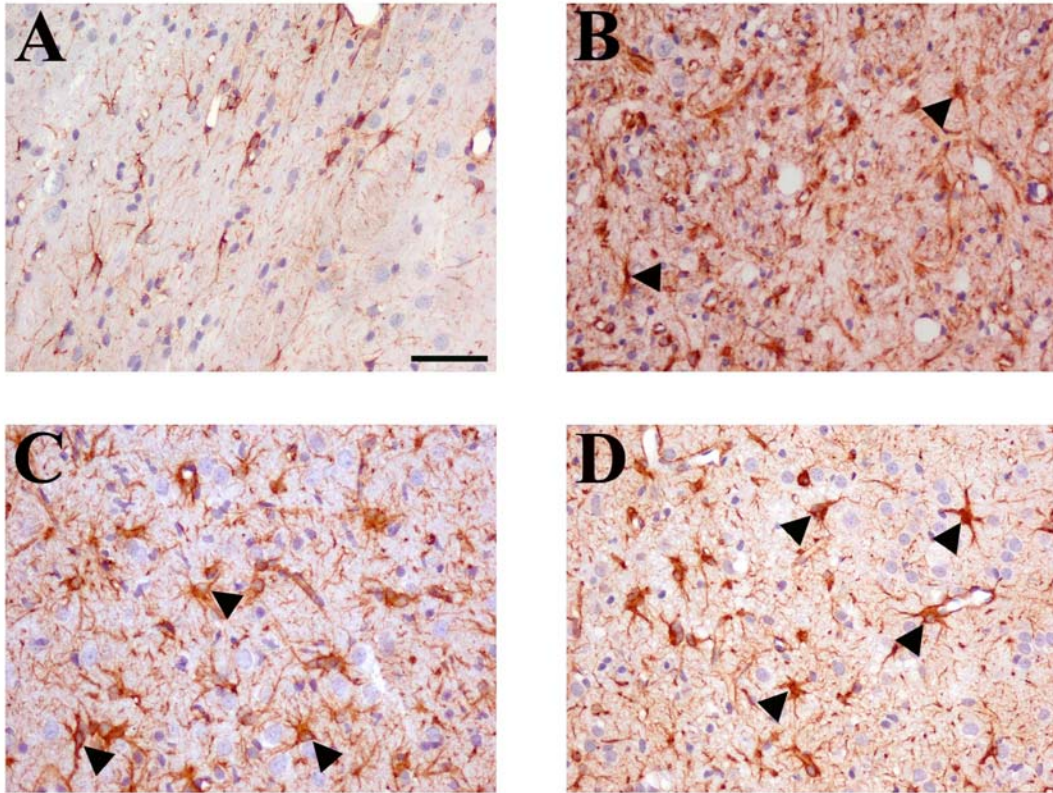


Figure 8.14 Capsaicin pre-treatment – GFAP immunoreactivity within the infarct border zone at 7 d following stroke. GFAP stained sections (Bar = 100 μm).

Faint GFAP immunoreactivity was observed in sham tissue (A). At 7 d following stroke (B), an increase in GFAP immunoreactivity (arrowheads) was observed in the infarct border zone. Treatment with NAT exacerbated this response to injury (C) (arrows). Treatment with capsaicin (D) produced GFAP immunoreactivity within the infarct border zone comparable to vehicles.

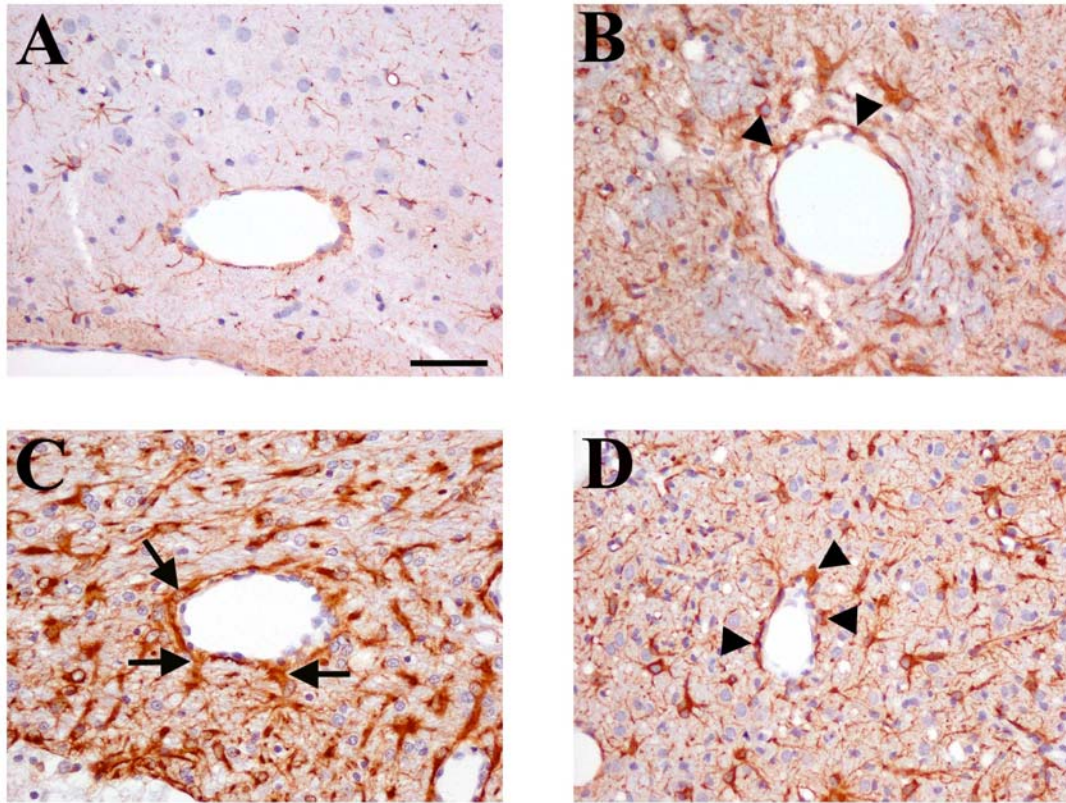


Figure 8.15 Capsaicin pre-treatment – Perivascular GFAP immunoreactivity at 7 d following stroke. GFAP stained sections (Bar = 100 μ m).

Faint GFAP immunoreactivity was observed in sham tissue (A). At 7 d following reperfusion (B), an increase in GFAP immunoreactivity (arrowheads) was observed within perivascular tissue in NAT-treated animals (C). Capsaicin treatment (D) produced a perivascular GFAP response comparable to vehicles.

Macrophage/Activated Microglia Response: ED-1 Immunohistochemistry

In sham tissue, no macrophages/activated microglia were observed within cortical tissue or in association with blood vessels, indicating that the surgical procedure did not elicit a significant inflammatory response (Figure 8.16). At 7 d following stroke, a profound influx of ED-1 positive cells was observed within the cortex of vehicle animals. NAT treatment produced a marked reduction in the number of macrophages/activated microglia observed within the cortex. A modest reduction in ED-1 positive cells within the cortex was also observed in capsaicin animals. With respect to the perivascular response, an increase in the number of macrophages/activated microglia observed in close association with blood vessels was seen at 7 d post-stroke (Figure 8.17) in vehicle-treated animals. This perivascular response to injury was not affected by treatment with either NAT or capsaicin.

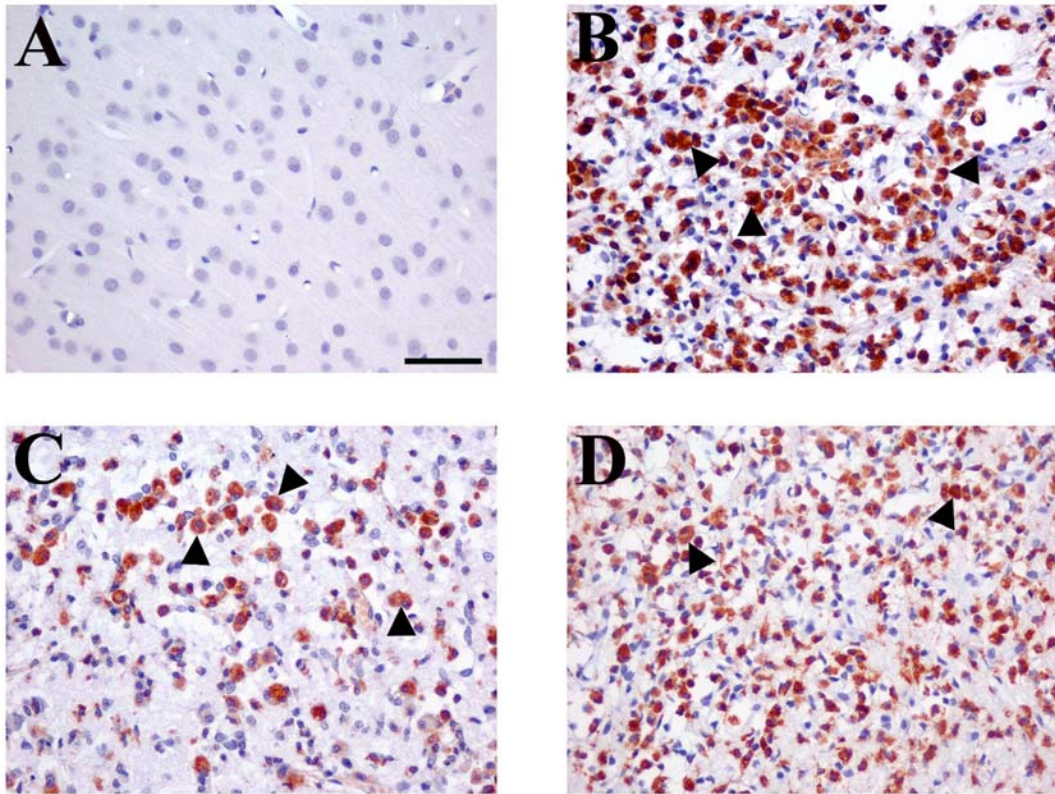


Figure 8.16 Capsaicin pre-treatment – Macrophage/Activated Microglia response within the infarct at 7 d post-reperfusion. ED-1 stained sections (Bar = 100 μm).

No macrophages/activated microglia were observed within the cortex of sham animals (A). At 7 d post-reperfusion (B), a profound influx of ED-1 positive macrophages/activated microglia (arrowheads) was observed within the cortex. Treatment with NAT (C) markedly reduced the number of ED-1 positive cells within the cortex. Treatment with capsaicin (D) resulted in a modest reduction in the number of ED-1 positive cells observed within the infarct.

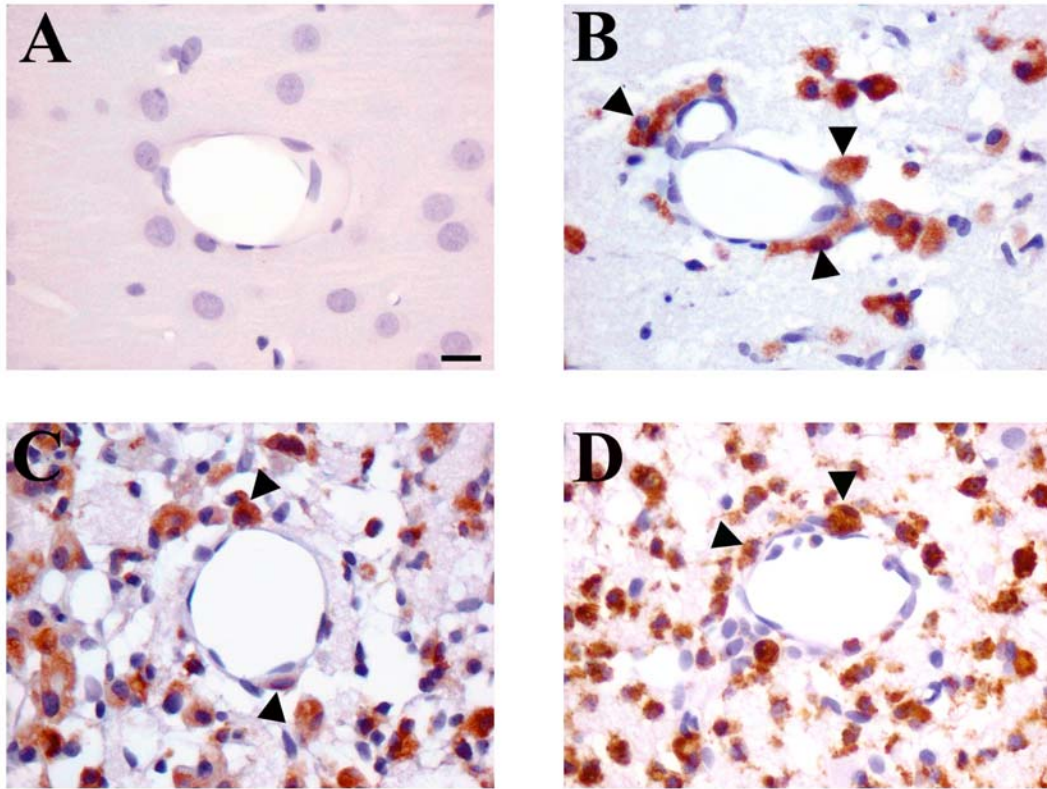


Figure 8.17 Capsaicin pre-treatment – Perivascular Macrophage/Activated Microglia immunoreactivity at 7 d following stroke. ED-1 stained sections (Bar = 100 μ m).

No macrophages/activated microglia were observed in association with blood vessels in sham tissue (A). At 7 d post-reperfusion (B), ED-1 positive macrophages/activated microglia (arrowheads) were observed in close association with blood vessels. This response was largely unaffected by treatment with NAT (C) or capsaicin (D).

8.4 Discussion

In the present study, we demonstrate that depletion of neuropeptides prior to cerebral ischaemia results in attenuation of functional deficits and a reduction in histological abnormalities. These findings represent one of the first investigations of the role of sensory neuropeptides in stroke. A role for neuropeptides and neurogenic inflammation in BBB dysfunction, cerebral oedema and functional deficits following TBI has recently been demonstrated (Donkin et al., 2007; Nimmo et al., 2004; Vink et al., 2003).

Although many studies have used capsaicin as an experimental tool to study neuropeptides (Dembinski et al., 2003; Turchanyi et al., 2005), few studies have investigated behavioural outcome end-points following capsaicin pre-treatment (Nimmo et al., 2004; Vink et al., 2003), and even fewer have studied cerebral ischaemia (Pegorini et al., 2005). Administration of capsaicin 5 mins after recirculation completely protected against global cerebral ischaemia, as indicated by a recovery in spontaneous motor activity, memory and hippocampal CA1 neuron density (Pegorini et al., 2005). However, these authors were unsure of the mechanism whereby capsaicin treatment provided protection but speculated that following ischaemia the release of neuropeptides from sensory nerves could be involved. Yet, the findings of the present thesis have clearly demonstrated a deleterious role for the neuropeptide SP following cerebral ischaemia. Our findings are consistent with those previously reported in TBI that reveal that capsaicin-induced neuropeptide depletion prior to injury is protective (Nimmo et al., 2004; Vink et al., 2003).

In the present study, pre-treatment with capsaicin was associated with an improvement in motor, sensory and neurological function and reduced hemiparesis. However, NAT treatment produced a more rapid recovery in motor and sensory function and a complete recovery in spontaneous exploratory behaviour following stroke, albeit not significantly in all tests when compared to capsaicin. Nonetheless, the overall trend was that NAT-treated animals demonstrated superior functional recovery when compared to capsaicin-treated animals. The fact that the functional recovery of the capsaicin group was not comparable to the NAT group in all tests of functional outcome suggests that one or more of the neuropeptides that was depleted with capsaicin pre-treatment may have been beneficial to outcome following stroke. As such, the removal of this neuropeptide by capsaicin pre-treatment may have negatively affected outcome. One candidate neuropeptide is CGRP, the most potent endogenous vasodilator, which acts to increase local blood flow (Dray, 1995). Intravenous administration of CGRP in a rat produces a transient increase in mean arterial blood pressure (Wimalawansa, 1996). However, CGRP is also involved in a number of other biological processes other than vascular regulation, including sensory transmission, neuromodulation at the neuromuscular junction and nociception (Wimalawansa, 1996). Indeed, protective roles for CGRP in ischaemia have previously been reported (Kjartansson, 1987). In a model of pancreatic ischaemia/reperfusion injury, the ablation of neuropeptides was found to aggravate the ischaemic damage (Dembinski et al., 2003), with the authors hypothesising that the lack of CGRP was detrimental to ischaemic tissue. CGRP has also been found to improve the survival of ischaemic surgical flap tissue (Kjartansson, 1987; Bucinskaite et al., 1998). The proposed mechanism of CGRP-induced protection was the promotion of angiogenesis, new blood vessel formation within the tissue.

Proliferation of endothelial cells and the migration of endothelial cells to injured monolayers of human microvascular endothelium has also been reported in response to CGRP (Datta et al., 1990). The release of CGRP has been proposed to be a generalised response of cerebral tissue to injury (Dragunow et al., 1992; Gherardini et al., 1996) and may directly promote the survival of damaged neurons (Bulloch et al., 1998; Dragunow et al., 1992) and nerve regeneration (Wimalawansa, 1996). Furthermore, CGRP immunoreactivity within the CA1 neurons of the hippocampus was associated with increased neuronal survival (Bulloch et al., 1998). In experimental SAH, infusion of CGRP prevented vasospasm and reduced the extent of the ischaemic lesion (Holland et al., 1994; Inoue et al., 1996). Taken together, these studies suggest a potential protective function for CGRP following ischaemia. In the context of the present study, this protective function may be quite modest.

When SP and CGRP are present in concert, CGRP may potentiate the effects of SP-induced neurogenic inflammation (Holzer, 1998). As such, antagonism of the SP pathway alone, through administration of an NK₁ receptor antagonist, may provide a favourable cerebral environment following stroke. Specifically, the action of SP is blocked so that deleterious neurogenic inflammation is circumvented, but the released CGRP may nonetheless have some favourable effects on the cerebral vasculature. This has not been observed in previous studies of TBI, although one could hypothesise that an improved vascular response would have little efficacy in TBI injuries without an ischaemic component. Nonetheless, similar findings have been reported following ischaemia/reperfusion injury in peripheral tissues. The absence of sensory neuropeptides was found to be beneficial in long durations of ischaemia (2 h) of skeletal muscle due to inhibition of neurogenic inflammation

(Turchanyi et al., 2005), whereas the absence of neuropeptides in shorter durations of ischaemia (1 h) was found to be unfavourable. This group suggested that this was due to a lack of vasodilator neuropeptides that improve microcirculation. Similarly, tissue damage following ischaemia/reperfusion injury of the pancreas was aggravated by neuropeptide depletion, presumably because of the lack of CGRP (Dembinski et al., 2003).

Recently the transient receptor potential V1 (TRPV1) receptor, where capsaicin is the ligand, has gained a lot of interest. Activation of TRPV1 leads to disruption of the BBB following cerebral ischemia/reperfusion injury (Hu et al., 2005). Capsazepine, a TRPV1 antagonist, reduced permeability levels, suggesting that TRPV1 is involved in BBB dysfunction in ischaemia. The fact that TRPV1 receptor activation stimulates neurogenic inflammation supports a neuropeptide-based mechanism for these events.

Axonal injury was a consistent feature of stroke in the present study, and as observed in the previous chapters, treatment with NAT was able to reduce the extent of axonal injury within the white matter. Similarly, capsaicin treatment also markedly reduced axonal injury, such that fewer and smaller retraction balls were observed. A reduction in axonal injury may have been one mechanism whereby treatment with capsaicin was able to convey some protection from the ischaemic insult. Although the mechanism whereby capsaicin was able to reduce axonal injury is unknown, this may partially account for improvement in functional outcome, and in particular motor deficits, observed in these animals.

A reduction in the influx of macrophages was also observed in both NAT- and capsaicin-treated animals, as evidenced by a reduced number of ED-1 positive cells within the infarct. The reduced macrophage response may be partially explained by the role of SP as a chemotactic factor for monocytes (Ruff et al., 1985). In addition, SP may induce the release of cytokines, eg IL-6, IL-1 β and TNF- α from inflammatory cells such as neutrophils and macrophages (Delgado et al., 2003). Therefore, NAT and capsaicin reduced the SP signal following stroke, thereby reducing the chemotactic signal for monocyte recruitment into the tissue. A reduced number of macrophages may have been beneficial as these inflammatory cells are major sources of pro-inflammatory cytokines such as IL-1 β (Mabuchi et al., 2000).

8.5 Conclusions

Ablation of neuropeptides by treatment with capsaicin provided protection from the ischaemic insult. Specifically, functional deficits were markedly reduced and histological abnormalities were reduced in the capsaicin group compared to vehicles. However, the overall trend was that NAT treated animals performed superior to the capsaicin pre-treated group, indicating that NAT administration is more effective in reducing functional deficits than capsaicin. These results indicated that one or more of the neuropeptides may have a beneficial role post-stroke, and this is most likely to be CGRP. Nevertheless, depletion of neuropeptides provides a degree of protection from the development of functional deficits and histological abnormalities following ischaemic stroke, confirming that sensory neuropeptides play a significant role in the post-ischaemic secondary injury process and may offer a novel target for development of interventional

pharmacological strategies. The further investigation of the role of CGRP in stroke is beyond the scope of the present thesis.

CHAPTER 9:
GENERAL DISCUSSION

The present thesis characterises the effects of an NK₁ receptor antagonist on BBB permeability, cerebral oedema, infarct volume, histological abnormalities and functional deficits following reversible ischaemic stroke. Few previous studies have examined the role of SP in cerebral ischaemia (Bruno et al., 2003; Stumm et al., 2001; Yu et al., 1997), and accordingly, the findings presented in this thesis represent the most extensive characterisation to date of an NK₁ receptor antagonist in stroke.

Initially, we were able to demonstrate that an increase in SP immunoreactivity occurred within the infarct border zone following stroke. Such an increase in SP immunoreactivity was a feature of ischaemic stroke with reperfusion and was not observed in permanent stroke where there was no reperfusion of the ischaemic territory. The latter finding was consistent with a previous study of cerebral ischaemia (Fu et al., 2004), where a lack of SP immunoreactivity was observed following pMCAO. The authors suggested that SP is not directly involved in the injury process that occurs following stroke without reperfusion, and our findings support this hypothesis. However, there was a striking difference in the pattern of SP immunoreactivity between the pMCAO and tMCAO groups that has not previously been reported. The increase in SP within the infarcted and reperfused hemisphere was observed as early as 5 h post-reperfusion, but was most pronounced at 24 h post-reperfusion. Specifically, increased SP was observed within glial and neuronal tissue within the cortex, but was particularly apparent at the perivascular level, with increased SP immunoreactivity observed around blood vessels within the ischaemic penumbra. The increase in SP levels was quantitatively confirmed using ELISA. These results indicated that the increase in SP observed by

immunohistochemistry was due to a significant increase in SP protein. These novel findings were consistent with previous studies (Bruno et al., 2003), which reported increased SP levels in the serum of SP patients with TIA or stroke, and suggesting that the cellular source of increased SP in cerebral ischaemia was the perivascular fibres. An increase in SP levels at 24 h following stroke is consistent with BBB opening and cerebral oedema at this time-point (Preston et al., 1993), supporting a role for SP in these events. Indeed, increased SP levels are associated with the development of neurogenic inflammation in peripheral tissues, characterised by increased vascular permeability and tissue swelling. Furthermore, previous studies from our laboratory have shown that SP release is a consistent feature of brain injury and that the NK₁ receptor contributes to BBB dysfunction, cerebral oedema and functional deficits (Donkin et al., 2007; Nimmo et al., 2004; Vink et al., 2003).

Having established that SP release was a feature of stroke with reperfusion, we next characterised the effects of antagonism of the SP pathway. As such, an NK₁ receptor antagonist (NAT) was used, with specific attention paid to its' effects on BBB permeability, cerebral oedema, functional outcome and histological abnormalities. Notably, NAT treatment was able to decrease brain SP levels to levels comparable to shams. Such findings prompted consideration of an autoreceptor for SP, documented by several research groups in the skin (Lever et al., 2003), gastrointestinal tract (Patacchini et al., 2000), spinal cord (Lever et al., 2003) and brain (Levesque et al., 2007). Autoreceptors are those neuronal receptors that respond to their agonist with alterations in transmitter release (Kalsner et al., 2000). For example, the NK₁ autoreceptor may respond to increased SP levels within the synapse by reducing SP release through negative feedback (Malcangio and Bowery,

1999). Consistent with this, the release of SP has been reported to be greater in NK₁ receptor knockout mice compared to wild type mice (Malcangio and Bowery, 1999). It has therefore been proposed that NK₁ autoreceptors may be relevant to the modulation of neurogenic inflammation and may participate in pathological events (Lever et al., 2003). The fact that an NK₁ receptor antagonist also modulated SP release in our studies supports the concept of an NK₁ autoreceptor. However, given that the NK₁ receptor antagonist decreases SP release suggests that it may not necessarily be a strictly negative feedback loop, and this requires further study.

A profound disruption of the BBB was observed at 24 h post-reperfusion and this was associated with significant cerebral oedema. These events also correlated with the increased SP seen at 24 h post-stroke. Administration of the NK₁ receptor antagonist completely ameliorated BBB dysfunction and cerebral oedema. Furthermore, these improvements in BBB status and brain water content were observed in the setting of reduced SP levels, as evidenced by the ELISA results, suggesting that SP is integral to these events post-stroke, and furthermore, that the oedema observed was of the vasogenic type. Studies in NK₁ receptor knockout mice have also shown that they are unable to produce oedema (Cao et al., 1999), confirming a potential role of the NK₁ receptor in oedema formation. Our findings represent the first investigation of an NK₁ receptor antagonist on cerebral oedema and BBB following stroke, and confirm the hypothesis that activation of NK₁ receptors on the vascular endothelium may contribute to tissue swelling (Stumm et al., 2001).

In addition to measurement of BBB permeability and oedema, we also characterised the effects of the NK₁ receptor antagonist on functional outcome. To date, no other studies have extensively evaluated functional outcome after stroke following NK₁ receptor antagonist administration. Functional outcome was assessed using a battery of functional outcome tests, purported to be the most effective means of assessing the functional capacity of animals following stroke (Corbett and Nurse, 1998; DeVries et al., 2001; Rogers et al., 1997; Schallert, 2006). The rotarod was used as the primary measure of motor function. The rotarod has been used with great success in models of TBI and stroke (Hunter et al., 2000; Nimmo et al., 2004; Smith et al., 1997; Vink et al., 2003; Zausinger et al., 2000) and is considered the most sensitive motor test due to its' incorporation of both gross and fine motor components (Hamm et al., 1994). NAT administration produced a profound recovery from rotarod deficits following stroke.

The bilateral asymmetry test was used as a measure of sensory function following stroke. This "sticky label" test is effective because it is resistant to practice (Modo et al., 2000b), and assesses tactile extinction while probing sensory neglect (Schallert et al., 1982). It is suitable for use in stroke since animals subject to right-side MCAO frequently experience sensory deficits on the contralateral (LHS) side (Modo et al., 2000b), as was consistently observed in the present study. NAT treatment produced a marked improvement in sensory function. Such an effect on sensory function has never before been documented following NK₁ receptor antagonist treatment in stroke. Another outcome test used in the present study was the open field test of spontaneous exploratory behaviour, as modified from that of Guilian and Silverman (Giulian and Silverman, 1975). Spontaneous exploratory

behaviour is an important indicator of the general well being of the animal. NAT produced a complete recovery in spontaneous exploratory behaviour. A modified neuroseverity score was also used to assess overall neurological function following stroke (Li et al., 2000b). Many experimental stroke studies use a basic neuroscore to assess animals (Bederson et al., 1986b; Mary et al., 2001; Yu et al., 1997; Zhang et al., 2003), and the present study also demonstrated that the NK₁ receptor antagonist was effective in significantly improving this outcome parameter. Finally, the angleboard was used to assess hemiparesis, which was particularly pronounced following stroke. Treatment with an NK₁ receptor antagonist completely ameliorated hemiparesis, which is a common long-term complication of human stroke (Plummer et al., 2007; Rijntjes, 2006). All of the behavioural measures used in the studies outlined in this thesis were easy to carry out, required no pre-training, were cost-effective and were not stressful to the animals. Taken together, they provided an accurate picture of the functional capacity of the animals, with respect to motor, sensory and neurological function, and the effectiveness of an NK₁ receptor antagonist to improve functional outcome following ischaemic stroke.

The attenuation of functional deficits following antagonism of the NK₁ receptor was observed in association with improvement in histological outcome. In particular, a marked reduction in the extent of axonal injury within the white matter was observed. APP is a particularly sensitive marker of axonal injury, as documented in many studies of TBI (Blumbergs et al., 1995; Povlishock, 1992; Povlishock, 1993; Van Den Heuvel et al., 1999; Van Den Heuvel et al., 1998). NAT treatment markedly reduced the number of axonal swellings within the white matter. Although the mechanism whereby the NK₁ receptor antagonist was able to afford a

degree of protection from axonal injury following stroke is unclear, it may partially account for the improvements in functional outcome observed.

Increases in GFAP immunoreactivity within the infarct border zone and within perivascular tissue were also apparent following stroke. Interestingly, NAT treatment further increased this response to ischaemia. Although the interpretation of this result is not readily apparent, given that the role of astrocytes following stroke is ill defined, it suggests a potential protective function of astrocytes. An increase in GFAP staining within the infarct boundary zone may be a protective function whereby the brain is trying to re-establish the integrity of the BBB. The more immature phenotype displayed by these astrocytes, as evidenced by their elongated processes, may provide an environment that is conducive to regeneration and neurogenesis (Cramer and Chopp, 2000). Also, astrocytes are able to produce a number of trophic factors that may facilitate neuronal survival, such as bFGF, TGF- β , NGF and VEGF, amongst others (Chen and Swanson, 2003).

The inflammatory response following stroke has been well characterised and involves the complex interaction of inflammatory cells and mediators. ED-1 staining revealed a profound macrophage/activated microglia response to ischaemia, which was markedly reduced in NAT-treated animals. This was not altogether surprising seeing as SP is known to initiate and regulate the immune response (Guo et al., 2004). As such, a dampened SP response in the setting of an NK₁ receptor antagonist would reduce inflammatory cell recruitment and pro-inflammatory cytokines within the brain tissue. This may have been reflected in the modest preservation of tissue observed in these animals.

No reduction in infarct volume was observed in these studies despite functional improvement across a number of behavioural measures. However, this is not an unusual observation. Many experimental studies have now reported remarkable improvements in functional outcome which were not accompanied by a reduction in lesion volume or histological abnormalities (Grotta et al., 1988; Grotta et al., 1990; Aronowski et al., 1994; Aronowski et al., 1996; van der Staay et al., 1996). This is despite the extensive reporting of infarct volume as an outcome parameter in the early stroke literature. As such, ischaemic lesion volume may not be as accurate a predictor of functional capacity as the early literature suggests. Hence, many researchers are now suggesting that it is the synaptic plasticity of the peri-lesional area that is important for functional recovery, rather than salvage of the tissue volume per se (Gladstone et al., 2002). Indeed, one could argue that it is the functional capacity of an individual that is most important following stroke. Consistent with this, clinical studies use functional measures and quality of life scales to determine the functional capacity of individuals following stroke, not infarct volumes (Foell et al., 2003; Jennett and Bond, 1975; Panicker et al., 2003). Accordingly, it was extremely important to extensively evaluate the functional capacity of animals following stroke throughout the present studies.

Taken together, the findings of chapter 4 clearly demonstrated that an NK₁ receptor antagonist can improve functional outcome following stroke, presumably through maintenance of normal BBB status, brain water content and by improving a number of histological abnormalities. Although these findings were extremely encouraging, it was essential to determine whether an NK₁ receptor antagonist was suitable for use in conjunction with thrombolytic agents, as thrombolysis with tPA is currently

the only approved therapy for ischaemic stroke (Kwiatkowski et al., 1999). As such, chapter 5 evaluated the efficacy of combination treatment using an NK₁ receptor antagonist with tPA. These studies demonstrated that adjunctive therapy with NAT was a highly efficacious therapeutic intervention following ischaemic stroke. Profound improvements in functional outcome were observed in association with a reduction in the extent of histological abnormalities. As NK₁ receptor antagonists have not previously been investigated in conjunction with tPA, the mechanism of action is unknown. However, it is likely that NAT was able to afford protection from the neurotoxic actions of tPA, including those involving MMPs at the level of the BBB (Kaur et al., 2004). Indeed, previous studies have reported on the efficacy of adjunctive therapies on reducing the toxicity of tPA (Asahi et al., 2000; Lapchak et al., 2002; Wang and Lo, 2007), although the mechanism of such actions were also unknown. The present study demonstrated effects on axonal injury, reactive gliosis and reversible cellular injury. In particular, the combination therapy of NAT/tPA markedly reduced the degree of cortical and white matter damage, as evidenced by a reduction in axonal injury, less extensive reactive gliosis and a preservation of normal cerebral architecture. Furthermore, the risk of haemorrhagic transformation and death were markedly reduced, common complications of thrombolytic therapy.

Having established that NAT was effective in improving outcome following stroke and that it could be used safely and effectively in conjunction with tPA, it was essential to determine the therapeutic window for administration, and this was addressed in chapter 6. Many other potential neuroprotective agents have been investigated in experimental stroke (Gladstone et al., 2002; Heiss et al., 1999).

Unfortunately, none of these have been successful in the setting of clinical stroke. Functional and histological outcome studies showed that NAT could be administered up to 8 h after the onset of ischaemia with significant improvements in motor, sensory and neurological outcome, as well as histological outcome. This 8 h treatment window represents a significant therapeutic window, that is 2.5 times greater than that of the current therapy, tPA. It is essential for any potential neuroprotective agent to have a clinically relevant treatment window. Despite this, some previous studies have only demonstrated efficacy of a compound when administered before or shortly after the onset of ischaemia (Green, 2002). Indeed, tPA is often criticised for its' short therapeutic window and the increased incidence of ICH associated with its' use (Hill and Hachinski, 1998; Lees, 2000). However, with advanced imaging and the use of effective adjunctive treatments, many of these problems may be overcome. Whether an NK₁ receptor antagonist can increase the window for tPA therapy remains to be seen, and this was beyond the scope of the present study. The effects on BBB status, cerebral oedema and functional outcome are likely to account for the efficacy of the NK₁ receptor antagonist adjunctive therapy.

As stroke is an extremely heterogenous condition, encompassing many sub-types and severities, it was important to ascertain whether an NK₁ receptor antagonist was effective in different severities of stroke. As such, chapter 7 evaluated the administration of NAT following mild, moderate and severe ischaemia. In these studies, NAT was administered at the upper end of the therapeutic window, 8 h after stroke onset. Consistent with previous studies (Garcia et al., 1995a), mild ischaemia produced only minor histological abnormalities and modest functional deficits. As

such, NAT treatment did not produce a profound improvement, due to the relatively benign level of injury observed in these animals. However, a modest reduction in the number of degenerating and injured cells was observed in the mild ischaemia group following NAT treatment. The most profound histological abnormalities were observed in the moderate and severe ischaemia groups, indicating that 1.5 h is the duration of ischaemia where the ischaemic injury becomes more severe in nature. Similar findings have been reported in other studies of MCAO (Li et al., 1999). Significant improvements in motor, sensory and neurological function were observed in the moderate and severe groups following NK₁ receptor antagonist administration, indicating that the NK₁ receptor antagonist was particularly beneficial in stroke of a moderate to severe grade. Moreover, the NK₁ receptor antagonist was significantly effective in improving outcome at these levels of injury, even when administered 8 h after stroke onset.

Having clearly established a role for SP in stroke we were curious to determine whether other neuropeptides were involved. As such, in chapter 8 capsaicin pre-treatment was used to deplete all neuropeptides prior to MCAO, and observe the effect on post-stroke outcome. Depletion of sensory neuropeptides by capsaicin pre-treatment was found to be protective following stroke. However, treatment with the NK₁ receptor antagonist was generally superior in improving outcome following stroke, in regards to functional and histological outcome. Previous, studies of ischaemia/reperfusion injury in peripheral tissues have shown that ablation of all neuropeptides can exacerbate injury (Dembinski et al., 2003; Turchanyi et al., 2005). Depletion of all neuropeptides was not as effective as an NK₁ receptor antagonist in the present study and this may be due to the beneficial effects of one

or more of the neuropeptides in cerebral ischaemia acting via the NK₂ or NK₃ receptor (Figure 9.1). Although, previous studies from our laboratory have shown that neuropeptide depletion with capsaicin pre-treatment completely ameliorated the BBB dysfunction, cerebral oedema and functional deficits observed following TBI (Nimmo et al., 2004; Vink et al., 2003), a protective role for CGRP following ischaemia has previously been proposed (Gherardini et al., 1996). CGRP is a potent vasodilator with the capacity to increase cerebral blood flow (Wimalawansa, 1996). Although CGRP alone cannot induce neurogenic inflammation, in the presence of SP it is able to potentiate this process (Black, 2002; Richardson and Vasko, 2002). Thus, antagonising the SP response following stroke with the NK₁ receptor antagonist is preferable to ablation of all neuropeptides for several reasons. As our results have conclusively demonstrated, SP is deleterious following ischaemia and blocking its' action is beneficial. In contrast, CGRP may produce valuable vasodilatory actions in stroke without potentiation of neurogenic inflammation. Consistent with this, CGRP was found to improve the survival of ischaemic surgical flap tissue (Kjartansson, 1987; (Bucinskaite et al., 1998). Furthermore, the release of CGRP has been proposed to be a generalised response of cerebral tissue to injury (Dragunow et al., 1992; Gherardini et al., 1996) that may directly promote the survival of damaged neurons (Bulloch et al., 1998; Dragunow et al., 1992), as well as nerve regeneration (Wimalawansa, 1996). Finally, CGRP immunoreactivity within the CA1 neurons of the hippocampus has been associated with increased neuronal survival (Bulloch et al., 1998).

Heretofore, the findings of this thesis clearly demonstrate the efficacy of NK₁ receptor antagonists following stroke. It is apparent that SP has pleiotropic actions

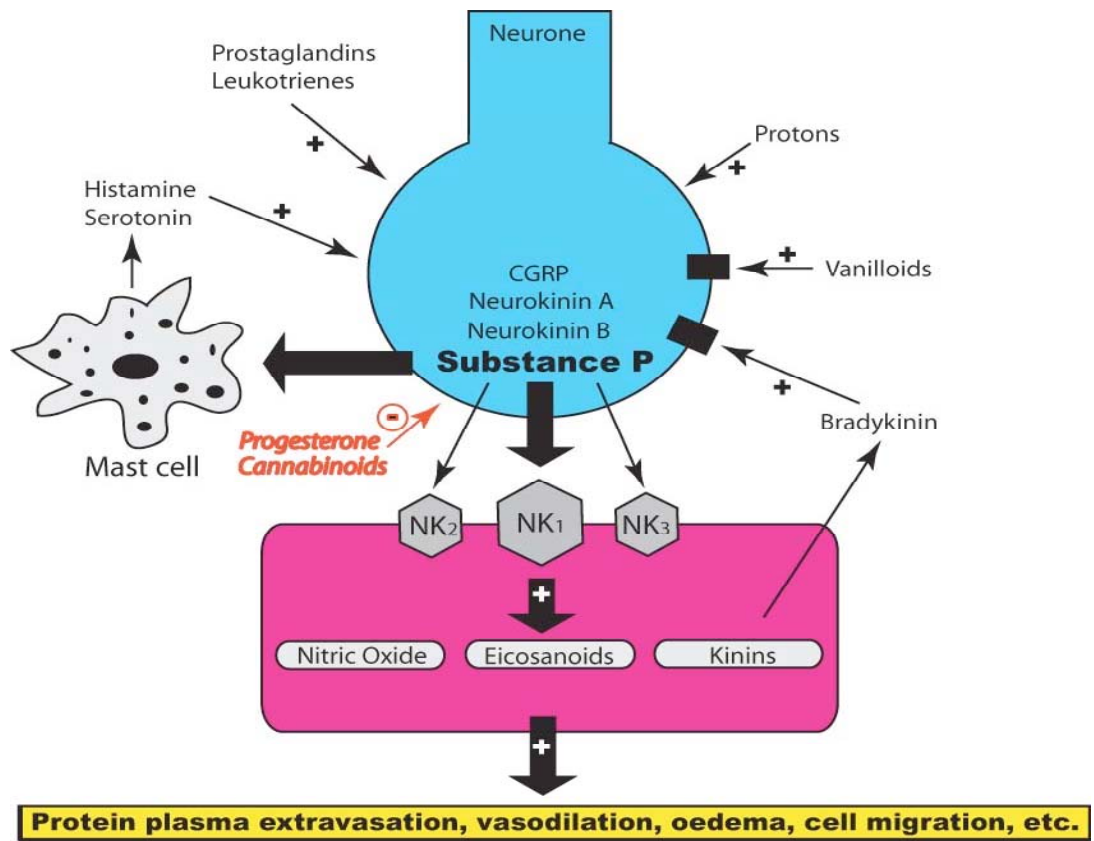


Figure 9. 1 The involvement of SP and neurogenic inflammation in CNS injury (Turner and Vink, 2007).

beyond that of neurogenic inflammation and increased vascular permeability at the BBB level, as evidenced by the improvements in functional outcome and histological abnormalities observed across the studies. Although the exact mechanisms whereby an NK₁ receptor antagonist was able to convey neuroprotection following stroke is unknown, there are a number of possibilities. With regard to the inflammatory response after ischaemia, it is well known that SP plays a role in the initiation and modulation of inflammation. Many studies have documented the effects of SP on inflammatory cells and their actions, including the fact that SP is a chemotactic factor for inflammatory cells including neutrophils (Braun et al., 1996) and monocytes (Ruff et al., 1985). As such, SP may be involved in the recruitment of inflammatory cells into the infarct, thus contributing to the inflammatory response. SP also induces the dose-dependent release of pro-inflammatory cytokines from inflammatory cells, such as IL-1, TNF- α (Lotz et al., 1988) and IL-6 (Brain, 1997; Yamaguchi et al., 2004), thereby potentiating the inflammatory response. SP has also been shown to stimulate superoxide production (Serra et al., 1988; Hafstrom et al., 1989), lysosomal enzyme release and phagocytic activity by polymorphonuclear cells (Bar-Shavit et al., 1980), thus exacerbating injury (Siesjo et al., 1996). As such, SP may play an important role in orchestrating the immune response following stroke.

Although a role for SP in the regulation of MMPs has not been proposed in the brain, a role for SP in extracellular matrix metabolism has previously been reported in peripheral tissues. In lung tissue, SP produces an upregulation of MMP-1 expression (Ramos et al., 2007) and has also been correlated with increased MMP-12 levels in chronic obstructive pulmonary disease (Xu et al., 2007). SP can also

induce the secretion of MMP-2 from human synovial fibroblasts, whilst increasing overall MMP activity. It is feasible that SP may also have similar effects on MMPs within the brain as it does in peripheral tissues, further implicating SP release in the barrier dysfunction observed following stroke. Any potential interaction of SP with these extracellular matrix proteases has implications for the stability and integrity of the blood vessels, and therefore on cerebrovascular permeability and haemorrhagic transformation, as these proteases are integral to BBB integrity. Interestingly, in lung tissue, infusion of SP induced a dose-dependent increase in tPA release and activity, accompanied by increased blood flow (Newby et al., 1999; Newby et al., 2001). Although there are no published reports of an association between SP and tPA within the brain, it is tempting to speculate that similar events may occur in the brain as in peripheral tissue, thus linking the later infusion of tPA to an increased incidence of haemorrhage. Clearly, the effects of SP on tPA and the plasminogen system, and their interaction in haemorrhagic transformation, requires further study.

In the present study, cell death pathways were clearly activated or potentiated by increased SP levels. It has recently been shown that SP induces a non-apoptotic form of programmed cell death (Castro-Obregon et al., 2002), and that this cell death pathway is mediated by the NK₁-SP receptor-ligand pair. Therefore, excess SP may have contributed to cell loss following cerebral ischaemia. SP and the excitatory neurotransmitter glutamate are closely linked (Afrah et al., 2001; Malcangio et al., 1998; Benoliel et al., 2000; Lieberman and Mody, 1998; Marvizon et al., 1997; Stacey et al., 2002). SP is able to exert feedback to promote glutamate release and therefore potentiate excitotoxicity (Lieberman and Mody, 1998; Stacey et al., 2002). Excessive glutamate release is associated with markedly increased

Ca²⁺ flux into cells and over-activation of the NMDA receptor, culminating in cell death (Choi, 1992; Rothman and Olney, 1987). The effects of the NK₁ receptor antagonist on neuronal cell death in the present study are consistent with these observations.

9.1 Conclusions

In the present thesis, I have demonstrated that an NK₁ antagonist is beneficial in reducing blood brain barrier permeability, cerebral oedema, functional deficits and neuronal damage following ischaemic stroke. In addition, combination of the NK₁ receptor antagonist with tPA reduced the haemorrhage and neurotoxicity associated with tPA administration, whilst also markedly improving functional and histological outcome. The NK₁ receptor antagonist was highly efficacious even when administered up to 8 h following stroke of moderate or severe grades. Nonetheless, as has become apparent in the study of many other neurological conditions, there is unlikely to be a single “magic bullet” that can result in significant improvement in outcome. Successful treatment strategies will be those that target a number of aspects of the injury (Ginsberg, 2003; Heiss et al., 1999). Future stroke treatments may include thrombolysis in conjunction with neuroprotective agents administered at various time points along the post-stroke course, followed by active rehabilitation programs. In the present study adjunctive therapy of an NK₁ receptor antagonist with tPA was able to reduce the neurotoxicity associated with tPA administration by reducing the risk of haemorrhage and death, reducing reperfusion injury, maintaining barrier integrity and improving functional outcome. As such, administration of an NK₁ receptor antagonist with tPA may represent a novel adjunctive therapy for the clinical management of acute ischaemic stroke.