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

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CHAPTER 4:
CHARACTERISATION OF N-Acetyl-L-Tryptophan
TREATMENT IN ACUTE ISCHAEMIC STROKE

4.1 Introduction

The experiments detailed in chapter 3 demonstrate that increased SP immunoreactivity occurs following acute ischaemic stroke, supporting the presence of neurogenic inflammation. As previously discussed in chapter 1, activation of NK_1 receptors leads to neurogenic inflammation, a process characterised by vasodilation, increased vascular permeability and tissue oedema (Black, 2002; Richardson and Vasko, 2002). The efficacy of NK_1 receptor antagonists in ameliorating neurogenic inflammation has been extensively demonstrated in peripheral tissues such as the skin (Holzer, 1998; Saria et al., 1983). However, since central NK_1 receptors are similar in function to those in the periphery (Stumm et al., 2001), neurogenic inflammation may also be involved in the genesis of cerebral oedema. Recent studies in TBI from our laboratory confirmed this, demonstrating that neurogenic inflammation is a key process in the breakdown of the BBB and in the development of oedema, particularly vasogenic oedema, ultimately leading to worsened functional outcome (Nimmo et al., 2004; Vink et al., 2003).

NK_1 receptor antagonists have previously been shown to be effective in a number of central pathologies including emesis and depression (Rupniak and Kramer, 1999). Administration of a SP antagonist following cerebral ischaemia also reduced infarct volume and associated neurological deficits as measured at 24 h (Yu et al., 1997). Similarly, neuropeptide depletion has previously been shown to be beneficial in reducing BBB dysfunction, cerebral oedema and functional deficits in a model of diffuse TBI (Nimmo et al., 2004; Vink et al., 2003). The present study aims to determine the effects of inhibition of neurogenic inflammation via administration of

an NK_1 receptor antagonist, on BBB permeability, cerebral oedema, infarct volume, functional outcome, histological outcome and SP tissue levels following tMCAO.

4.2 Study Design

Animals (n=91) were randomly assigned to sham, vehicle and NAT-treatment groups, and subject to 2 h tMCAO as required, followed by either 24 h or 7 d of reperfusion (as described in Chapter 2.2.2). Treated animals were then administered either 25 μmoles/kg of NAT or equal volume of saline vehicle via the tail vein at 4 h post-ischaemic onset (2 h post-reperfusion) under general anaesthetic. Drugs were prepared and administered as described in Chapter 2.3.

4.2.1 ELISA

As described in chapter 2.8, the levels of SP within the right hemisphere of sham, vehicle- and NAT-treated animals was determined using ELISA. Briefly, at 24 h post-reperfusion animals were anaesthetised and their brains rapidly removed. The level of SP protein within the right hemisphere was then determined by ELISA using an anti-SP antibody.

4.2.2 Blood Brain Barrier Permeability

At 24 h following reperfusion, brains were removed and processed using the Evan's Blue extravasation protocol to determine the degree of blood brain barrier permeability within the right hemisphere of sham, vehicle- and NAT-treated animals. Details are as described in Chapter 2.7.

4.2.3 Oedema

At 24 h following reperfusion, brains were removed and processed by the wet weight dry weight method to determine the brain water content of the right hemisphere of sham, vehicle- and NAT-treated animals. Details are as previously described in Chapter 2.6. The brain water content was then calculated using the wet weight dry weight formula.

4.2.4 Infarct Volume

As described in detail in chapter 2.7, the degree of infarction in vehicle- and NAT animals at 24 h post-reperfusion was determined using TTC staining. Briefly, at 24 h post-reperfusion animals were anaesthetised and their brains rapidly removed. The brain was cut into 2mm slices and incubated in 3% TTC solution to reveal the extent of the infarction.

4.2.5 Functional Outcome

The functional outcome of animals was assessed using the rotarod, bilateral asymmetry test, neuroscore, open field and angleboard as described in detail in chapter 2.4. Animals were assessed daily for a 7 d period following ischaemia, commencing at 24 h post-surgery.

4.2.6 Histological Outcome

At the pre-determined time-point animals were perfusion fixed with 10% formalin and their brains processed for immunohistochemistry (as detailed in Chapter 2.5). Tissue sections were stained for H&E, SP, APP, GFAP, ED-1 and FJC, and assessed using light and fluorescence microscopy, as appropriate.

4.2.7 Statistical analysis

All parametric data was analysed using analysis of variance followed by individual Student Newman-Keuls post-hoc tests. The neuroscore data was analysed, using the Kruskal-Wallis test followed by Dunn's Multiple Comparison Test. The mortality data was analysed using Fisher's exact test. All data are expressed as mean ± SEM, with the exception of the neuroscore data that is expressed as the median. The level of significance as taken at $p<0.05$.

4.3 Results

4.3.1 Mortality

No mortality was observed in the sham group (uninjured). In vehicle-treated animals, survival over a 7 d period was 50%. In contrast, there was a trend towards an improved survival rate in the NAT-treated group compared to the vehicle-treated group (Figure 4.1), with 80% of the NAT group surviving over 7 d. Power analysis revealed that this difference in mortality rates would have been significant with an n of 18/group.

4.3.2 ELISA (24 h post-reperfusion) - Effect of NAT treatment on SP protein levels

A significant increase in SP protein was observed by ELISA determination, within the infarcted hemisphere at 24 h post-reperfusion (Figure 4.2). Such an increase in SP levels has previously been observed in the serum of patients with TIA and stroke (Bruno et al., 2003). Subsequent administration of NAT significantly lowered SP protein levels within the infarcted hemisphere ($p<0.001$ versus vehicles) to below that observed in shams.
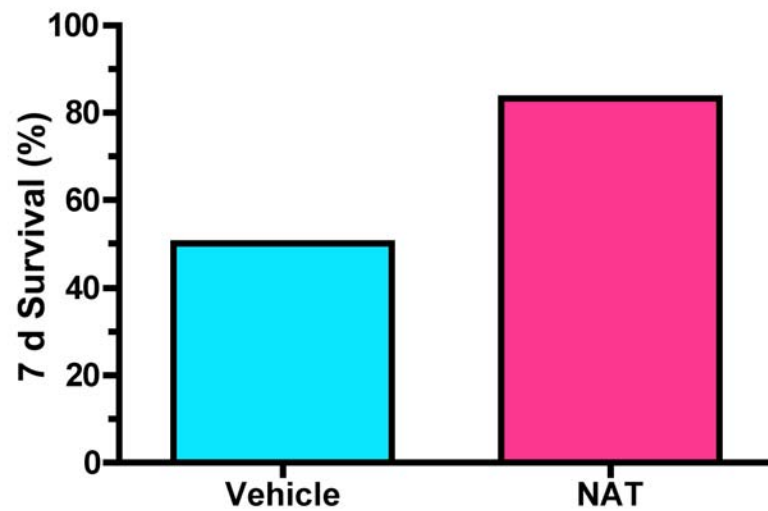
Figure 4.1 NK$_1$ receptor antagonist treatment. Survival at 7 d post-stroke.

At 7 d post-stroke only 50% of the vehicle-treated group survived, whereas 80% of the NAT-treated group survived (p>0.05), however this difference was not statistically significant.
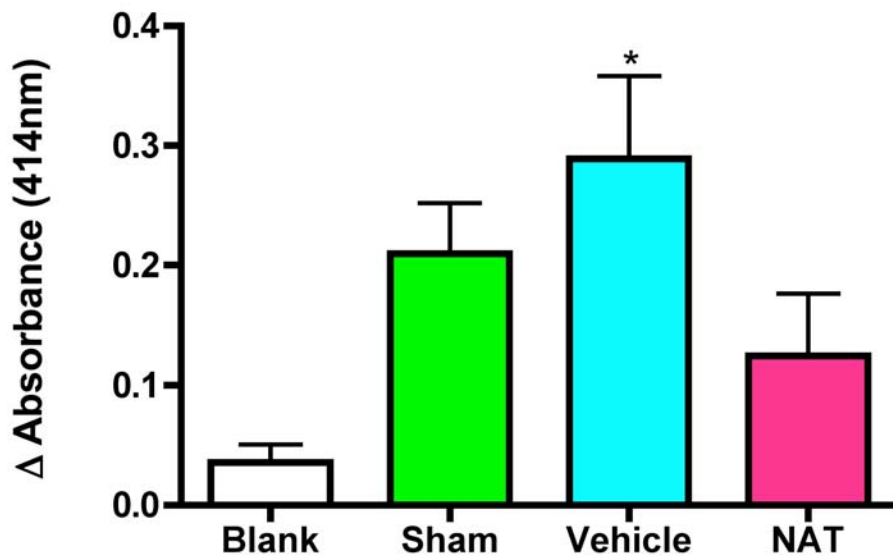
Figure 4.2 NK$_1$ receptor antagonist treatment. Level of SP within the infarcted hemisphere measured at 24 h post-reperfusion, as assessed by ELISA.

Vehicle-treated animals had a significantly greater ($p<0.05$) level of SP within the right hemisphere compared to shams. NAT-treated animals showed a trend towards a lower level of SP within the infarcted hemisphere as compared to vehicle-treated animals, however this was not statistically significant (*denotes $p<0.05$ versus sham; Blank n=2; Sham n=9; Vehicle n=8; NAT n=11).

4.3.3 Blood Brain Barrier (24 h post-reperfusion) - Effect of NAT treatment on blood brain barrier permeability

As shown in Figure 4.3 the extravasation of EB in sham animals was approximately 4.17 ng/mg of brain tissue. This was consistent with the BBB being essentially intact in these animals. The extravasation of EB was significantly ($p < 0.001$) increased in vehicle animals following stroke, increasing to 5.9 ng/mg brain tissue at 24 h post-reperfusion. In contrast, administration of NAT at 4 h post-stroke significantly ($p < 0.001$) reduced EB extravasation to 3.6 ng/mg brain tissue as compared with vehicle animals. This figure was also significantly ($p < 0.001$) lower than the EB extravasation observed in sham animals. These results demonstrate that there was significant opening of the BBB after 2 h tMCAO followed by 24 h of reperfusion, and that administration of NAT was able to significantly reduce the extent of barrier opening following stroke.

4.3.4 Cerebral Oedema (24 h post-reperfusion) - Effect of NAT treatment on brain water content

The hemispheric water content of sham animals was $80.27 \pm 0.70\%$ (Figure 4.4). Following stroke there was a significant ($p < 0.001$) increase in the water content of the ipsilateral (infarcted) hemisphere of vehicle animals to $83.87 \pm 1.79\%$. A non-significant increase in the water content of the contralateral (non-infarcted) hemisphere was also observed in vehicle animals following stroke (results not shown). Administration of NAT decreased the hemispheric water content to $81.12\% \pm 0.87\%$, significantly ($p < 0.001$) lower than vehicle-treated animals. Indeed, the water content in NAT-treated animals was not significantly different
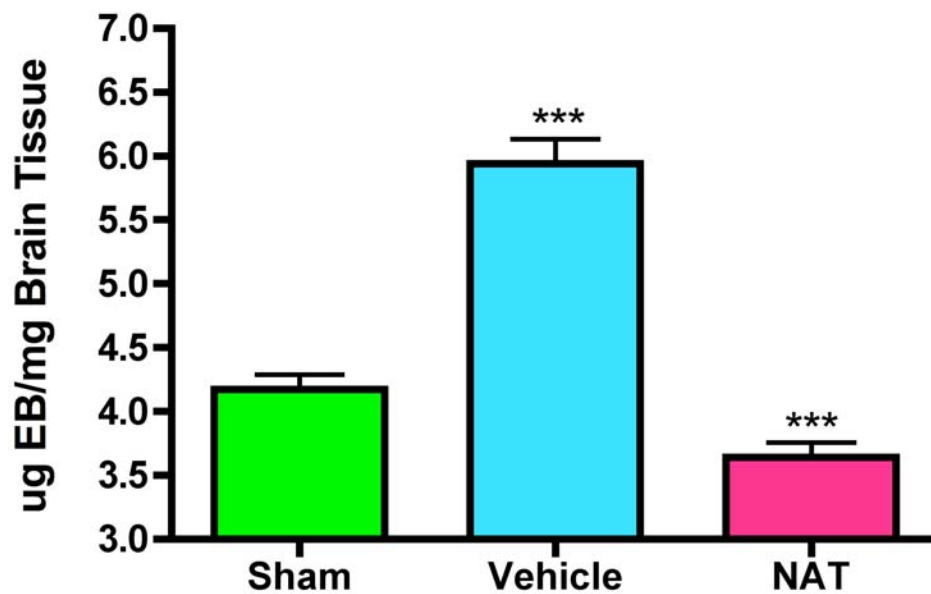
Figure 4.3 NK₁ receptor antagonist treatment.   Blood brain barrier permeability of the infarcted hemisphere measured at 24 h post-reperfusion, as assesses by Evan's Blue extravasation.

Following stroke, there was a significant increase ($p < 0.001$) in the permeability of the BBB to EB.   Treatment with NAT significantly ($p < 0.001$) reduced the permeability of the BBB to levels comparable to shams (**denotes $p < 0.01$; Sham n=7; Vehicle n=7; NAT n=5).
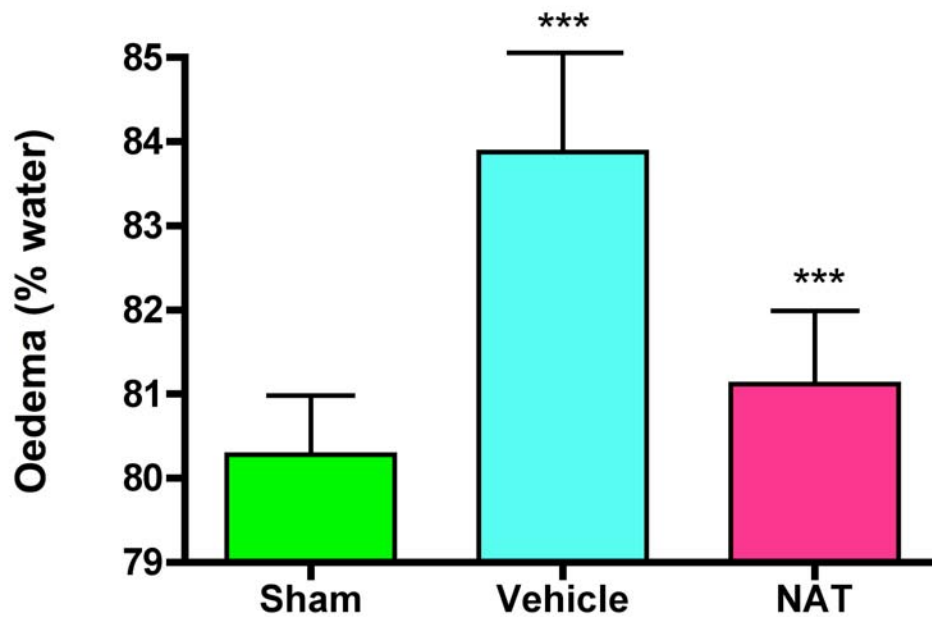
Figure 4.4 NK$_1$ receptor antagonist treatment. Oedema within the infarcted hemisphere measured at 24 h post-reperfusion, as assessed by wet weight dry weight.

Following stroke, there was a significant increase (p<0.001) in the brain water content of the right hemisphere, as compared to sham animals. Treatment with NAT significantly reduced (p<0.001) the brain water content to levels comparable to shams (p>0.05) (***denotes p<0.001; Sham n=7; Vehicle n=7; NAT n=7).

(p>0.05) from sham animals. Hence, NAT-treatment profoundly attenuated oedema formation following ischaemic stroke.

4.3.5 Infarct Volume (24 h post-reperfusion) – Effect of NAT treatment on infarct volume

At 24 h following stroke, extensive infarction of the right hemisphere was observed (Figure 4.5). The infarcted volume occupied approximately 43% of the cortex and 84% of the striatum.   When combined, this represented 48% of the total hemispheric volume. Treatment with NAT did not significantly change the degree of infarction, with 43% cortical, 78% striatal and 46% total infarction observed. Statistical analysis revealed that there was no significant (p>0.05) difference between the vehicle and NAT-treated groups with respect to the degree of infarction.

4.3.6 Functional Outcome

Assessment of Motor Outcome: Rotarod

Sham animals typically demonstrated no motor deficits over the 7 d assessment period, confirming that the surgical procedure did not affect motor function. On day 1 following stroke, vehicle-treated animals were able to complete approximately 30 s out of a maximum of 120 s on the rotarod (Figure 4.6). Over the 7 d assessment period, these animals showed no significant improvement in their ability to perform on the rotarod and did not reach normal functional levels at any time during the post-stroke period. Conversely, NAT-treated animals were able to complete approximately 70 s on the rotarod on day 1 post-stroke and showed a
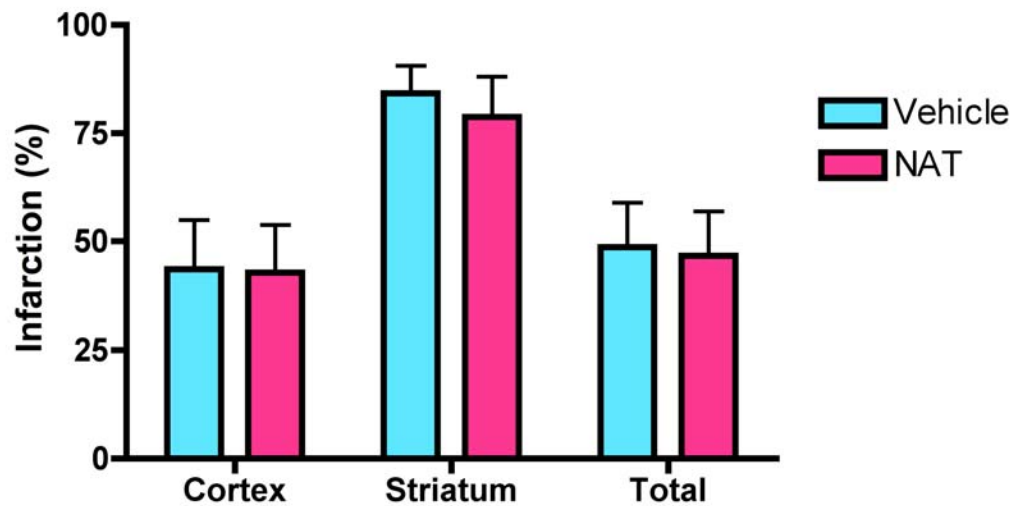
Figure 4.5 NK$_1$ receptor antagonist treatment. Percentage of infarction within the cortex and striatum as assessed by TTC staining.

The percentage of infarction within the cortex and striatum was comparable in both vehicle- and NAT-treated animals. No significant (p>0.05) difference between the degree of infarction was observed between the vehicle- and NAT-treated groups (Vehicle n=7; NAT n=9).

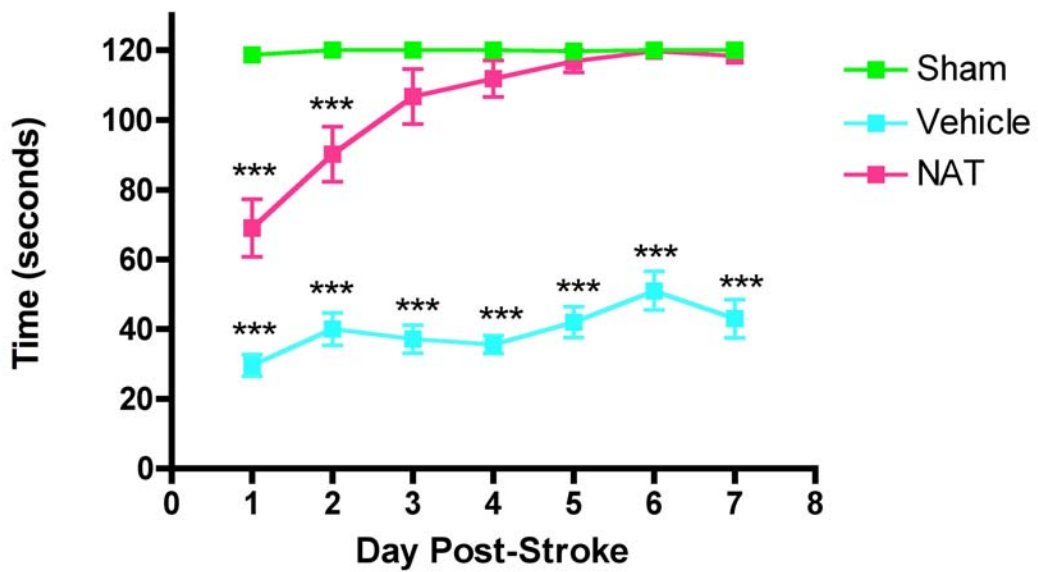**Figure 4.6 NK$_1$ receptor antagonist treatment. Motor performance as assessed by the rotarod.**

Sham animals (green) recorded no motor deficit. Following stroke, vehicle-treated animals (blue) showed profound motor deficits, performing at levels significantly lower (p<0.001) than shams on all days post-stroke. NAT-treated animals (pink) initially displayed motor deficits, scoring significantly lower (p<0.001) than shams on days 1 and 2 post-stroke. However, by day 3 they were performing at levels not significantly (p>0.05) different to sham animals (***denotes p<0.001 versus shams) (Sham n=6; Vehicle n=12; NAT n=6).

rapid improvement in their ability to perform on the rotarod device. By day 4 following stroke, the NAT-treated animals were able to complete the 2 min rotarod task and hence had reached normal functional levels. At all post-stroke time-points, NAT-treated animals performed significantly ($p<0.001$) better than vehicle-treated animals.

Assessment of Tactile Extinction: Bilateral Asymmetry Test

Sham animals remonstrated normal sensory function, rapidly removing the tape on all assessment days. The latency of vehicle-treated animals to remove the tape from their forepaws did not improve over the 7 d assessment period (Figure 4.7). Conversely, NAT-treated animals were able to rapidly remove the tape from their forepaws and by day 4 had reached normal functional levels (latency ≤ 20 s). The differences between the bilateral asymmetry test latencies of the NAT- and vehicle-treated groups were significant ($0.001<p<0.001$) from day 4 post-stroke onwards.

Assessment of Spontaneous Exploratory Activity: Open Field

Sham animals recorded normal activity levels in the open field, indicating that sham surgery had no effect on spontaneous activity. Also noted, some habituation occurred in the sham group over time, and this has been previously well documented in uninjured animals (McIlwain et al., 2001; Paylor et al., 2006; Stohr et al., 1998). On day 1 following stroke, vehicle-treated animals transversed less than 50 squares in the open field and their activity decreased further over the assessment period (Figure 4.8). At no time following stroke did vehicle-treated animals reach normal levels of activity in the open field (≥ 100 squares). In comparison, NAT-treated animals showed relatively normal levels of open field
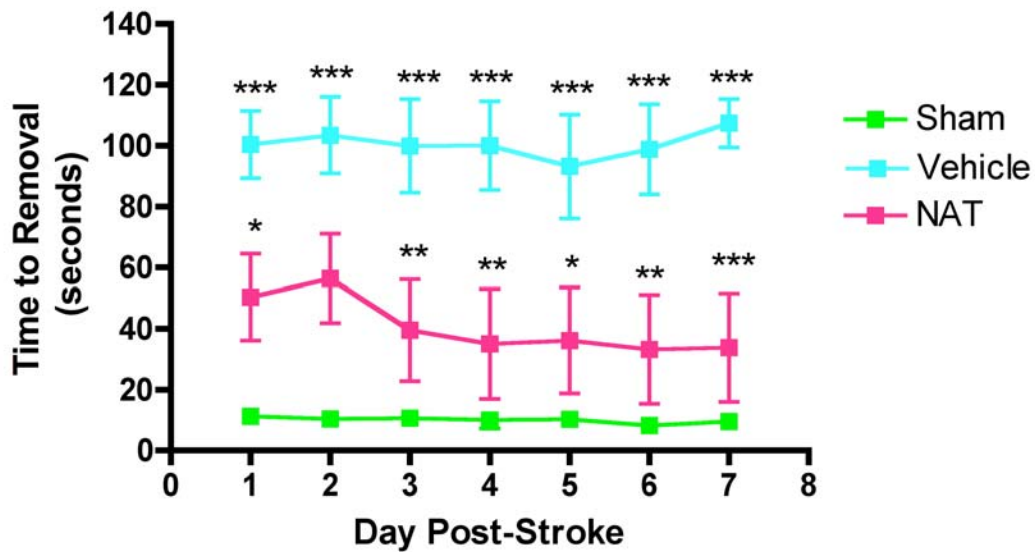
Figure 4.7 NK₁ receptor antagonist treatment. Sensory function as assessed by the bilateral asymmetry test.

Sham animals (green) rapidly removed the tape on all the assessment days and displayed no sensory deficits. Following stroke, vehicle-treated animals (blue) had a significantly increased latency to remove the tape. However, NAT-treated animals (pink) had a significantly lower ($0.05 < p < 0.001$) latency to remove the tape on days 1, 3-7 post-stroke, performing at levels comparable to shams ($p > 0.05$) (*denotes $p < 0.05$; **denotes $p < 0.01$; ***denotes $p < 0.001$; Sham n=6; Vehicle n=12; NAT n=6).
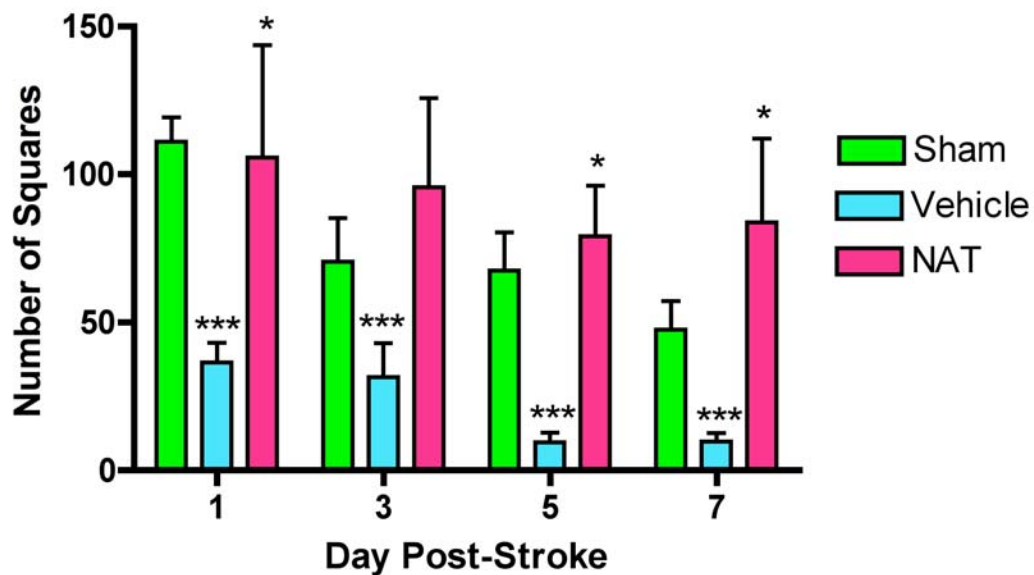
Figure 4.8 NK$_1$ receptor antagonist treatment. Stress and anxiety as assessed by the open field.

Sham animals (green) displayed normal activity levels in the open field. Following stroke vehicle-treated animals (blue) transversed a significantly lower ($p<0.001$) number of squares as competent to shams. NAT-treated animals (pink) showed a significant increase ($p<0.05$) in the number of squares transversed compared to vehicle-treated animals on days 1, 5 and 7 post-stroke, performing at levels comparable to shams ($p>0.05$) (*denotes $p<0.05$ versus vehicle; ***denotes $p<0.001$ versus shams; Sham n=6; Vehicle n=12; NAT n=6).

activity. On day 1 post-stroke, animals in the NAT group transversed approximately 100 squares in the open field and their activity over the assessment period decreased slightly with habituation. Their performance was consistently better than vehicle-treated animals and statistical analysis of the number of squares transversed by the vehicle-treated and NAT-treated animals revealed that there was a significant ($p<0.001$) between group difference. However, the large variability in the treated animals prevented the identification of significant differences within groups.

Assessment of Neurological Function: mNSS

The neurological severity score is the summation of a series of simple motor and sensory tasks and tests of basic reflexes, where a score of 1-4 denotes mild injury, 5-8 denotes moderate injury and 9-12 denotes severe injury. Following stroke, there was a marked difference in the neurological ranking of the saline-treated vehicle animals compared to the NAT-treated animals (Figure 4.9). At all time points following stroke, NAT-treated animals were ranked as having a mild injury, of less than 4, and by day 5 were ranked to have no observable deficit. Conversely, vehicle-treated animals were ranked as having a moderate injury, ranging between 6 and 8, following stroke, this ranking did not improve throughout the 7 d assessment period. As such, there was a significant difference between the median neuroscore ranking of the NAT- and vehicle-treated groups following stroke ($p<0.001$) at all time points following stroke.
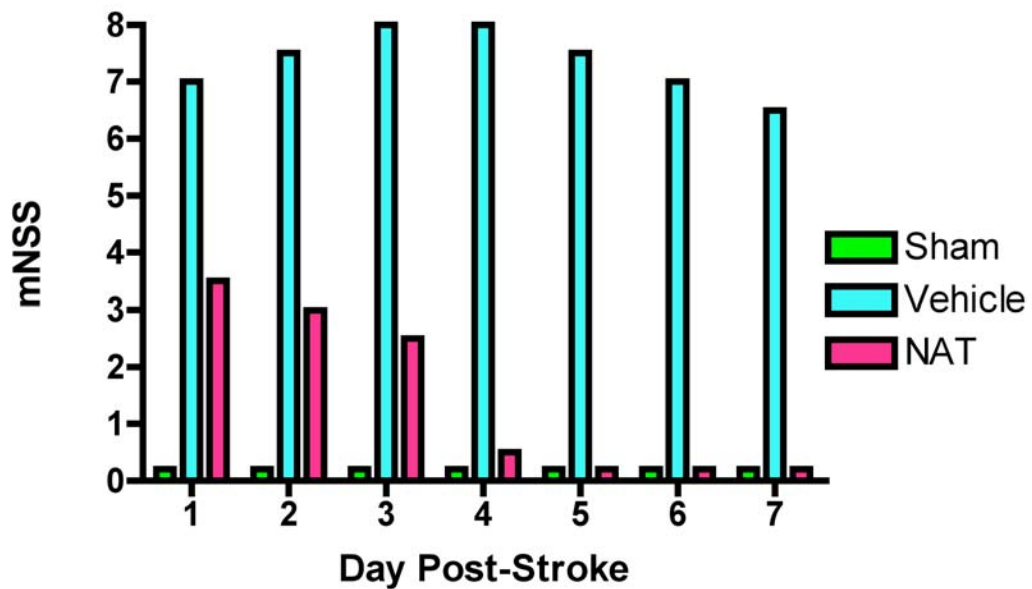
Figure 4.9 NK₁ receptor antagonist treatment. **Neurological function as assessed by the modified neuroseverity score.**

Sham animals (green) recorded no observable neurological deficit on all assessment days post-surgery. Following stroke, vehicle-treated animals (blue) showed significant neurological deficits indicative of severe injury that persisted for the 7 d assessment period. NAT-treated animals (pink) initially demonstrated mild neurological deficits that resolved by day 5 post-stroke (Sham n=6; Vehicle n=12; NAT n=6).

Assessment of Hemiparesis: Angleboard

Sham animals showed no hemiparesis, indicating that the surgical procedure had no effect on muscle strength and balance. Vehicle-treated animals demonstrated profound hemiparesis that persisted for the 7 d assessment period. NAT-treatment resulted in a reduction in the degree of hemiparesis observed. Although NAT-treated animals were able to stay on the angleboard at higher inclines than the vehicle animals at all time-points following stroke, these differences were only significant ($p < 0.05$) on day 4 post-stroke (Figure 4.10).
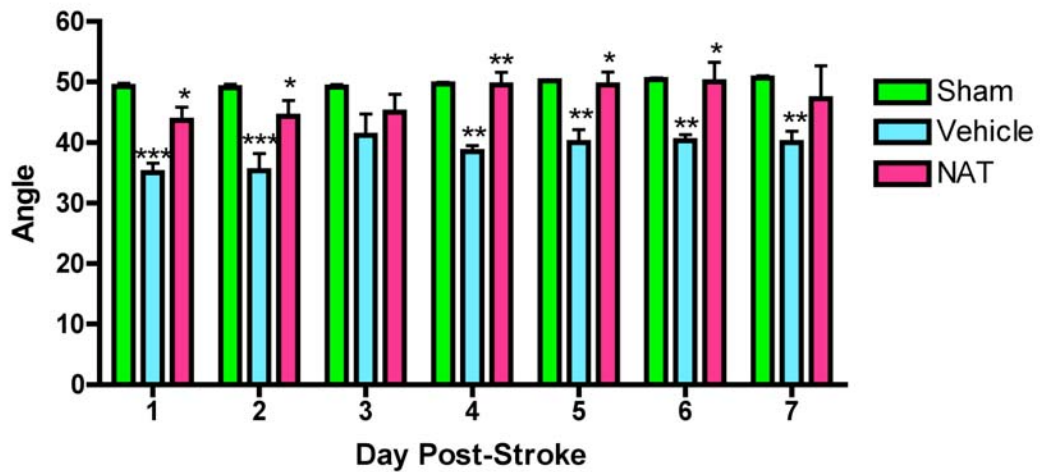
Figure 4.10 NK$_1$ receptor antagonist treatment. Hemiparesis as assessed by the angleboard.

Sham animals (green) showed no signs of hemiparesis. Vehicle-treated animals (blue) showed a marked degree of hemiparesis, scoring significantly lower ($0.01 < p < 0.01$) than shams on days 1-2, 4-7 post-stroke. NAT-treated animals (pink) demonstrated a recovery in angleboard performance, scoring significantly better ($0.05 < p < 0.01$) than vehicle-treated animals on 1-2, 3-6 post-stroke (*denotes $p < 0.05$; ** denotes $p < 0.01$; ***denotes $p < 0.001$; Sham n=6; Vehicle n=12; NAT n=6).

4.3.7 Histological Outcome (24 h, 7 d post-reperfusion) - Effect of NAT treatment on histological outcome

Following MCAO, all the hallmarks of ischaemia such as neuronal loss, oedema and the influx of inflammatory cells were observed in 24 h and 7 d tissue. This has already been described in chapter 3, and the effects of NAT will be the focus on the current chapter.

H&E - General Pathology

H&E staining was used to assess general pathology and to determine whether NAT treatment affected the degree of infarction and cell injury following stroke. TTC staining revealed no reduction in the degree of infarction following NAT treatment (see Figure 4.5). In vehicle-treated animals, shrunken, retracted cells and marked vacuolation of the parenchyma was observed. By 7 d post-reperfusion, extensive reactive gliosis occupied the cortex (Figure 4.11). NAT treatment resulted in preservation of cells and cortical tissue architecture, in addition to reduced parenchymal vacuolation. A modest preservation of cortical architecture and a reduction in the reactive gliosis was still apparent in NAT-treated animals at 7 d after stroke.

No abnormalities were observed within the white matter of sham tissue (Figure 4.12). Following stroke, extensive vacuolation and shrunken cells were observed at 24 h, and this was largely unaffected by NAT administration. By 7 d post-stroke, the vacuolation and cell injury of the white matter was more advanced. Administration of NAT resulted in a modest preservation in white matter architecture.
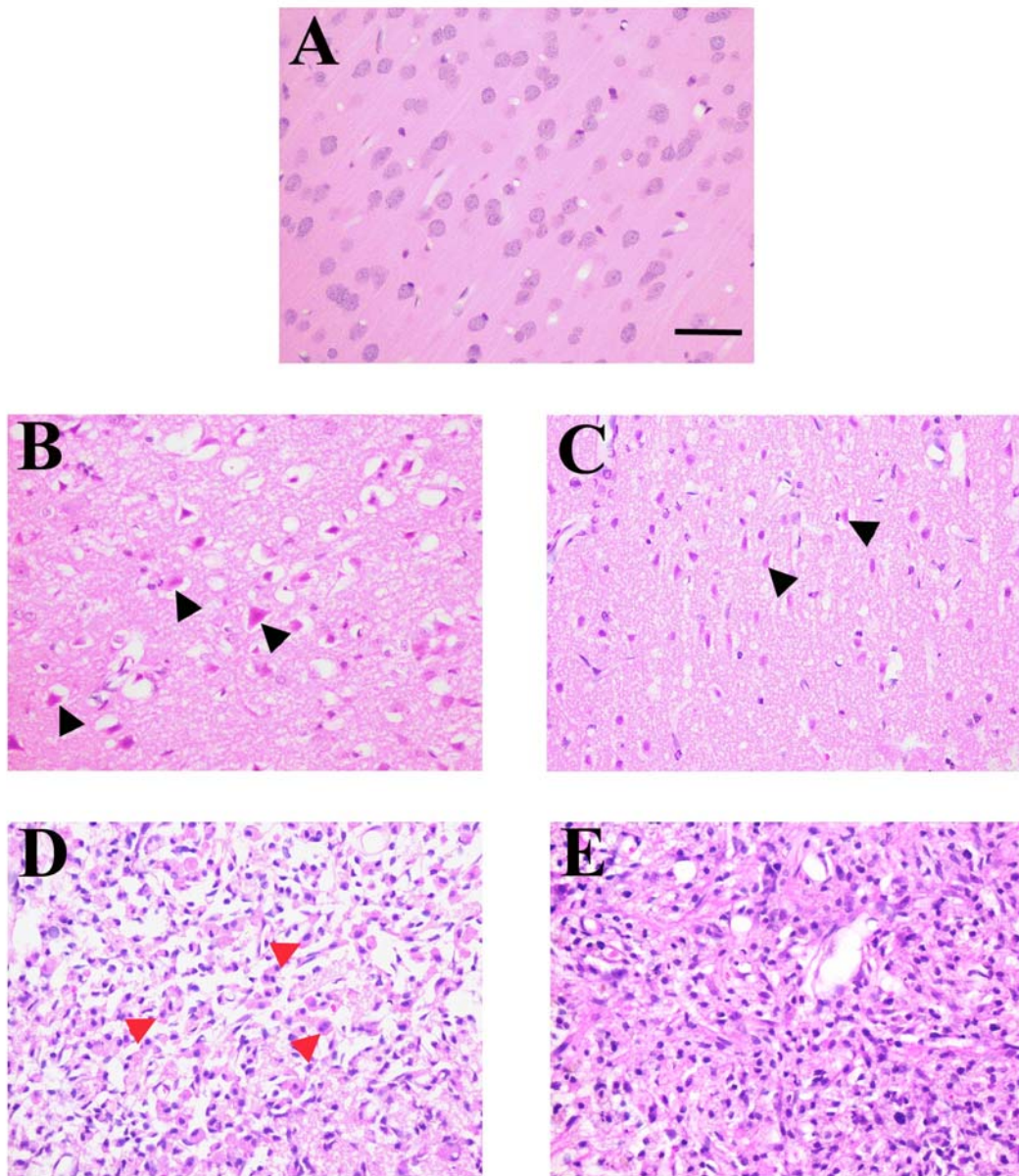
Figure 4.11 NK$_1$ receptor antagonist treatment. Cortex – H&E stained sections (Bar = 100 μm).

No abnormalities were observed in the cortex of sham animals (A). At 24 h following reperfusion (B), extensive infarction characterised by cell injury (black arrowheads) and vacuolation was observed. However, preservation of cortical architecture and cell survival was observed in NAT-treated animals. By 7 d post-reperfusion extensive reactive gliosis occupied the infarct (D), with an influx of macrophages (red arrowheads) also noted. However, treatment with NAT resulted in a modest reduction in the reactive gliosis and a degree of preservation in tissue architecture.
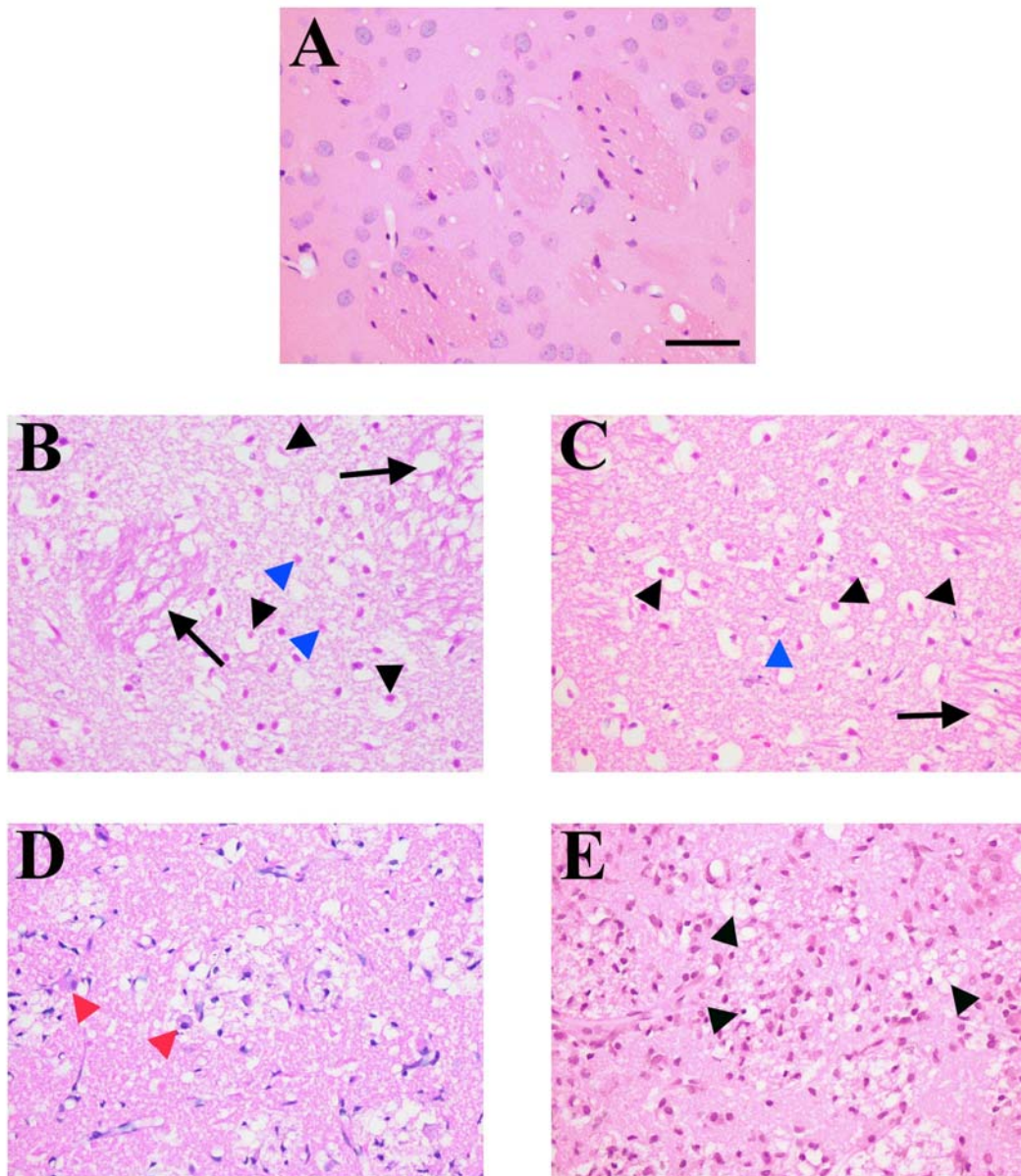
Figure 4.12 NK$_1$ receptor antagonist treatment. White Matter – H&E stained sections (Bar = 100 μm).

No abnormalities were observed in the white matter of sham animals (A). At 24 h post-reperfusion (B) extensive vacuolation of the white matter bundles (arrows) And parenchyma (black arrowheads) were observed, in addition to cell injury (blue arrowheads). Treatment with NAT (C) did not appear to reduced the amount of cell injury or vacuolation within the white matter. By 7 d post-reperfusion (D), profound loss of architecture with evidence of cell loss were observed. Treatment with NAT (E) resulted in a modest preservation in white matter architecture, although tissue vacuolation was still apparent.

SP Immunoreactivity – SP response

SP immunohistochemistry was carried out to determine the effect of NAT treatment on SP immunoreactivity following stroke. In sham animals faint SP immunoreactivity was observed around blood vessels (Figure 4.13). NAT treatment altered the SP response to ischaemia. Although SP immunoreactivity was still observed within perivascular tissue of the infarcted hemisphere it was reduced compared to vehicle-treated animals. By 7 d post-reperfusion, extensive reactive gliosis dominated the infarct, as such, perivascular SP immunoreactivity was not observed in either the vehicle or NAT treatment groups.

The cortex of sham animals (Figure 4.14) showed very faint SP immunoreactivity, consistent with previous reports (Ribeiro-da-Silva and Hokfelt, 2000). At 24 h post-reperfusion there was a marked increase in the SP immunoreactivity of the parenchyma and cortical neurons. Treatment with NAT resulted in a modest reduction in cortical and parenchymal SP immunoreactivity. By 7 d post-reperfusion, extensive reactive gliosis occupied the infarct, such that SP immunoreactivity was not observed in either the vehicle or NAT groups. Nevertheless, NAT administration reduced SP immunoreactivity within cortical and perivascular tissue post-stroke.
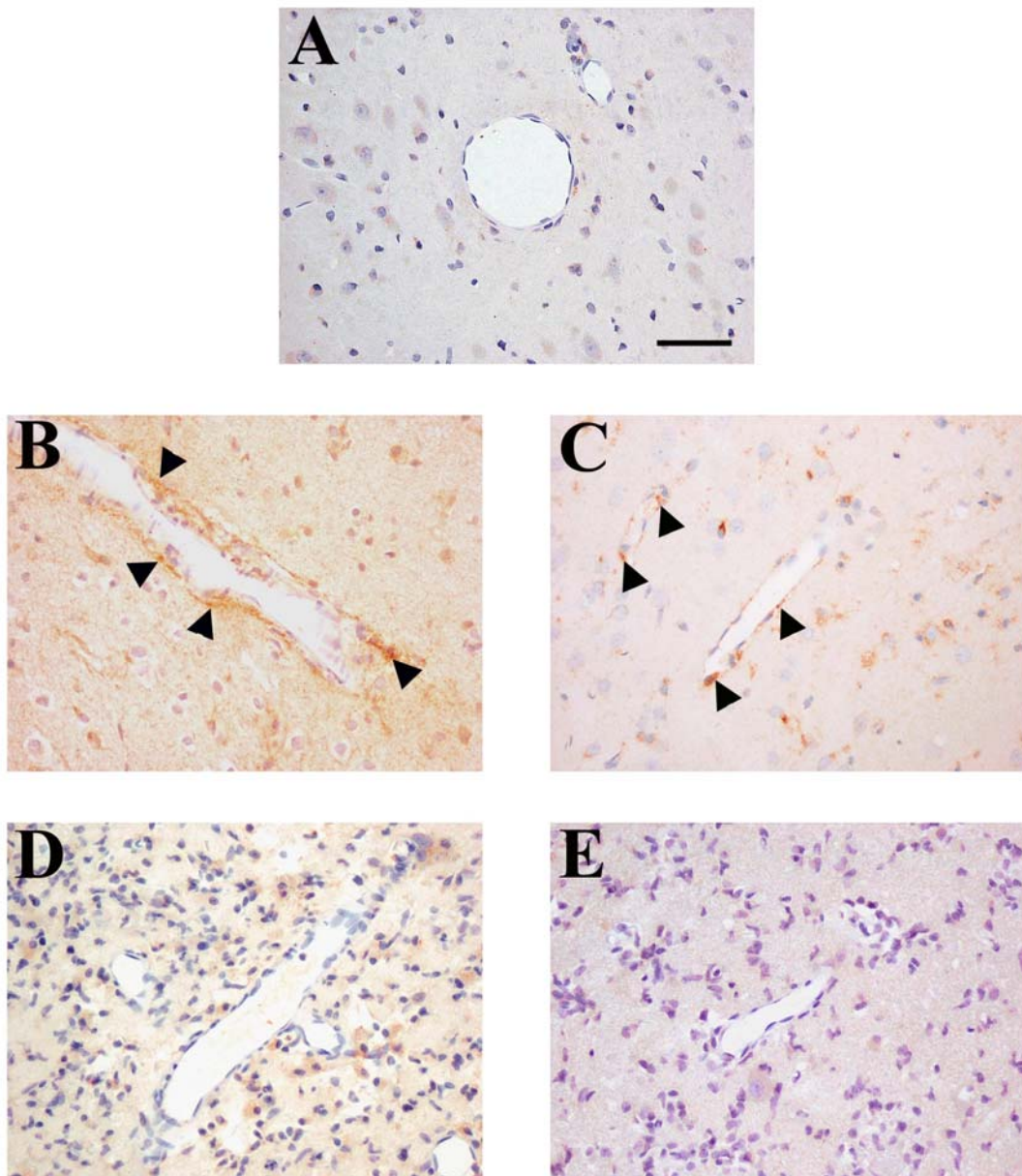
Figure 4.13 NK$_1$ receptor antagonist treatment. Perivascular SP response – SP stained sections (Bar = 100 μm).

Faint SP immunoreactivity was observed in sham tissue (A). At 24 h post-reperfusion (B) a profound increase in perivascular SP immunoreactivity (arrowheads) was observed. Following NAT treatment (C) the perivascular SP immunoreactivity was decreased. By 7 d post-reperfusion (D) extensive reactive gliosis occupied the infarct and perivascular SP was not observed and this was unaffected by NAT treatment (E).
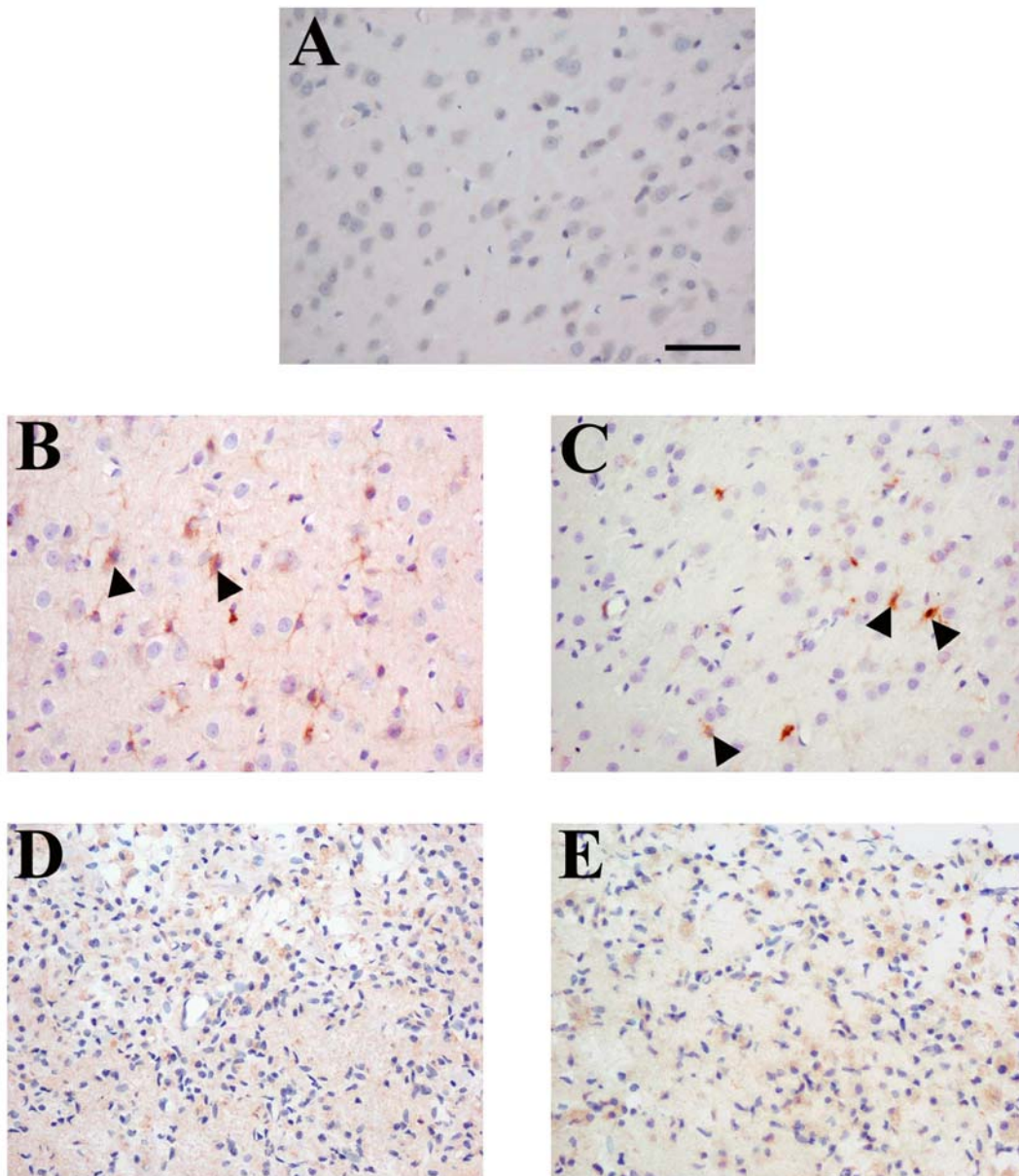
Figure 4. 14 NK$_1$ receptor antagonist treatment. Cortical SP response – SP stained sections (Bar = 100 μm).

Faint SP immunoreactivity was observed in the cortex of sham animals (A). At 24 h post-reperfusion (B) there was an increase in the SP immunoreactivity within the cortex (arrowheads), an increase in parenchymal staining was also observed. NAT treatment (C) resulted in a modest decrease in SP immunoreactivity within the cortex, decreased parenchymal staining was also observed. By 7 d post-reperfusion (D) extensive reactive gliosis occupied the cortex and SP immunoreactivity was not observed. Treatment with NAT (E) did not affect this.

APP Immunoreactivity - APP response

APP immunohistochemistry was carried out to determine the effect of NAT on axonal injury and APP immunoreactivity following stroke. No axonal injury was observed in sham tissue (Figure 4.15). At 24 h post-reperfusion, florid axonal injury was observed within the white matter, seen as intense orange-brown stained retraction balls. This is consistent with axonal injury being a consequence of ischaemic injury to axons (Lewis et al., 1996). Subsequent administration of NAT resulted in a modest reduction in axonal injury, as evidenced by a reduced number of retraction balls. At 7 d post-reperfusion, axonal injury persisted within the white matter, with larger retraction balls present. Treatment with NAT resulted in a reduction in the number and size of retraction balls.

Light APP staining was observed within cortical neurons in sham animals (Figure 4.16). At 24 h post-reperfusion, an increase in parenchymal APP immunoreactivity was observed in addition to shrunken and retracted neurons that were intensely APP immunoreactive. Administration of NAT reduced both the degree of parenchymal and cortical neuron APP immunoreactivity. In summary, NAT treatment reduced APP immunoreactivity, at both the axonal and neuronal level following stroke.
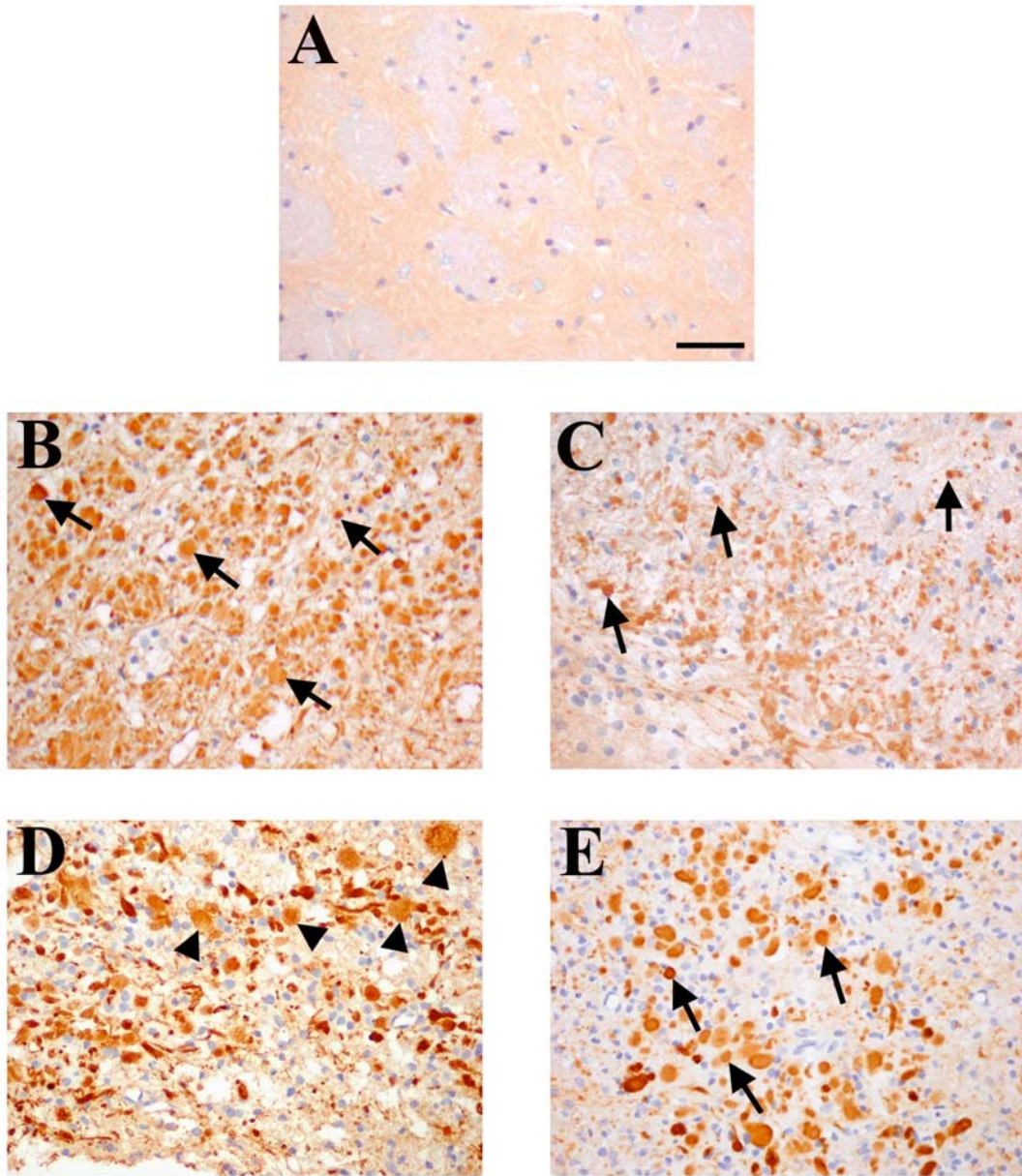
Figure 4.15 NK$_1$ receptor antagonist treatment. Axonal Injury – APP stained sections (Bar = 100 μm).

No axonal injury was observed in sham tissue (A). At 24 h post-reperfusion (B) florid axonal injury (arrows) was observed within the white matter. Treatment with NAT (C) resulted in a modest reduction in the degree of axonal injury. By 7 d post-reperfusion (D) profound axonal injury was still seen, observed as large retraction balls (arrowheads). Treatment with NAT (E) resulted in a modest reduction in the axonal injury, with smaller and fewer retraction balls observed.
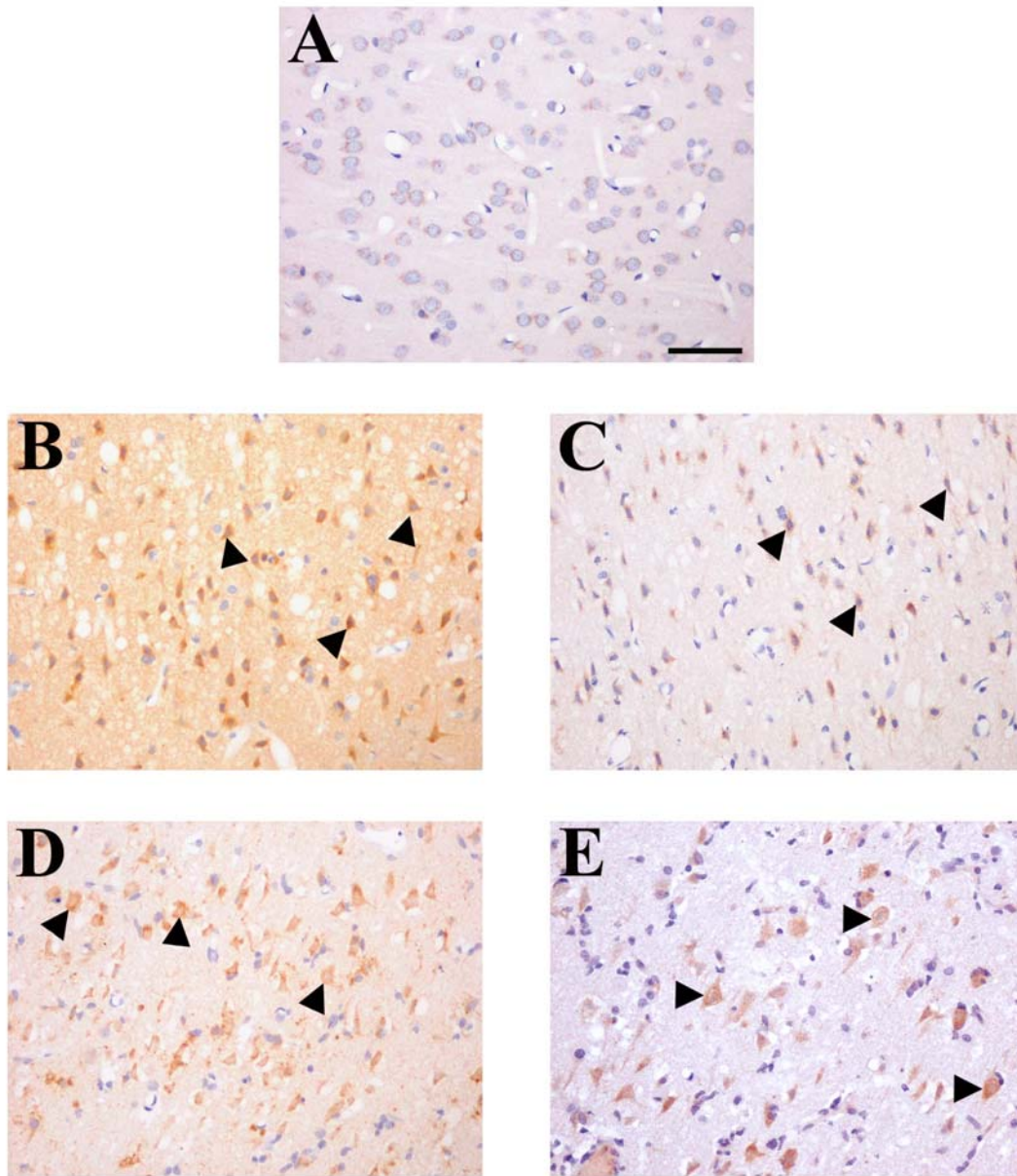
Figure 4.16 NK$_1$ receptor antagonist treatment. Cortex – APP stained sections (Bar = 100 μm).

Moderate APP immunoreactivity was observed in sham tissue (A). At 24 h post-reperfusion (B) darker APP immunoreactivity (arrowheads) was observed within shrunken, injured neurons. Following NAT treatment (C) a decrease in neuronal APP immunoreactivity was observed. By 7 d post-reperfusion (D) profound APP immunoreactity of coritcal neurons was observed, which was modestly reduced by NAT treatment (E).

Fluoro Jade C – Degenerating Neurons

FJC staining was carried out to ascertain whether NAT treatment affected the number of degenerating neurons following stroke. No degenerating neurons were observed in sham tissue (Figure 4.17), consistent with the surgical procedure having no deleterious effects on neuronal survival. At 24 h post-reperfusion, shrunken and retracted FJC positive cells were present throughout the cortex, in addition to marked vacuolation of the parenchyma. Although NAT-treated animals still showed FJC positive cells throughout the cortex, the neurons were less shrunken and the parenchyma was less vacuolated. After 7 d of reperfusion, extensive reactive gliosis occupied the cortex and no FJC positive cells were observed as a result. NAT treatment produced a modest reduction in the reactive gliosis and some FJC positive cells were observed.

No FJC positive cells were observed within the white matter of sham animals (Figure 4.18). After 24 h of reperfusion, degenerating neurons were present throughout the white matter and were observed in association with extensive vacuolation. NAT treatment reduced both the tissue vacuolation and the number of degenerating neurons within the white matter. After 7 d of reperfusion, reactive gliosis was observed and as such, few degenerating neurons were seen. This reaction was largely unaffected by NAT treatment.
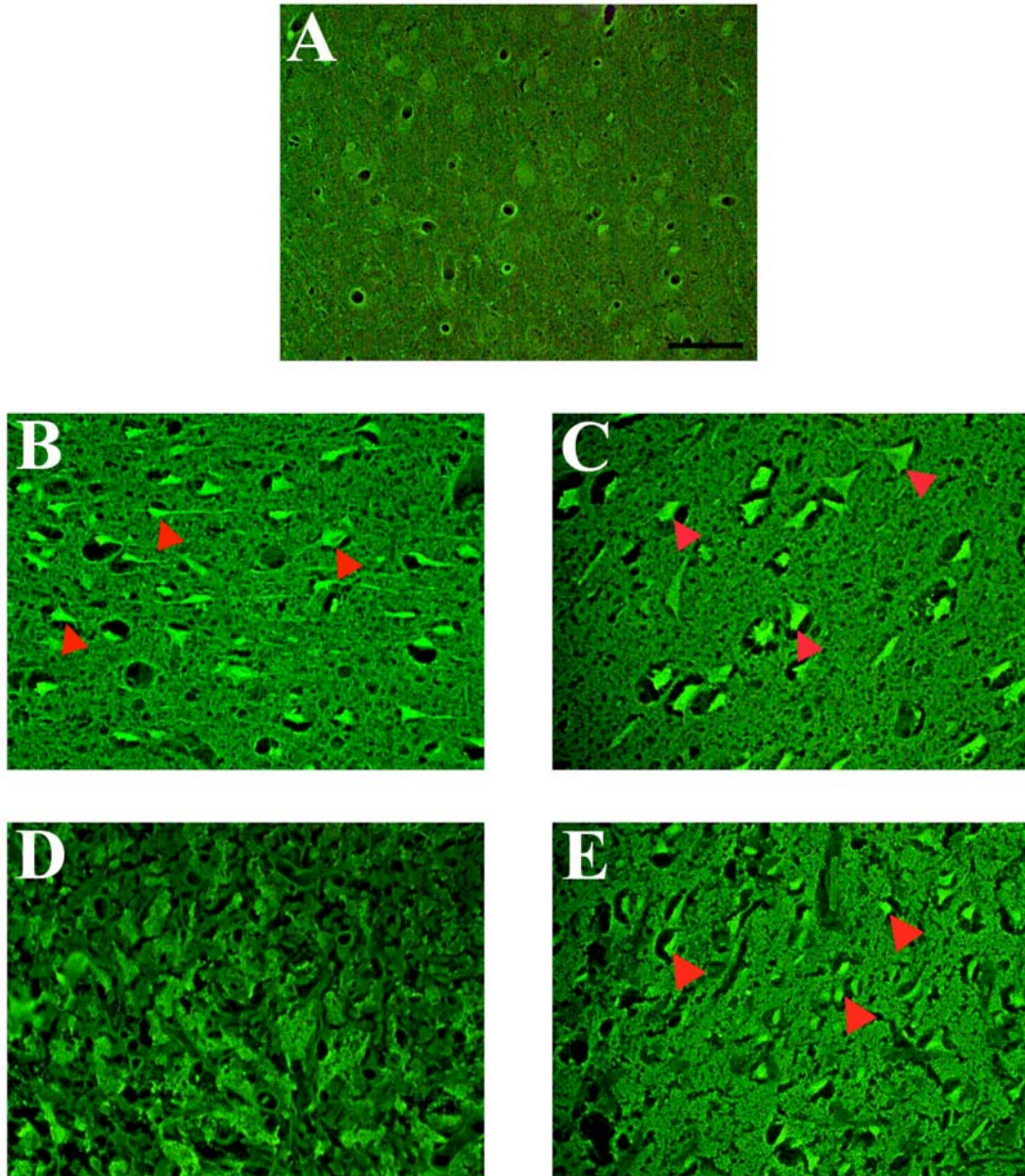
Figure 4.17 NK$_1$ receptor antagonist treatment. Degenerating Neurons Cortex – FJC stained sections (Bar = 100 μm).

No degenerating neurons were seen in sham tissue (A). Comparable amounts of degenerating neurons (arrowheads) were seen in vehicle- (B) and NAT-treated (C) animals at 24 h post-reperfusion. However the parenchyma was less vacuolated and the neurons less shrunken in the NAT-treated group. By 7 d post-reperfusion (D) extensive reactive gliosis occupied the cortex and degenerating neurons were not observed. In NAT-treated animals (E) the reactive gliosis was less extensive and some degenerating neurons were observed.
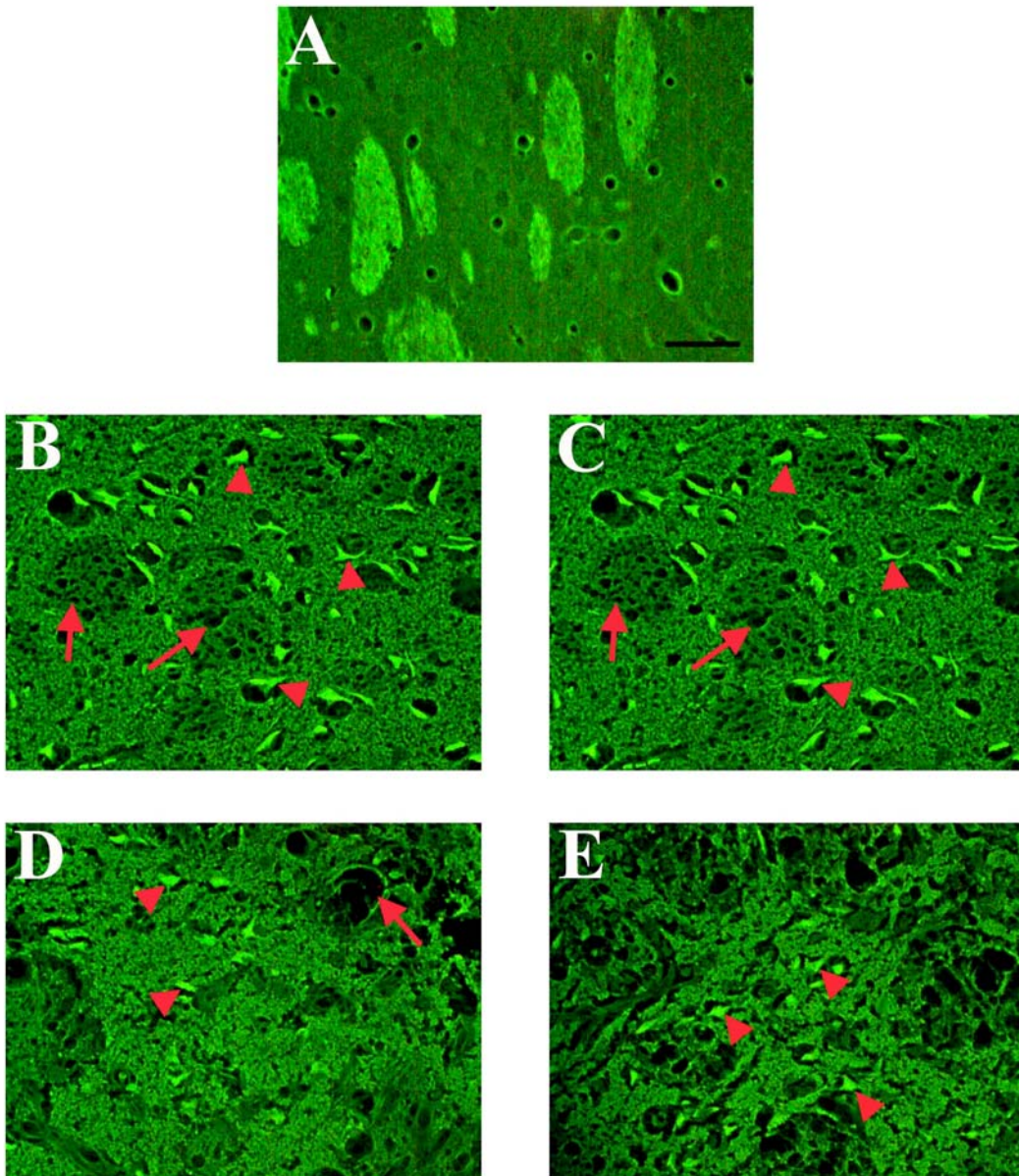
Figure 4.18 NK$_1$ receptor antagonist treatment. Degenerating Neurons White Matter – FJC stained sections (Bar = 100 μm).

No degenerating neurons were observed in sham tissue (A). At 24 h post-reperfusion (B) FJC positive cells (arrowheads) were observed throughout the white matter, destruction of the white matter bundles (arrows) was also apparent. NAT treatment (C) resulted in a modest reduction in FJC positive cells. At 7 d post-reperfusion (D) few FJC positive cells were observed as reactive gliosis occupied the white matter. This was largely unaffected by NAT treatment (E).

ED-1 – Macrophage/Activated Microglia response

ED-1 immunohistochemistry was carried out to determine whether NAT treatment affected the degree of the macrophage/microglial response to ischaemia. No macrophages/activated microglia were observed in sham tissue (Figure 4.19). At 7 d following stroke, there was a profound influx in ED-1 positive macrophages/activated microglia. Subsequent treatment with NAT resulted in a reduced infiltration of ED-1 positive cells into the infarct and this was associated with a modest preservation in tissue architecture.

No macrophages/activated microglia were observed in association with blood vessels within sham tissue (Figure 4.20). At 7 d following stroke, macrophages/activated microglia were observed in close association with blood vessels, and this response was largely unaffected by NAT treatment.

GFAP – Astrocyte response

GFAP immunohistochemistry was used to determine the effect of NAT treatment of the astrocytic response to stroke. As previously outlined in chapter 3, MCAO resulted in a loss of GFAP staining within the infarct core and an increase in the infarct boundary zone. In sham tissue, light GFAP immunoreactivity was observed around blood vessels (Figure 4.21). At 7 d following stroke, an increase within perivascular GFAP immunoreactivity was observed. NAT treatment resulted in a further and more profound increase in GFAP immunoreactivity in perivascular tissue.

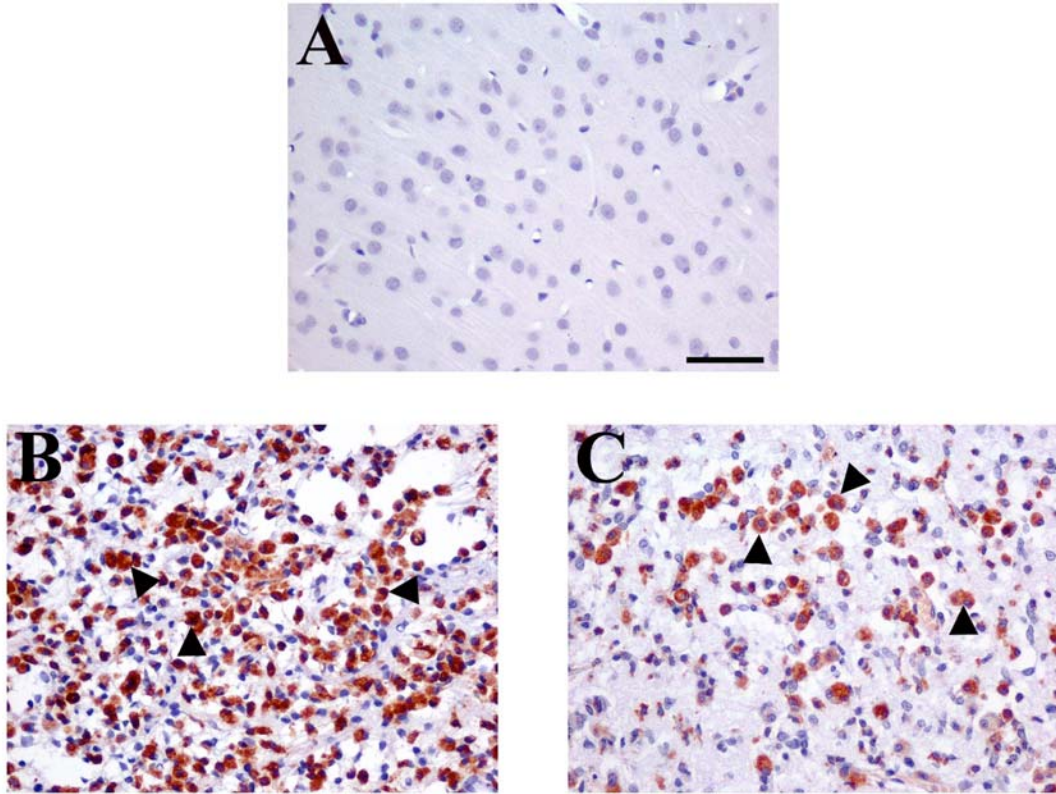**Figure 4.19 NK₁ receptor antagonist treatment. Cortical Macrophage/ Activated Microglia Response – ED-1 stained sections (Bar = 100 μm).**

No macrophages were seen in sham tissue (A). A massive influx of macrophages/activated microglia (arrowheads) was seen in the vehicle-treated group (B). More tissue preservation is apparent in the NAT-treated group (C), and less macrophages/activated microglia were present.
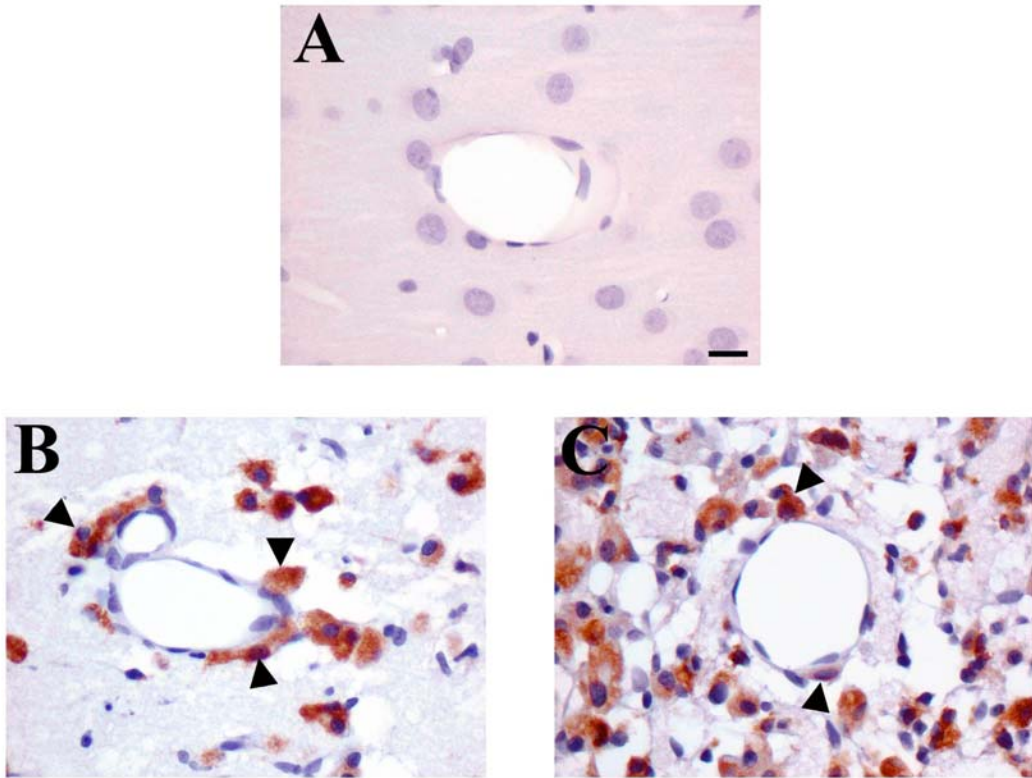
Figure 4.20 NK₁ receptor antagonist treatment. Perivascular Macrophage/ Activated Microglia response ED-1 stained sections (Bar = 100 μm).

Sham tissue showed no macrophages/activated microglia associated with vessels (A). Macrophages/activated microglia (arrowheads) were seen around blood vessels in vehicle-treated (B) and NAT-treated animals (C).
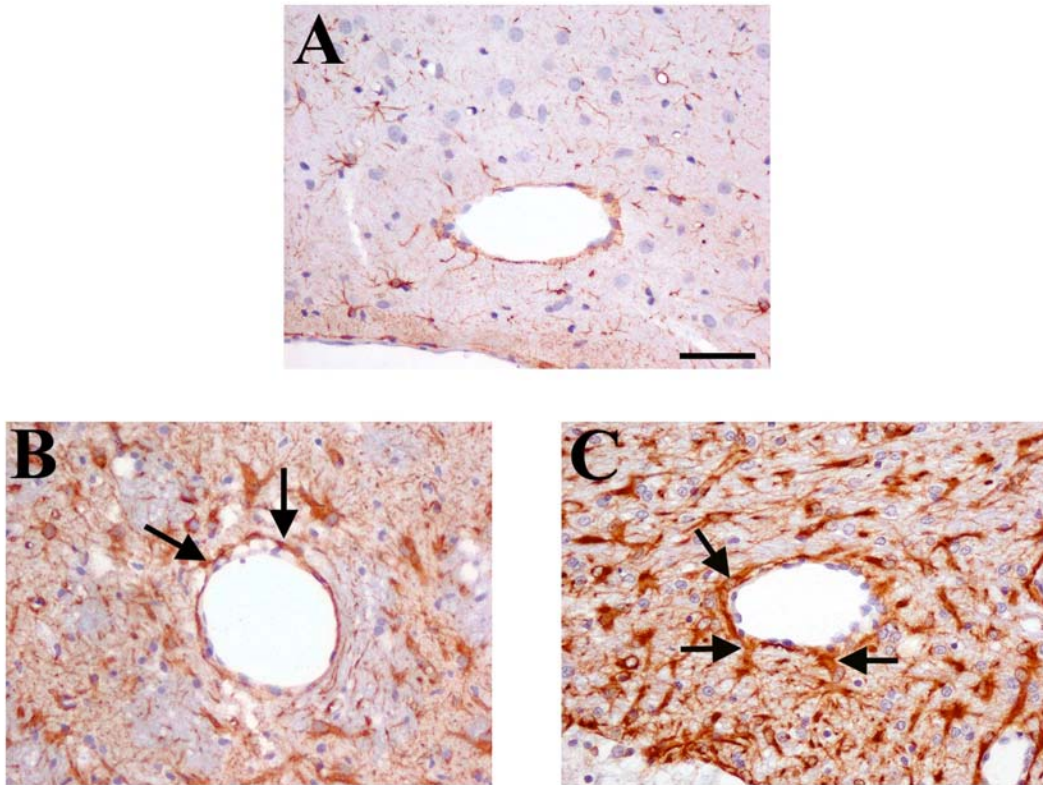
Figure 4.21 NK$_1$ receptor antagonist treatment.   GFAP Perivascular – GFAP stained sections (Bar = 100 μm).

In sham tissue, light GFAP staining was observed around vessels (A).   At 7 d post-stroke (B) there was an increase in GFAP immunoreactivity (arrows) around vessels.   A further increase was observed following NAT-treatment (C).

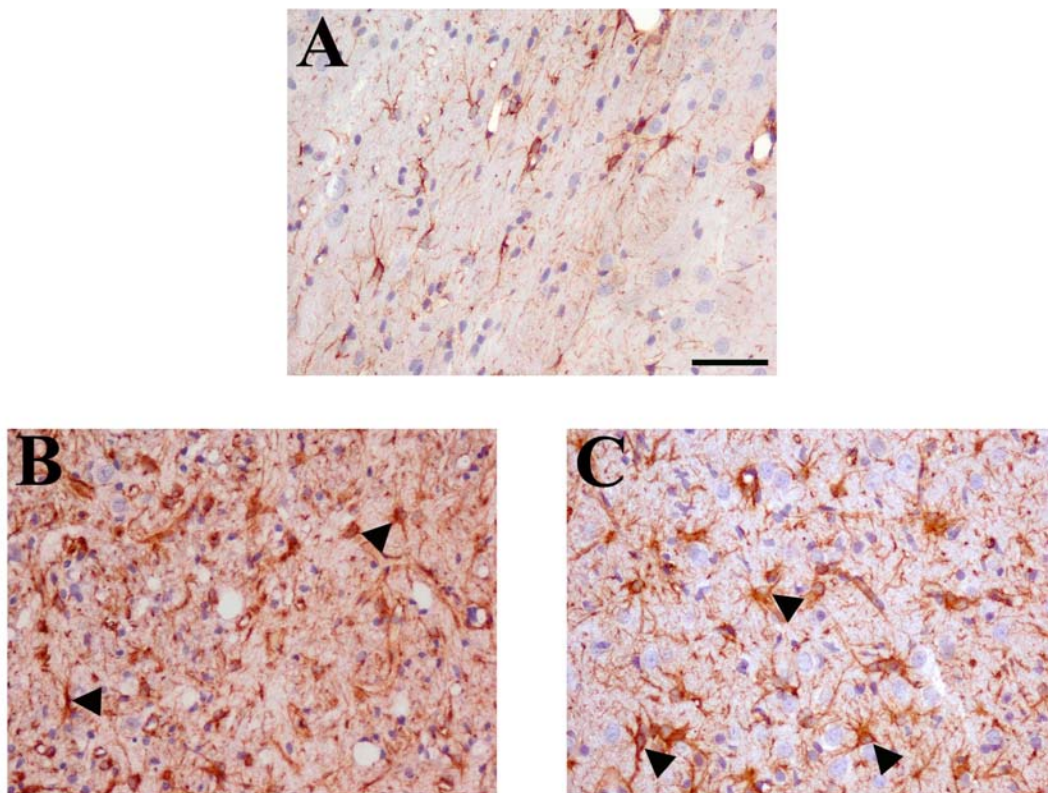**Figure 4.22 NK$_1$ receptor antagonist treatment. GFAP Penumbra – GFAP stained sections (Bar = 100 μm).**

In sham animals, GFAP positive cells were scattered throughout the tissue (A). At 7 d following stroke an increase in the number of GFAP positive cells (arrowheads) was observed (B). A further increase was observed in the NAT-treated group (C). Following stroke, the GFAP positive cells displayed an activated morphology.

In sham tissue, light GFAP immunoreactivity was observed in astrocytes displaying a resting morphology. At 7 d following stroke, there was an increase in GFAP immunoreactivity, as well as astrocyte hyperplasia and hypertrophy, which is a stereotypic response following stroke (Raivich et al., 1999). NAT treatment further exacerbated the astrocytic response to ischaemia with profound hypertrophy and hyperplasia of astrocytes observed.

4.4 Discussion

In the present study, we demonstrate that inhibition of neurogenic inflammation by post-ischaemic administration of the NK_1 receptor antagonist, NAT, significantly reduced BBB permeability, cerebral oedema, functional deficits and SP protein levels within the infarcted hemisphere. Such marked improvement in these variables was not associated with a reduction in infarct volume or histological outcome, with only modest reductions in axonal swellings and ED-1 positive cells observed. Nevertheless, such findings confirm a role for SP in these events following ischaemia.

Prior to this study, few research groups had investigated the role of SP in cerebral ischaemia (Bruno et al., 2003; Stumm et al., 2001; Yu et al., 1997). Those that did investigate SP did not characterise the role of SP in neurogenic inflammation in cerebral ischaemia. Indeed, the study of sensory neuropeptides in CNS injury has been mostly confined to isolated reports in peripheral nerve injury (Malcangio et al., 2000, spinal cord injury (Sharma et al., 1993), brain ischaemia (Bruno et al., 2003; Stumm et al., 2001; Yu et al., 1997) and those of our research group in TBI (Donkin et al., 2007; Nimmo et al., 2004; Vink et al., 2003). In contrast, the role of sensory

neuropeptides has been extensively characterised in the PNS, with SP in particular implicated in the pathophysiology of a number of conditions including asthma, emesis, dental pain, osteoarthritis and neuropathic pain, as well as the CNS conditions of migraine, movement disorders and depression (Hokfelt et al., 2000; Kramer et al., 1998; Rupniak and Kramer, 1999).

The increase in BBB permeability observed at 24 h post-reperfusion supports previous findings of a delayed opening of the BBB following stroke (Preston et al., 1993). The alterations we observed in BBB permeability were also associated with profound cerebral oedema formation. The fact that cerebral oedema occurred in the setting of increased BBB permeability, and in association with increased SP levels, suggests that the oedema was vasogenic in origin. Subsequent administration of an NK_1 receptor antagonist at 4 h post-stroke onset completely attenuated both the increased BBB permeability and the cerebral oedema, suggesting a direct role for SP in these post-stroke events. Although this is the first study to demonstrate neurogenic inflammation in cerebral ischaemia, our laboratory has previously reported a role for neurogenic inflammation in the BBB dysfunction and cerebral oedema that occurs following TBI (Donkin et al., 2007; Nimmo et al., 2004; Vink et al., 2003). Other studies in NK_1 receptor knockout mice have shown also that they are unable to produce oedema (Cao et al., 1999), confirming a potential role of the NK_1 receptor in oedema formation. Specifically, the application of SP produced plasma extravasation and oedema formation in a dose-dependent manner in wild-type mice, implying that activation of the NK_1 receptor by SP leads to oedema formation.

As discussed at length in chapter 1, a number of factors contribute to the injury pathways that lead to neuronal injury and death following stroke. Because such injury pathways evolve over space and time there is a window available for therapeutic intervention to potentially limit the ischaemic damage and improve outcome. Although the exact mechanisms whereby NK_1 receptor antagonists elicit neuroprotection are unknown, there are a number of pathways that could be affected by NAT administration. SP is involved in an array of processes, aside from neurogenic inflammation, that may contribute to tissue damage following stroke.

SP is known to activate the immune system and to modulate immune responses and inflammation (Guo et al., 2004). Specifically, SP can act as a chemotactic factor for inflammatory cells, including neutrophils (Braun et al., 1996) and monocytes (Ruff et al., 1985). Furthermore, SP can induce the dose-dependent release of cytokines, including IL-1, TNF-α (Lotz et al., 1988) and IL-6 (Brain, 1997; Yamaguchi et al., 2004). In addition, SP may 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). Such production of free radicals by neutrophils is known to exacerbate injury (Siesjo et al., 1996). Therefore, SP may initiate/propagate the inflammatory response to cerebral ischemia, thereby contributing to tissue injury. The fact that the brain is immune privileged may further contribute to the progression of ischaemic damage (Barone and Kilgore, 2006) although the timing of the inflammatory response is important as it is likely that acute responses are destructive, whereas chronic responses are beneficial (Leker and Shohami, 2002).

Studies in TBI have found that SP can stimulate the production of NO by endothelial cells (Persson et al., 1991). NO is a known injury factor in cerebral ischaemia with increased levels occurring following stroke. Such aberrant NO production through the iNOS and nNOS pathways leads to neuronal damage (Moro et al., 2004). However NO produced through the eNOS pathway is likely to be beneficial due to its vasodilating effects (Leker and Shohami, 2002).

In response to elevated SP levels after injury, astrocytes can develop an activated morphology and are stimulated to produce cytokines, prostaglandins and other pro-inflammatory agents (Marriott et al., 1991; Palma et al., 1997). Indeed, following stroke, marked hypertrophy and hyperplasia of astrocytes was observed within the infarct boundary zone, characteristic of MCAO (Chen et al., 1993; Li et al., 1995). The loss of GFAP staining within the infarct core may be partially explained by the fact that protoplasmic astrocytes do not express enough GFAP to be detected, meaning that most astrocytes within the grey matter are GFAP negative with conventional histological methods (Chen and Swanson, 2003). Administration of NAT at 4 h post-stroke onset further increased GFAP staining, suggesting a protective role of NAT. This was an interesting observation considering that NK_1 receptors are not found on activated astrocytes and do not play a direct role in their activation following pMCAO (Stumm et al., 2001). However, the role of astrocytes following ischaemia is ill defined. Reactive gliosis is a key component of the cellular response to CNS injury (Stoll et al., 1998; Stoll and Schroeter, 2000), functioning to contain the injured site and prevent further spread (Trendelenburg and Dirnagl, 2005). Although some view it as a process where inert scar tissue is laid down, dead neurons are not replaced, and regeneration of axons does not occur

as it inhibits neurite outgrowth (Bovolenta et al., 1992). However, the role of astrocytes following ischaemia is likely to represent a double-edged sword, playing both reparative and deleterious roles depending upon the location and timing (Nedergaard and Dirnagl, 2005; Trendelenburg and Dirnagl, 2005). Astrocytes are able to produce a number of trophic factors for neurons including bFGF, TGF-B, NGF, VEGF amongst others (Chen and Swanson, 2003). In addition, the elongated processes exhibited by astrocytes following ischaemia suggests a more immature phenotype, which may be more conducive to regeneration and neurogenesis (Cramer and Chopp, 2000). Nevertheless, the role of astrocytes in ischaemia requires further study.

In the present study, marked infiltration of ED-1 positive cells was observed in the infarct. Although macrophages and microglia have been reported to contribute to the gradual expansion of the infarct following cerebral ischaemia through the production of IL-1β (Mabuchi et al., 2000) systemic depletion of macrophages was shown to have no effect on infarct volume (Schroeter et al., 1997). Similarly, blocking neutrophil migration failed to reduce infarct volume after pMCAO (Garcia et al., 1996). Nevertheless, the reduced ED-1 staining may in part be accounted for by a reduction in SP levels. As SP can act as a chemotactic factor for monocytes (Ruff et al., 1985), lowered SP levels may have resulted in a dampened chemotactic signal and a reduced infiltration of monocytes into the brain.

As discussed in chapter 3, APP is a sensitive marker of axonal injury (Blumbergs et al, 1995). Following MCAO, florid axonal injury was observed. Administration of an NK$_1$ receptor antagonist resulted in a modest reduction in axonal swellings, as

assessed at 24 h and 7 d post-reperfusion. The exact mechanism whereby NAT was able to afford a degree of axonal protection is unknown. However, the preservation of axons may partially account for the functional improvement, in particular motor improvement, observed within the NAT-treated group.

The findings of the present study show that despite a remarkable recovery of function, as assessed by a number of outcome measures, infarcts were still extensive within the NAT treatment group. Such findings suggest that infarct volume may not be as accurate a predictor of outcome as the literature suggests. Unfortunately, a number of experimental studies use infarct volume as the primary outcome measure despite the fact that clinical studies use functional/neurological testing as the primary outcome measure (Gladstone et al., 2002). Nevertheless, an increasing number of researchers are reporting improvements in functional outcome without improvements in infarct volume or histological outcome (Grotta et al., 1988; Grotta et al., 1990; Aronowski et al., 1994; Aronowski et al., 1996; van der Staay et al., 1996). For example, bFGF administration was associated with marked functional improvement without an effect on infarct size. Similarly, a marked recovery in memory following administration of a Ca^{2+} blocker was observed despite observing no differences on light microscopy between the treatment and vehicle groups (Grotta et al., 1988). Some studies report the opposite, with improvements in infarct size in the absence of functional improvement (Reglodi et al., 2000). Regardless, it is clear that the relationship between infarct volume and functional outcome is not as causal as the early literature suggests.

It has previously been suggested that neuroprotective therapy may produce more functional improvement than histological improvement and that behavioural tests

may detect pharmacoprotection not evident by light microscopic evaluation (Aronowski et al., 1994; Aronowski et al., 1996). Sensorimotor impairments can't be predicted from infarct volume (van der Staay et al., 1996). Consistent with this, previous studies in TBI have suggested that behavioural testing is the most reliable indicator of outcome in TBI (Dixon et al., 1991). In stroke patients meaningful function, especially motor, is of paramount importance, rather than lesion volume. However, the improvement in function seen may reflect the changes at the subcellular level, for example changes in synaptic and electrophysiologic function (Gladstone et al., 2002). Recent reports have proposed that it is the synaptic plasticity of the ischaemic penumbra that is the key to explaining the lack of reduction in infarct volume despite marked functional recovery (Duffau et al., 2006). Therefore, it is not so much salvaging the penumbra per se, but improving the functionality of the peri-lesional tissue that is important. Thus, the improvements in functional outcome observed in the current study are likely to reflect the ability of the NK_1 receptor antagonist to improve the plasticity of the penumbra, such that other areas of the brain are able to take over the function of those areas that were damaged. In contrast, high levels of sensory neuropeptide receptors within the striatum and hippocampus (Huston and Hasenohrl, 1995), known to be integral to learning and memory, supports a role for SP in learning and memory. Indeed, alterations in SP, CGRP and NPY have been shown to affect cognition in mice (Bracci-Laudiero et al., 1999). Thus, the protective effects of NAT may be regionally specific.

One caveat regarding the infarct volume is the specificity of the TTC staining. TTC reacts with an enzyme within active mitochondria, and hence stains viable cells.

However, the staining does not accurately represent the heterogenous nature of the infarct. Hence, it may have masked any modest effect of NAT on the degree of infarction. Indeed, FJC staining showed modest reduction in the number of degenerating neurons, which was not reflected by the TTC staining.

An increase in SP levels within the infarcted hemisphere was observed at 24 h post-reperfusion by both immunohistochemical and ELISA detection methods. Such an increase has also been detected in the serum of patients with stroke and TIA (Bruno et al., 2003). Increases in SP levels have also been observed in other CNS disorders including depression (Bondy et al., 2003) and TBI (Donkin et al., 2007). It was not expected that NAT would reduce tissue levels of SP, as assessed by ELISA, but simply render SP ineffective at the NK_1 receptor. However, it may not be an altogether surprising finding given the presence of an autoreceptor for SP (Lever et al., 2003; Malcangio and Bowery, 1999). Blocking the activity of the autoreceptor may presumably prevent positive feedback on SP release. A decrease in SP immunoreactivity was also noted following NAT treatment, in cortical and perivascular tissue. The reduction in SP levels was associated with significant preservation of the BBB and reduced cerebral oedema. These results further confirm a role for SP in BBB dysfunction and cerebral oedema following stroke.

Taken together, the present findings suggest that the release of SP may be an early pathological event associated with cerebral ischaemia. These results are consistent with the observations of Yu et al (Yu et al., 1997), who previously reported that increased SP exacerbated tissue damage following cerebral ischaemia.

Accordingly, further studies are required to elucidate the exact mechanisms whereby NK$_1$ antagonists provide protection from cerebral ischaemia.

4.5 Conclusions

Administration of NAT is a highly effective means of reducing/preventing functional deficits, cerebral oedema and blood brain barrier opening that occur following reversible ischaemic stroke. These results show that SP plays a major role in post-ischaemic injury processes and may therefore provide a novel target for interventional pharmacologies. Whether such strategies can be used in combination with the current stroke treatment, tPA, remains to be seen, and is explored in Chapter 5.