

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

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**CHAPTER 6:**  
**THERAPEUTIC WINDOW FOR ADMINISTRATION**  
**OF AN NK<sub>1</sub> RECEPTOR ANTAGONIST FOLLOWING**  
**ISCHAEMIC STROKE**

## **6.1 Introduction**

The previous chapters have demonstrated that SP release is central to the injury processes that occur following cerebral ischaemia, and that antagonism of this pathway significantly reduces functional deficits, BBB instability, cerebral oedema and histological abnormalities. Such improvements in post-stroke outcome were all demonstrated when the SP antagonist, NAT, was administered at 4 h following stroke onset. Whilst these findings are extremely encouraging, it is essential to determine the therapeutic window for treatment, particularly when considered in the context of tPA which is currently administered up to 3 h post-stroke onset. Reports from our laboratory using a model of traumatic brain injury have shown that an NK<sub>1</sub> receptor antagonist may be administered up to 12 h following TBI with significant improvements in motor function (Donkin, 2006). As such, the aim of the present study was to determine the therapeutic window for the administration of the NK<sub>1</sub> receptor antagonist, NAT, following acute ischaemic stroke.

## **6.2 Study Design**

Animals (n=15) were subject to MCAO as described in detail in Chapter 2. Following induction of MCAO, animals were randomised into either the functional outcome group or the histological outcome group. At 8 or 12 h following the onset of stroke, animals received 25 µmoles/kg of NAT intravenously. Drug preparation and administration was as per outlined in chapter 2. Results obtained were compared to animals treated with NAT at 4 h after stroke onset, equal volume vehicle, or sham animals, as described in chapter 4.

### **6.2.1 Functional Outcome**

Animals were assessed daily for a 7 d period following stroke using the rotarod, bilateral asymmetry test, open field, modified neuroseverity score and angleboard. Details of the testing paradigm are as outlined in detail in Chapter 2.

### **6.2.2 Histological Outcome**

At 7 d following stroke, animals were perfused with 10% formalin and their brains processed as described in detail in Chapter 2. Sections were then stained for H&E, SP, APP, FJC, GFAP and ED-1 as described in Chapter 2. Sections were then assessed using light microscopy or fluorescence microscopy, as appropriate.

### **6.2.3 Statistical Analysis**

All parametric data was analysed using an analysis of variance (ANOVA) followed by individual Bonferroni Post-tests. The modified neuroseverity score data was analysed using a Kruskal-Wallis test followed by Dunn's Multiple Comparison Tests. A p value of 0.05 was considered significant.

## **6.3 Results**

### **6.3.1 Functional Outcome**

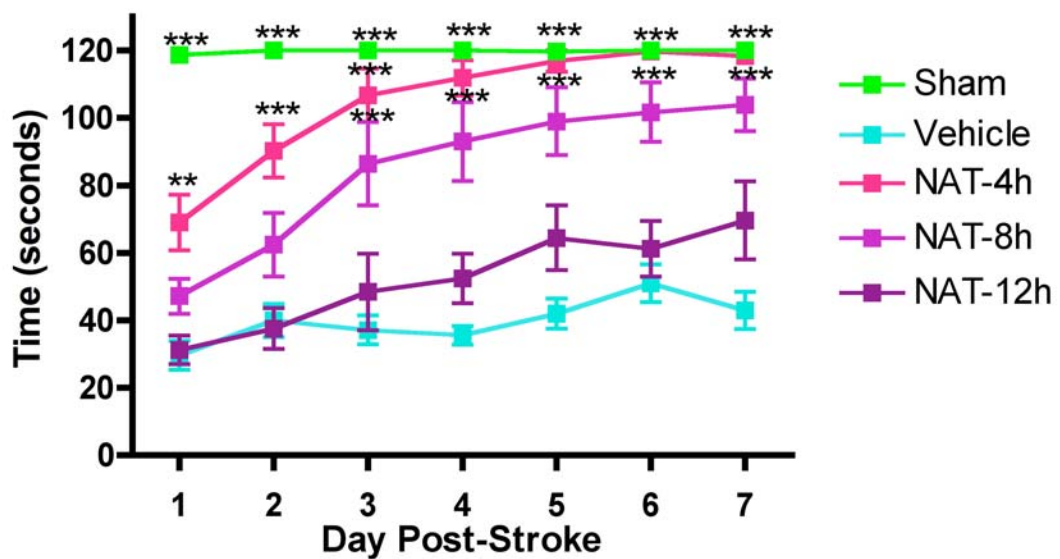
#### ***Motor Function: Rotarod***

Sham animals recorded no motor deficit and were able to successfully complete the 2 min rotarod task on all assessment days, confirming that the surgical procedure had no effect on motor function. Following stroke, vehicle-treated animals were unable to complete the 2-min task on any day, demonstrating profound motor deficits that did not improve over the 7 d assessment period ( $p < 0.001$  versus shams;

Figure 6.1). As previously described, the NAT-4 h animals demonstrated a steady improvement in their ability to perform the rotarod task, achieving normal ( $p > 0.05$  versus shams) functional levels by day 3 post-stroke. Similarly, NAT-8h animals also showed a significant recovery of motor function over the assessment period when compared to vehicle treated animals ( $p < 0.001$ ). Nonetheless, their performance was still significantly different from normal functional levels by 7 d post-stroke. This is in contrast to the NAT-12 h group, which did not demonstrate any recovery of motor function during the 7 d assessment period when compared to vehicle treated animals. Accordingly, NAT resulted in an improved motor outcome when administered up to 8 h post stroke onset, but not 12 h after stroke.

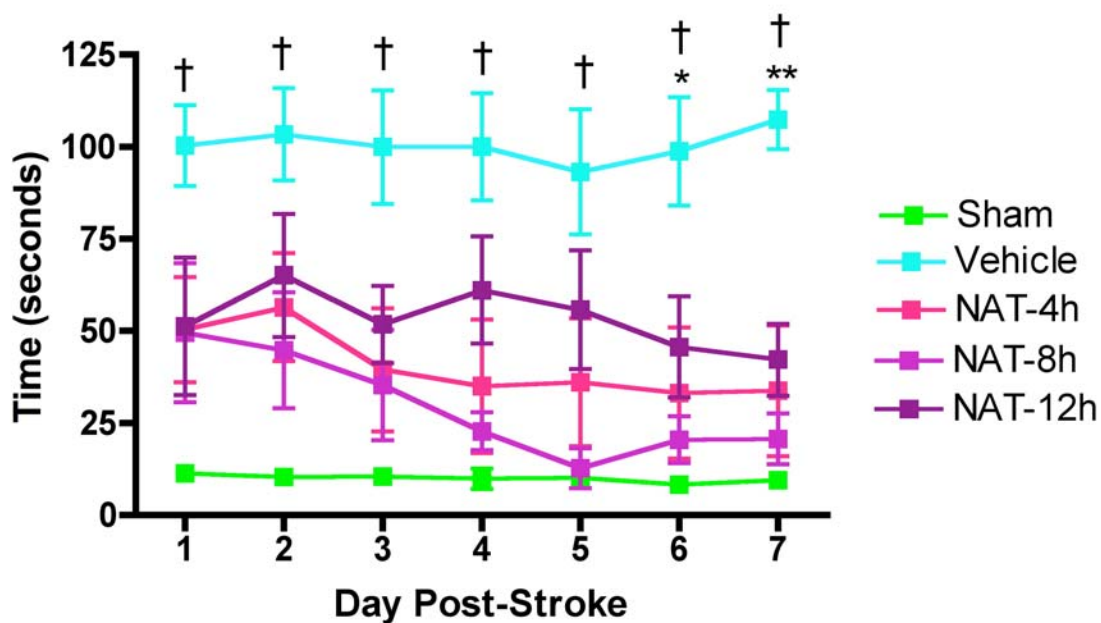
#### ***Sensory Function: Bilateral Asymmetry Test***

As expected, no sensory deficits were observed in sham animals. Following stroke, vehicle-treated animals demonstrated an increased latency to sense the tape and coordinate its removal on all assessment days (Figure 6.2). As such, the time to removal for this group did not drop below 95 s on any assessment day, which was significantly ( $p < 0.001$ ) higher than in sham animals. As previously described, the NAT-4 h group showed an improved latency to remove the tape, with time latencies decreasing steadily over the assessment period. While this group did not reach normal functional levels at any stage post-stroke, these animals did record latencies that were significantly ( $p < 0.001$ ) less than that observed in vehicle treated animals. Similarly, NAT-8 h animals showed improved latencies over the 7 d assessment period, performing at levels significantly ( $p < 0.001$ ) better than vehicles. However, this group also did not reach normal functional levels ( $p < 0.05$  versus shams). The



**Figure 6.1 Therapeutic window - Motor performance as assessed by the rotarod.**

Sham animals (green) showed no motor deficits. Vehicle animals (blue) had persistent motor deficits throughout the assessment period. NAT treatment at 4 h (pink) post-stroke onset significantly improved ( $p < 0.001$ ) motor function. NAT treatment at 8 h (magenta) post-stroke onset followed a similar pattern of recovery, improving ( $p < 0.001$ ) motor function. NAT treatment at 12 h (purple) post-stroke onset did not result in any significant improvement in motor function (\*\* denotes  $p < 0.01$  versus vehicle; \*\*\* denotes  $p < 0.001$  versus vehicle) (Sham  $n = 6$ ; Vehicle  $n = 6$ ; NAT-4h  $n = 6$ ; NAT-8h  $n = 7$ ; NAT-12h  $n = 8$ ).



**Figure 6.2 Therapeutic Window – Sensory function as assessed by the bilateral asymmetry test.**

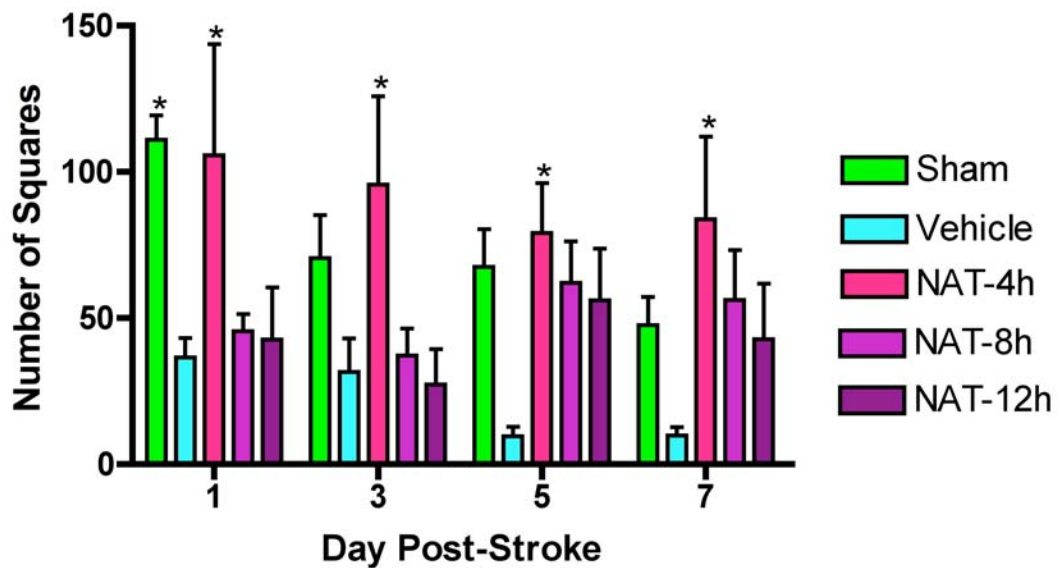
Sham animals (green) had no sensory deficits. Vehicle animals (blue) had persistent sensory deficits. Treatment with NAT at 4 h (pink) or 8 h (magenta) post-stroke onset resulted in a recovery of sensory function. Treatment with NAT at 12 h (purple) post-stroke onset resulted in a modest improvement in sensory function however this was significantly different than shams (†denotes  $0.001 < p < 0.05$  versus shams, NAT-4h and NAT-8h; \*denotes  $p < 0.05$  versus NAT-12h; \*\*denotes  $p < 0.01$  versus NAT-12h; †denotes  $0.01 < p < 0.05$  versus sham, NAT-4h, NAT-8h) (Sham n=6; Vehicle n=6; NAT-4h n=6; NAT-8h n=7; NAT-12h n=8).

NAT-12 h group showed reduced sensory deficits ( $p < 0.001$  versus vehicles) over the assessment period but also did not reach normal functional levels ( $p < 0.001$  versus shams). Whilst there was a clear trend supporting the beneficial effects of earlier administration of the NK<sub>1</sub> antagonist (Figure 6.2), there were no significant differences between the NAT treatment groups.

### ***Spontaneous Exploratory Behaviour: Open Field***

Sham animals demonstrated normal activity levels in the open field, indicating that the surgical procedure had no significant effect on spontaneous exploratory behaviour. Nonetheless, a decline in activity was noted over the 7 d assessment period, most likely reflecting habituation, which has been well documented in this test in uninjured animals (McIlwain et al., 2001; Paylor et al., 2006; Stohr et al., 1998). Following stroke, vehicle animals showed a profound decline in spontaneous activity (Figure 6.3), with the number of squares traversed significantly lower than shams on day 1. In contrast, NAT-4 h animals demonstrated significantly increased spontaneous activity levels on all assessment days ( $p < 0.001$ ). Despite a clear trend towards an improved level of spontaneous activity in the NAT-8 h group, the activity levels in this group did not significantly differ from vehicles on any assessment day, most likely because of the large variation in the test outcomes amongst animals. Similarly, NAT-12 h animals had no significant increase in spontaneous exploratory activity when compared to vehicles, although clear trends towards increased open field activity were apparent. Notably there was a decreased tendency toward improvement in this test with increasing delay of treatment, which was particularly apparent at day 7 (Figure 6.3).





**Figure 6.3 Therapeutic Window – Stress and anxiety as assessed by the open field.**

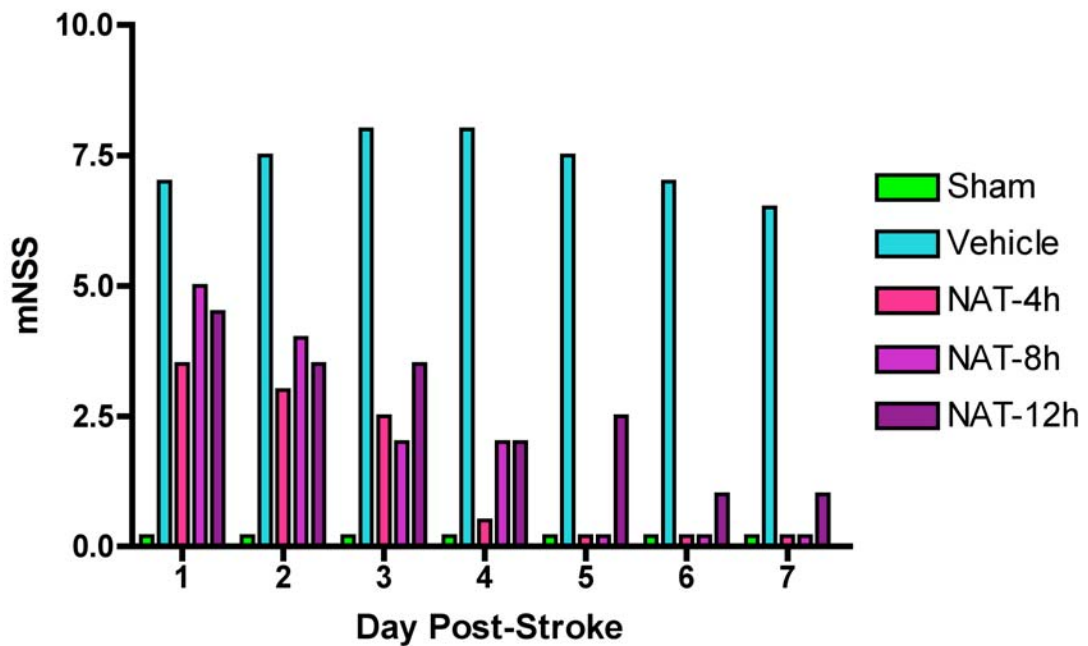
Sham animals (green) had normal functional levels in the open field. Vehicle animals (blue) had a significant decline in open field activity following stroke. NAT at 4 h (pink) post-stroke restored open field activity to normal levels. NAT at 8 h (magenta) and NAT at 12 h (purple) post-stroke generally showed a trend towards increasing open field activity (\* denotes  $p < 0.05$  versus vehicle) (Sham  $n=6$ ; Vehicle  $n=6$ ; NAT-4h  $n=6$ ; NAT-8h  $n=7$ ; NAT-12h  $n=8$ ).

### ***Neurological Function: Modified Neuroseverity Score***

Sham animals had no observable neurological deficits on any assessment day. Following stroke, vehicle animals consistently demonstrated profound sensory deficits that ranked in the moderate injury group (mNSS 5-8) for the 7 d assessment period (Figure 6.4). In contrast, NAT-4 h animals showed a steady improvement in neurological function, recording a ranking of mild injury on day 1 post-stroke, and no observable neurological deficits by day 5 post-stroke. NAT-8 h animals also demonstrated a marked recovery of neurological function over the 7 d assessment period. The injury ranking of these animals reduced from moderate over the first 3 days to no observable deficit by day 5 post-stroke. The neurological function of NAT-12 h animals also improved over the assessment period, however these animals still had a ranking of mild injury (mNSS 1-4) by day 7 post-stroke.

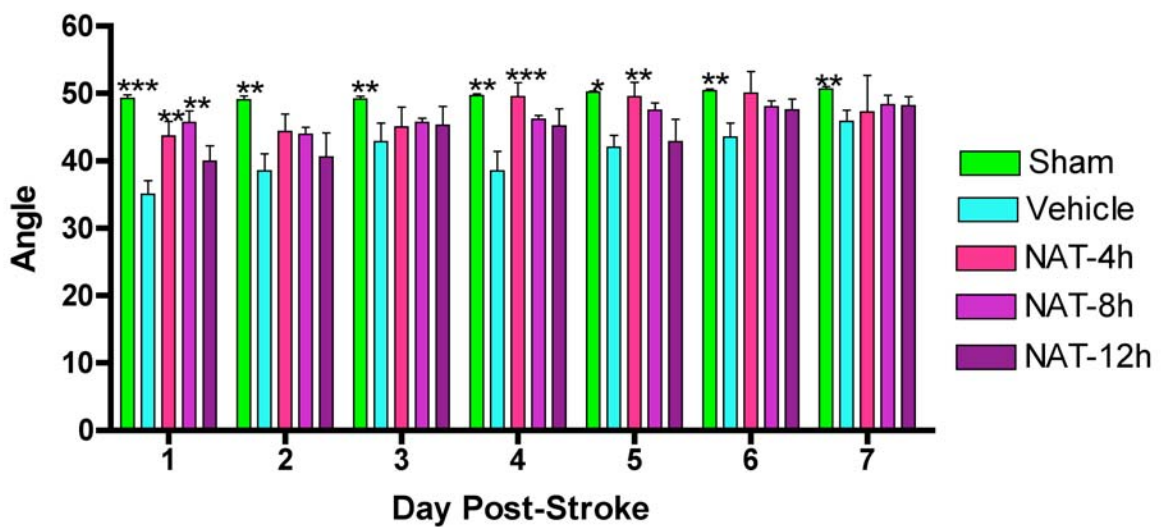
### ***Hemiparesis: Angleboard***

No hemiparesis was observed in sham animals, once again confirming that the surgical procedure had no effect on muscle strength and balance. Following stroke, vehicle animals demonstrated profound hemiparesis (Figure 6.5), scoring significantly lower on the angleboard test than shams on days 1-2 and 4-5 post-stroke. In contrast, NAT-4 h animals showed no significant hemiparesis on any of the assessment days, recording angleboard scores comparable to shams. However these scores were only significantly better than vehicle treated animals on days 1 and 4 post-stroke ( $0.05 < p < 0.01$ ). Similarly, the NAT-8 h group displayed no significant hemiparesis, with angleboard scores comparable to shams on all assessment days. However, such scores were only significantly better than vehicles on day 1 post-stroke ( $p < 0.05$ ). NAT-12 h animals showed signs of hemiparesis, with



**Figure 6.4 Therapeutic Window – Neurological function as assessed by the modified neuroseverity score.**

Sham animals (green) had no neurological deficits. Vehicle animals (blue) had persistent functional deficits. NAT at 4 h (pink) and NAT at 8 h (magenta) resulted in a steady improvement in neurological function. NAT at 12 h (purple) post-stroke onset resulted in a reduction in neurological deficits, however these persisted for the 7 d assessment period (Sham n=6; Vehicle n=6; NAT-4h n=6; NAT-8h n=7; NAT-12h n=8).



**Figure 6.5 Therapeutic Window – Hemiparesis as assessed by the angleboard.**

Sham animals (green) Vehicle animals (blue) showed significant hemiparesis. NAT treatment at 4 h (pink), 8 h (magenta) or 12 h (purple) improved angleboard performance (\* denotes  $p < 0.05$  versus vehicle; \*\* denotes  $p < 0.01$  versus vehicle; \*\*\* denotes  $p < 0.001$  versus vehicle) (Sham  $n=6$ ; Vehicle  $n=6$ ; NAT-4h  $n=6$ ; NAT-8h  $n=7$ ; NAT-12h  $n=8$ ).

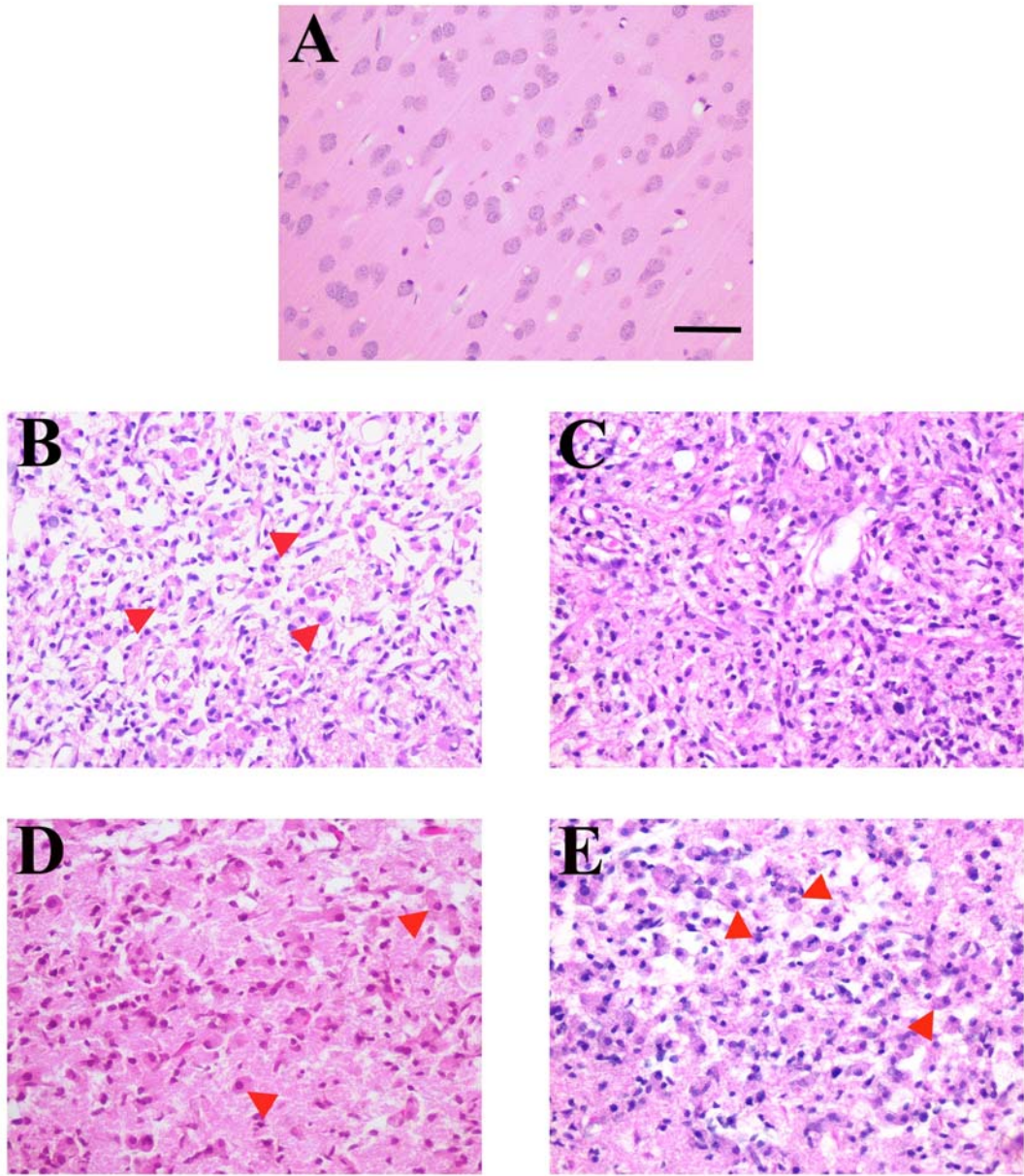
scores significantly lower ( $p < 0.01$ ) than shams on days 1 and 2 post-stroke. Despite a trend towards improved angleboard performance, these improvements were never statistically significant compared to vehicle treated animals on any day post-stroke.

### **6.3.2 Histological Outcome**

#### ***General Pathology: H&E***

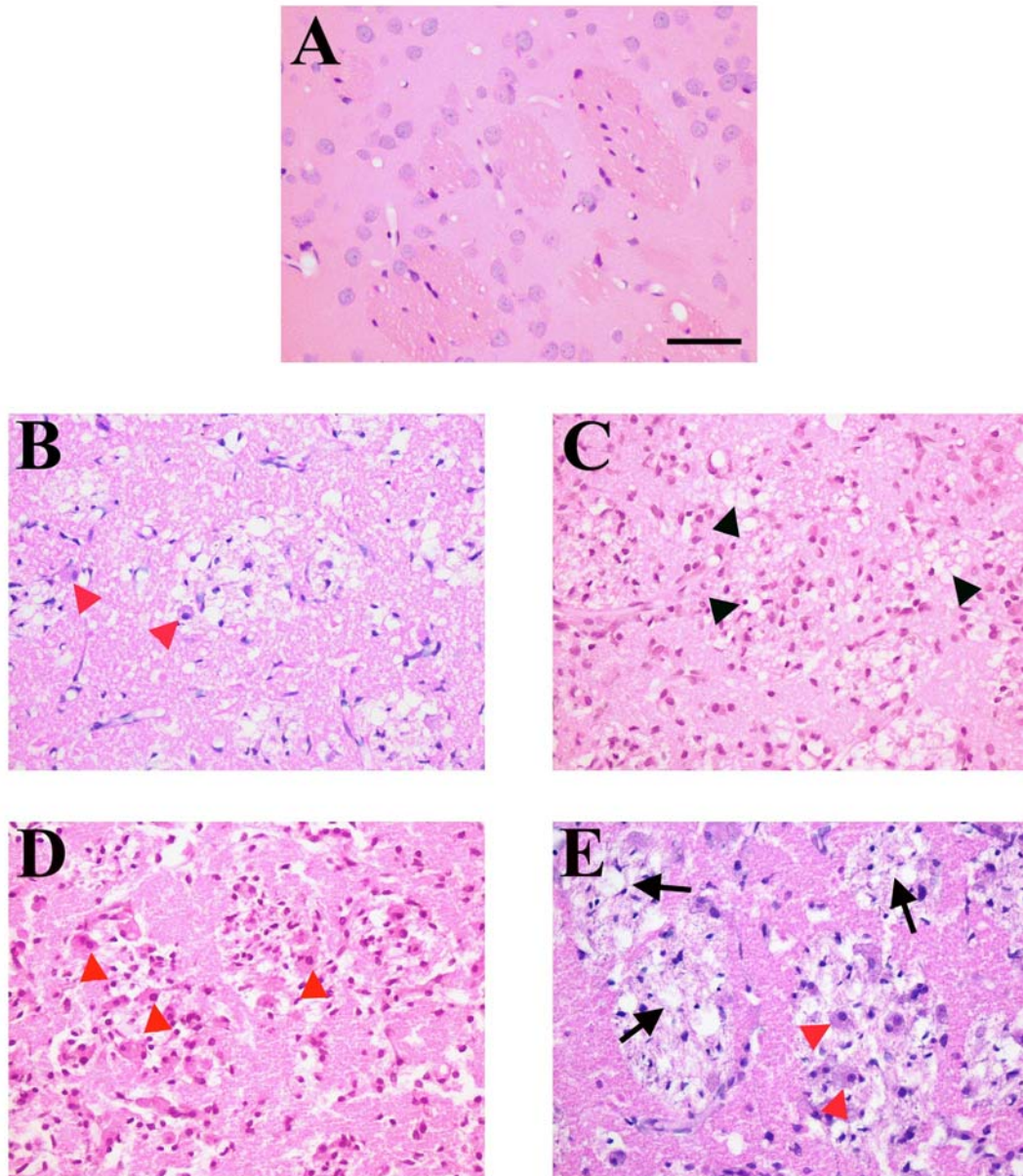
No abnormalities were observed within the cortex of sham animals, confirming that the surgical procedure had no effect on neuronal survival. At 7 d following reperfusion, marked reactive gliosis occupied the infarct (Figure 6.6), sparing little of the cortex. Treatment with NAT at 4 h or 8 h following stroke resulted in a modest preservation in cortical tissue, as evidenced by a reduction in the extent of the reactive gliosis observed. However, treatment with NAT at 12 h following stroke had no effect on the degree of reactive gliosis in cortical tissue.

No abnormalities were observed in white matter of sham animals, consistent with that observed in the cortex. At 7 d following stroke in vehicle treated animals, extensive reactive gliosis was observed occupying the white matter (Figure 6.7), as well as marked vacuolation of the tissue. Treatment with NAT at either 4 h or 8 h following stroke resulted in a modest reduction in the degree of reactive gliosis as well as producing some tissue preservation. However, treatment with NAT at 12 h following stroke did not affect the degree of white matter injury that was observed.



**Figure 6.6 Therapeutic Window – Cortex at 7 d post-reperfusion. H&E stained sections (Bar = 100  $\mu$ m).**

No abnormalities were observed in sham tissue (A). At 7 d post-reperfusion (B), profound reactive gliosis occupied the cortex, and an influx of macrophages (red arrowheads) was also observed. Treatment with NAT at 4 h (C) or 8 h (D) post-stroke onset resulted in a degree of tissue preservation as reduced reactive gliosis was observed. Treatment with NAT at 12 h post-stroke (E) onset did not affect cortical tissue.



**Figure 6.7 Therapeutic Window – White matter at 7 d post-reperfusion. H&E stained sections (Bar = 100  $\mu$ m).**

No abnormalities were observed in sham tissue (A). At 7 d post-reperfusion, profound destruction of the white matter (arrows) was apparent, including tissue vacuolation (black arrowheads) and an influx of macrophages (red arrowheads). Treatment with NAT at 4 h (C) or 8 h (D) post-stroke onset resulted in a modest preservation in tissue architecture, with less vacuolation observed. Treatment with NAT at 12 h (E) post-stroke onset did not affect the degree of white matter damage.

### ***SP Response: SP Immunohistochemistry***

As previously shown in chapter 3, faint SP immunoreactivity was observed in the perivascular tissue of sham animals. By 7 d following stroke, little perivascular SP immunoreactivity was observed (Figure 6.8) as extensive reactive gliosis occupied the infarct. This response was largely unaffected by treatment with NAT at 4 h, 8 h or 12 h following stroke.

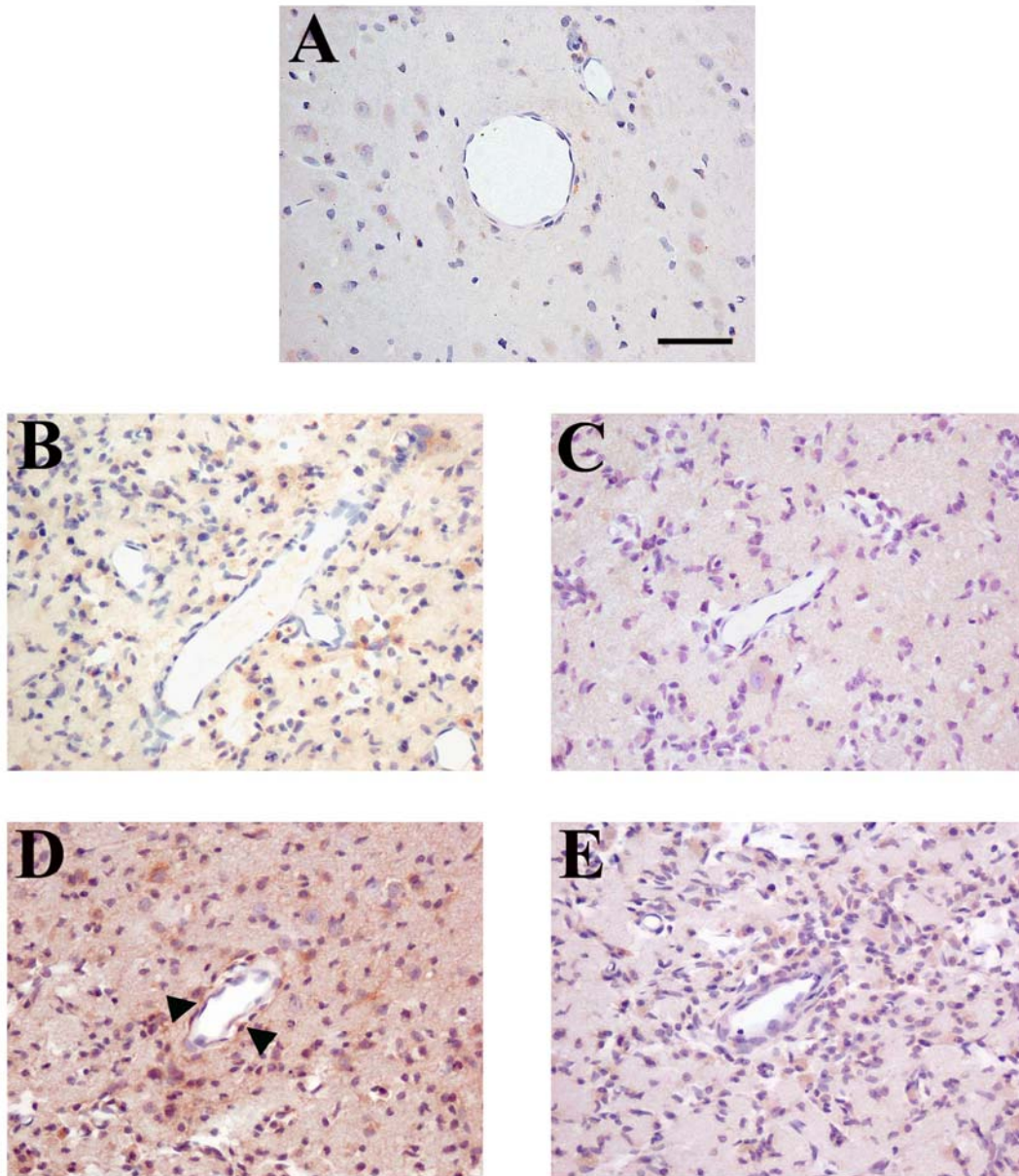
In the cortex of sham animals, faint neuronal SP immunoreactivity was observed, consistent with previous reports (Ribeiro-da-Silva and Hokfelt, 2000). At 7 d following stroke, reactive gliosis occupied the cortex and as a result, little cortical SP immunoreactivity was apparent (Figure 6.9). The SP immunoreactivity of cortical neurons was largely unaffected by treatment with NAT at 4 h, 8 h or 12 h post-stroke.

### ***Axonal Injury: APP Immunohistochemistry***

No axonal injury was observed in sham tissue, confirming that the surgical procedure had little effect on white matter tissue integrity. At 7 d post-stroke, florid axonal injury was observed throughout the white matter (Figure 6.10). Administration of NAT at either 4 h, 8 h or 12 h post-stroke resulted in a marked reduction in the degree of axonal injury, as evidenced by a reduced number of APP immunoreactive axonal swellings.

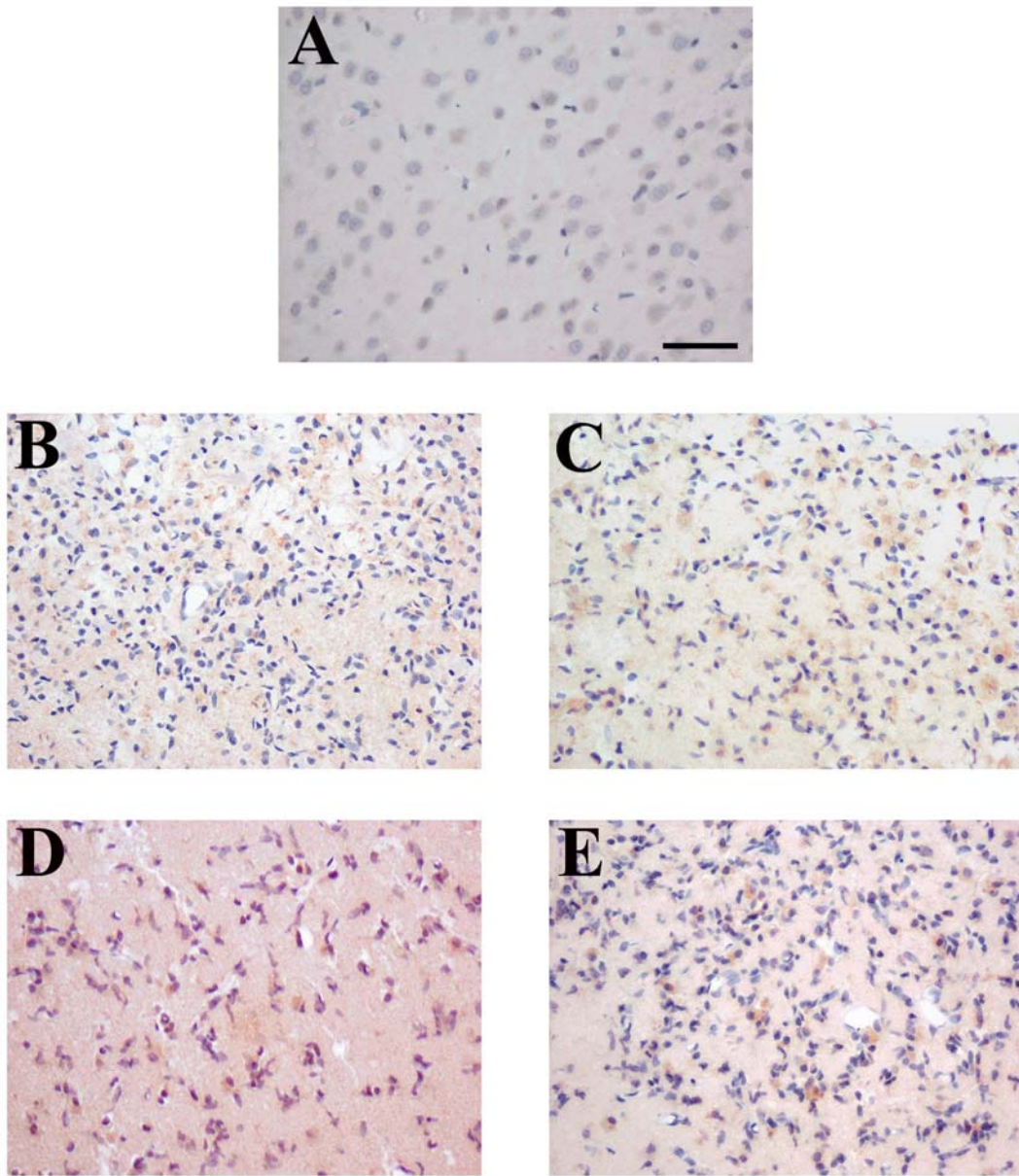
In the cortex of sham animals, faint neuronal APP immunoreactivity was observed. At 7 d following stroke, an increase in neuronal APP immunoreactivity (Figure 6.11) was observed within the cortex of vehicle treated animals. Treatment with





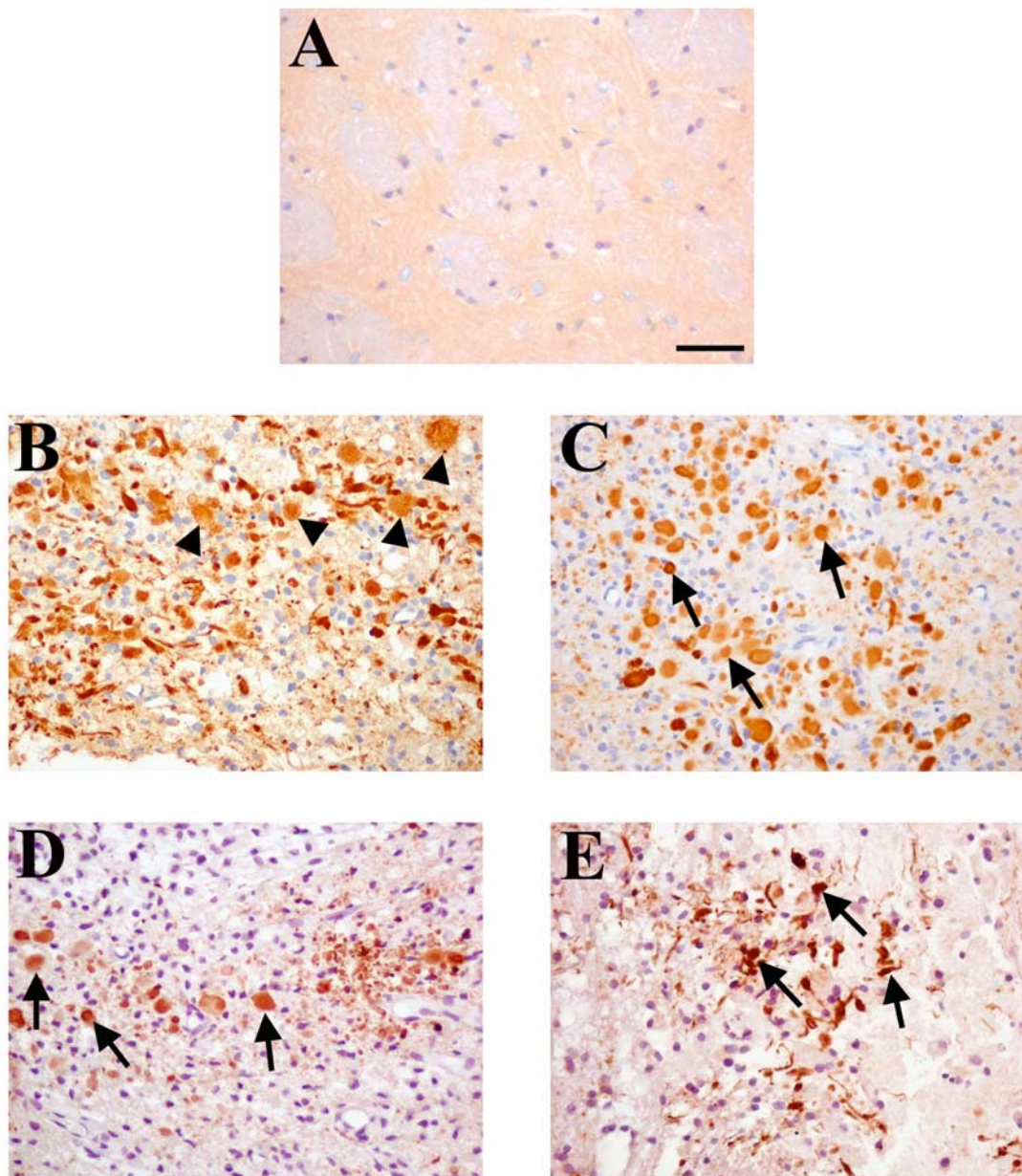
**Figure 6.8 Therapeutic Window – Perivascular tissue at 7 d post-reperfusion. SP stained sections (Bar = 100  $\mu$ m).**

Faint SP immunoreactivity was observed in the perivascular tissue of sham animals (A). At 7 d post-reperfusion, extensive reactive gliosis occupied the cortex and little perivascular SP immunoreactivity was observed. This was largely unaffected by treatment with NAT at 4 h (C), 8 h (D) or 12 h (E) post-stroke onset. However, some SP immunoreactivity (arrowheads) was observed in the NAT at 8 h group.



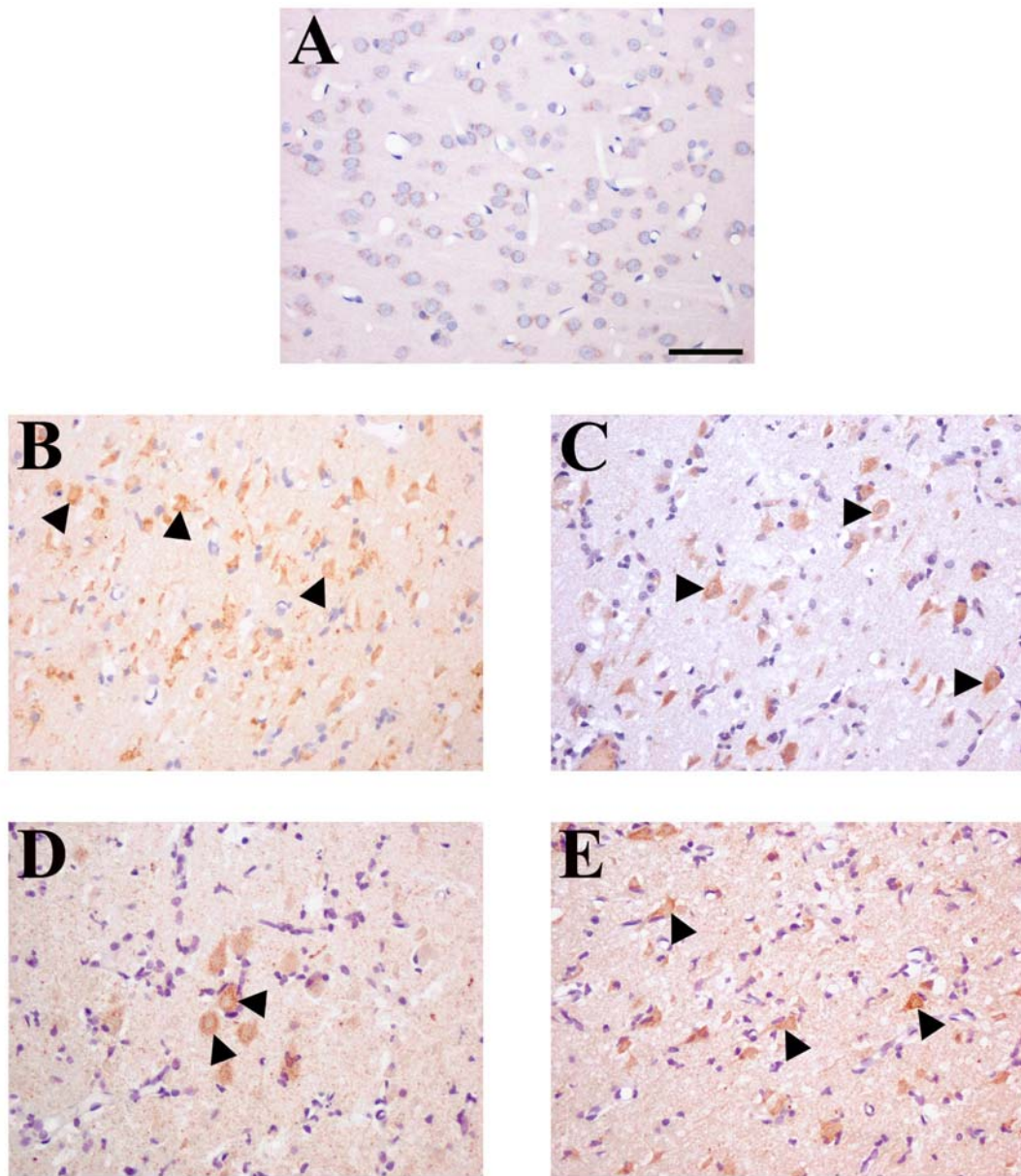
**Figure 6.9 Therapeutic Window – Cortex at 7 d post-reperfusion. SP stained sections (Bar = 100  $\mu$ m).**

Faint SP immunoreactivity was observed within the cortex of sham animals (A). At 7 d following stroke, reactive gliosis occupied the cortex and little SP immunoreactivity was observed. Treatment with NAT at 4 h (C), 8 h (D) or 12 h (E) post-stroke onset did not affect cortical SP immunoreactivity.



**Figure 6.10 Therapeutic Window – Axonal injury within the white matter at 7 d post-reperfusion. APP stained sections (Bar = 100  $\mu$ m).**

No axonal injury was observed in sham animals (A). At 7 d post-reperfusion, florid axonal injury was seen throughout the white matter (B), observed as large retraction balls (arrowheads). Treatment with Nat at 4 h (C) post-stroke onset resulted in a modest reduction in axonal injury (arrows). A further reduction was observed following NAT treatment at 8 h (D) or 12 h (E) post-stroke onset.



**Figure 6.11 Therapeutic Window – Cortex at 7 d post-reperfusion. APP stained sections (Bar = 100  $\mu$ m).**

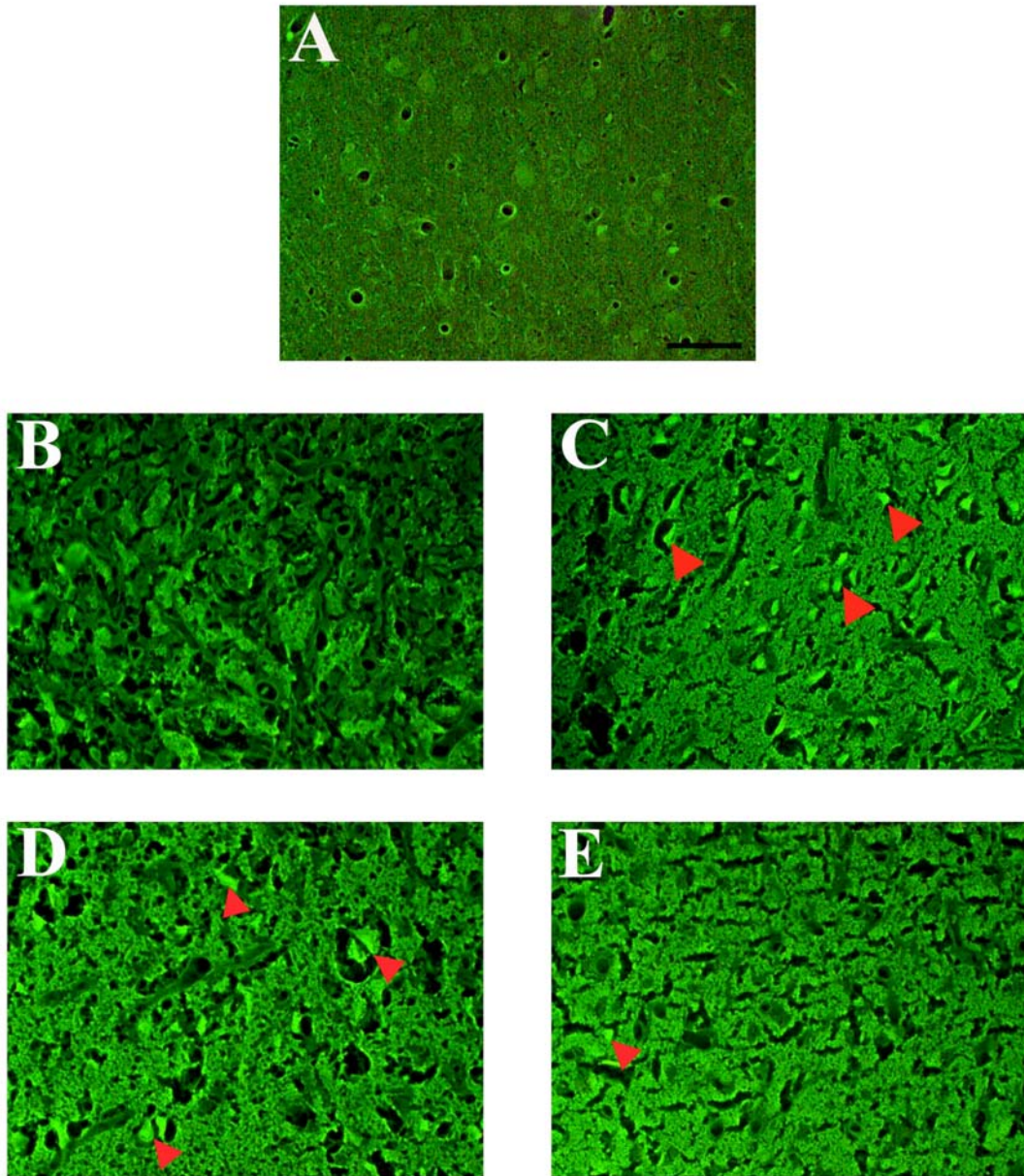
Faint APP immunoreactivity was observed in sham tissue (A). At 7 d following reperfusion (B), increased neuronal (arrowheads) and parenchymal APP immunoreactivity was observed. Treatment with NAT at 4 h (C), 8 h (D) or 12 h (E) post-stroke onset did not affect APP immunoreactivity within cortical neurons.

NAT at 4 h, 8 h or 12 h post-stroke did not affect neuronal APP immunoreactivity within cortical neurons.

***Degenerating neurons: FJC***

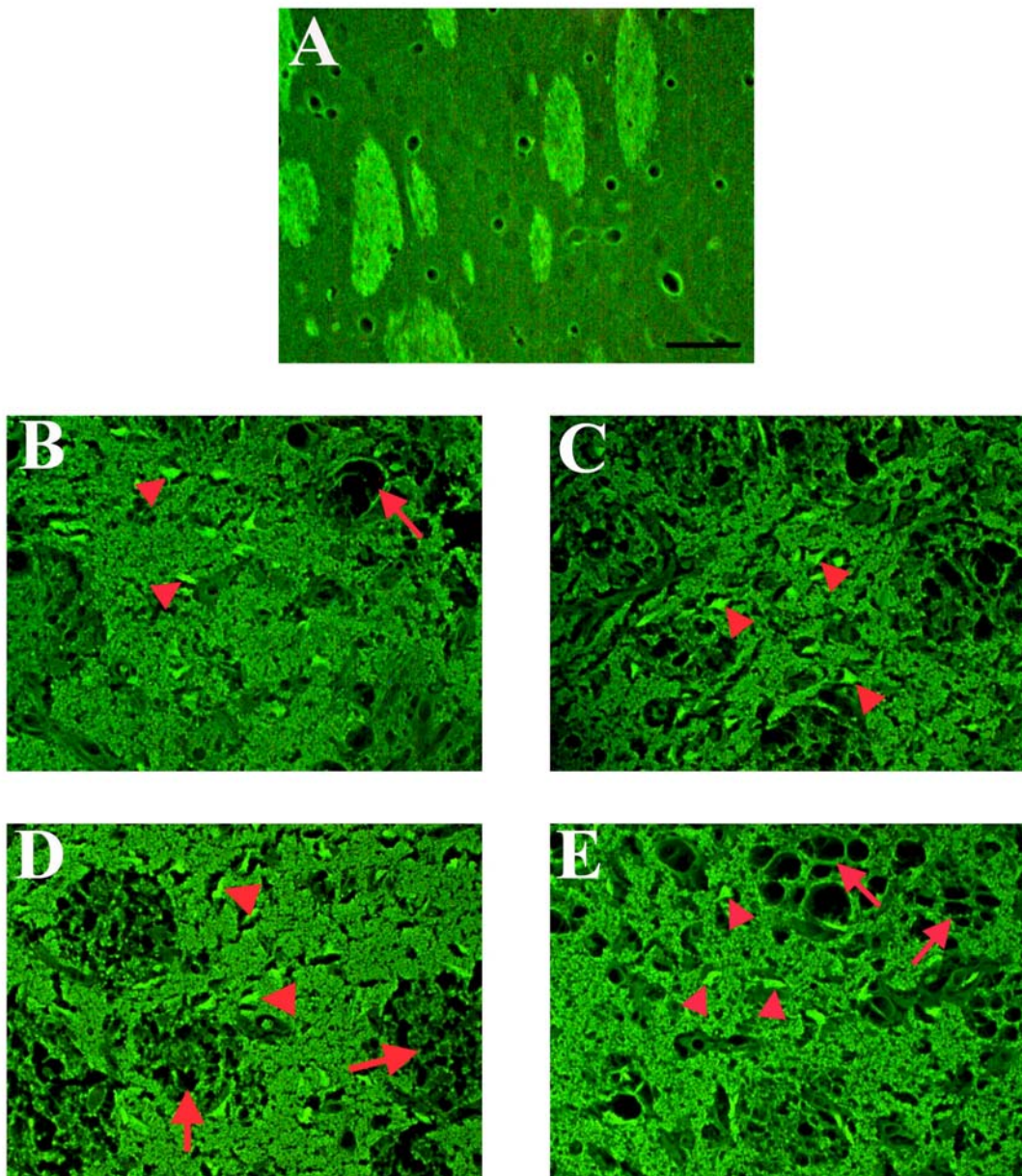
No degenerating neurons were observed within the cortex of sham tissue, consistent with the lack of degenerating neurons in the H&E stained sections. At 7 d post-reperfusion, few degenerating neurons were observed due to the extensive reactive gliosis that occupied the cortex. Following treatment with NAT at 4 h after stroke, a degree of tissue preservation, albeit with some degenerating neurons, could be seen. However, treatment with NAT at 8 h or 12 h after stroke did not appear to enhance tissue viability within the cortex.

No degenerating neurons were observed within the white matter of sham tissue, confirming the absence of histological injury by the surgical techniques used in the current study. At 7 d post-reperfusion, profound reactive gliosis occupied the white matter and as such, few degenerating neurons were observed (Figure 6.13). However, treatment with NAT at 4 h post-stroke resulted in a modest reduction in reactive gliosis and some reduction in degenerating neurons. Treatment with NAT at 8 h or 12 h post-stroke did not affect the viability of tissue within the white matter.



**Figure 6.12 Therapeutic Window – Degenerating neurons within the cortex at 7 d post-reperfusion. FJC stained sections (Bar = 100  $\mu$ m).**

No degenerating neurons were observed within the cortex of sham animals (A). At 7 d post-reperfusion (B), extensive reactive gliosis occupied the cortex and few degenerating neurons (arrowheads) were observed. Treatment with NAT at 4 h (C) post-stroke onset resulted in a modest reduction in the degree of reactive gliosis and some degenerating neurons were observed. However, NAT at 8 h (D) or 12 h (E) post-stroke onset had no effect on cortical tissue.



**Figure 6.13 Therapeutic Window – Degenerating neurons within the white matter at 7 d post-reperfusion. FJC stained sections (Bar = 100  $\mu$ m).**

No degenerating neurons were observed within the white matter of sham animals (A). At 7 d post-reperfusion (B), a marked loss of normal white matter architecture (arrows) with reactive gliosis occupying the tissue, such that few degenerating neurons (arrowheads) were observed. NAT treatment at 4 h (C) post-stroke onset produced a modest reduction in the extent of the reactive gliosis. Treatment with NAT at 8 h (D) or 12 h (E) post-stroke onset did not affect the degree of injury observed within the white matter.

### ***Astrocyte response: GFAP Immunohistochemistry***

As previously described in chapter 3, faint GFAP staining was observed in perivascular tissue of sham animals. By 7 d following stroke, an increase in GFAP immunoreactivity in perivascular tissue was observed in vehicle treated animals (Figure 6.14). Treatment with NAT at 4 h further increased this response, however, treatment with NAT at 8 h or 12 h following stroke produced no effect relative to vehicles.

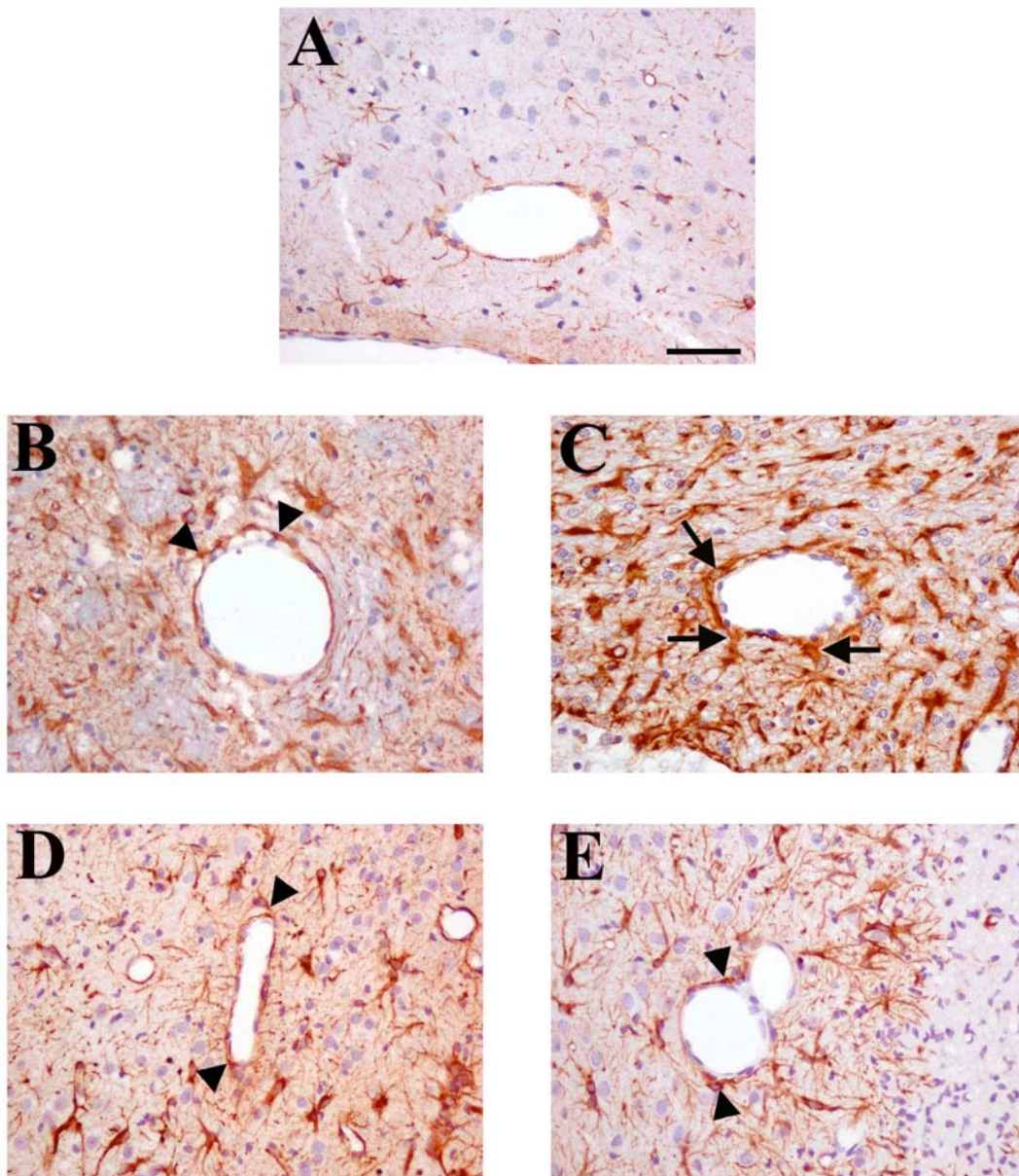
Faint GFAP staining was also observed within the cortex of sham animals, similar to that described in the perivascular tissue. At 7 d post-stroke, an increase in GFAP staining within the infarct boundary zone was observed, in addition to marked astrocyte hypertrophy and hyperplasia (Figure 6.15). This response was further increased by treatment with NAT at either 4 h or 8 h. However, no effect was observed with NAT treatment at 12 h compared to vehicle treated animals.

### ***Macrophage/Activated Microglia response: ED-1 Immunohistochemistry***

No macrophages/activated microglia were observed in association with blood vessels in sham tissue, confirming that the surgical procedure did not elicit an inflammatory response. As previously described for vehicle treated animals at 7 d following stroke, ED-1 positive cells were observed in close association with blood vessels (Figure 6.16). This response to ischaemia was largely unaffected by treatment with NAT at 4 h, 8 h or 12 h after stroke.

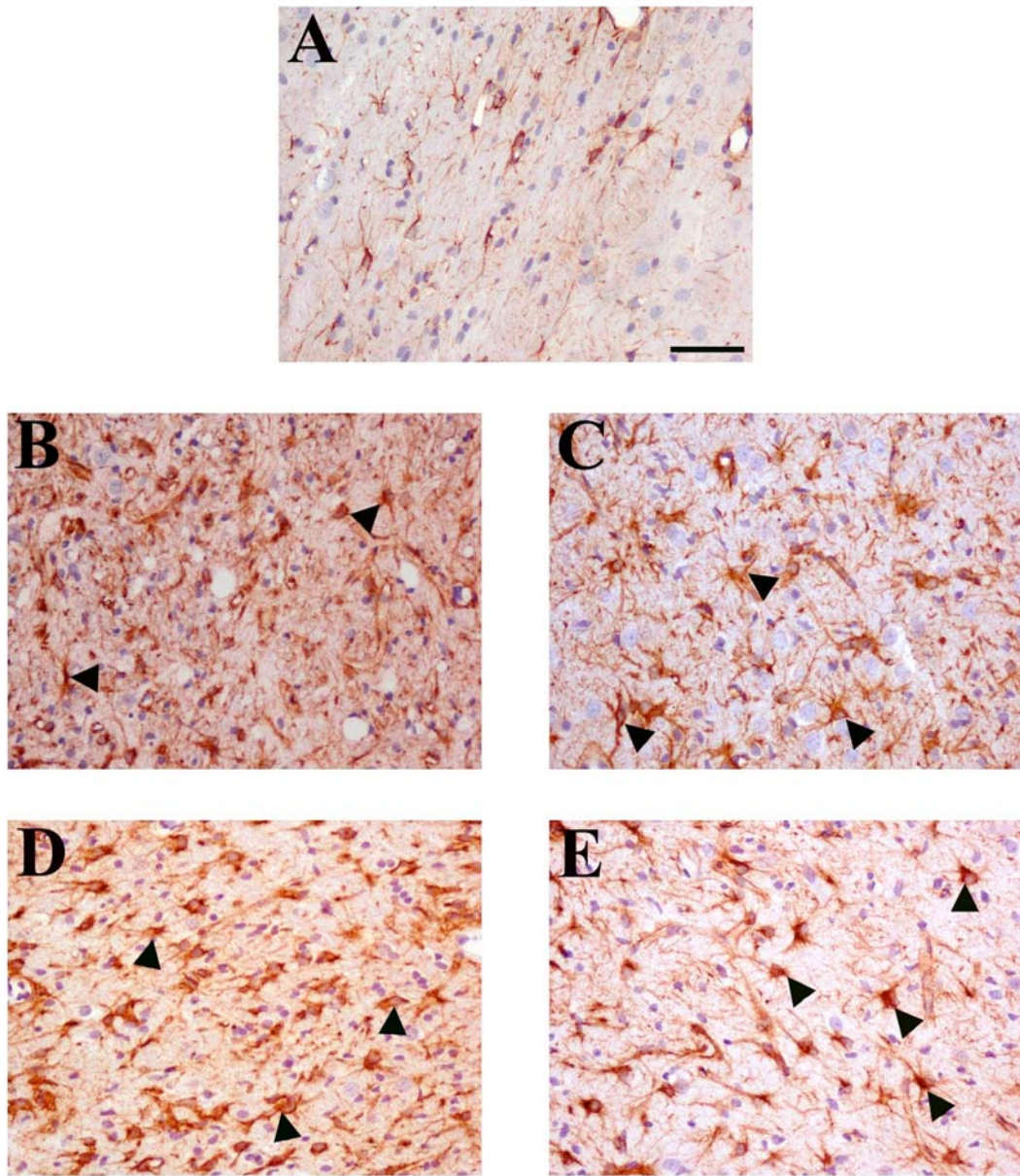
No macrophages/activated microglia were observed in the cortex of sham tissue, consistent with the lack of inflammatory response described for the blood vessels.





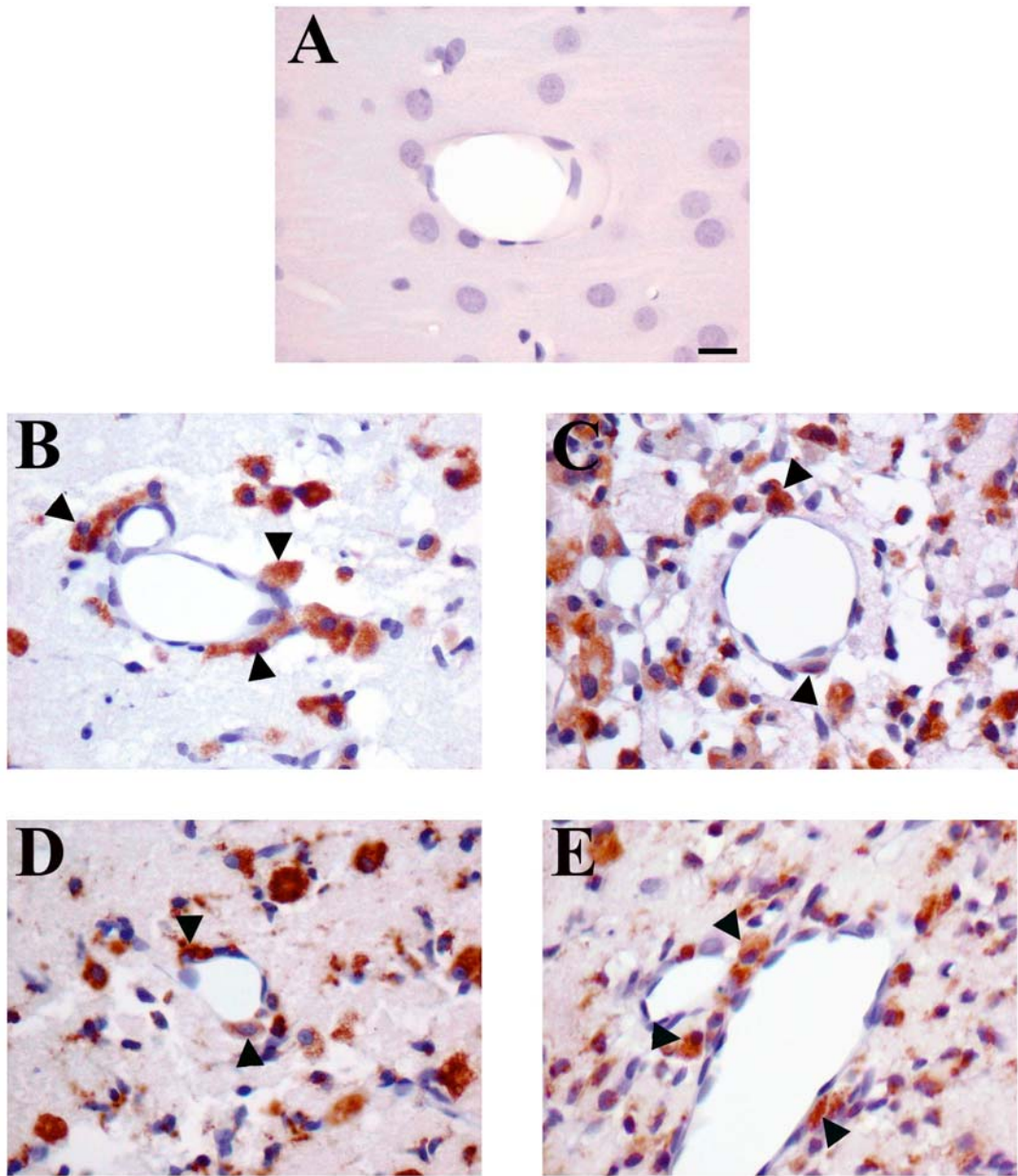
**Figure 6.14 Therapeutic Window – Perivascular astrocytic response at 7 d post-reperfusion. GFAP stained sections (Bar = 100  $\mu$ m).**

Faint GFAP immunoreactivity was observed within sham tissue (A). At 7 d post-reperfusion (B), an increase in GFAP immunoreactivity (arrows) was observed in all groups (Vehicle, NAT at 4h, 8 h and 12 h; B-E). However, the perivascular GFAP immunoreactivity was most profound in the NAT at 4 h (arrowheads) post-stroke onset group.



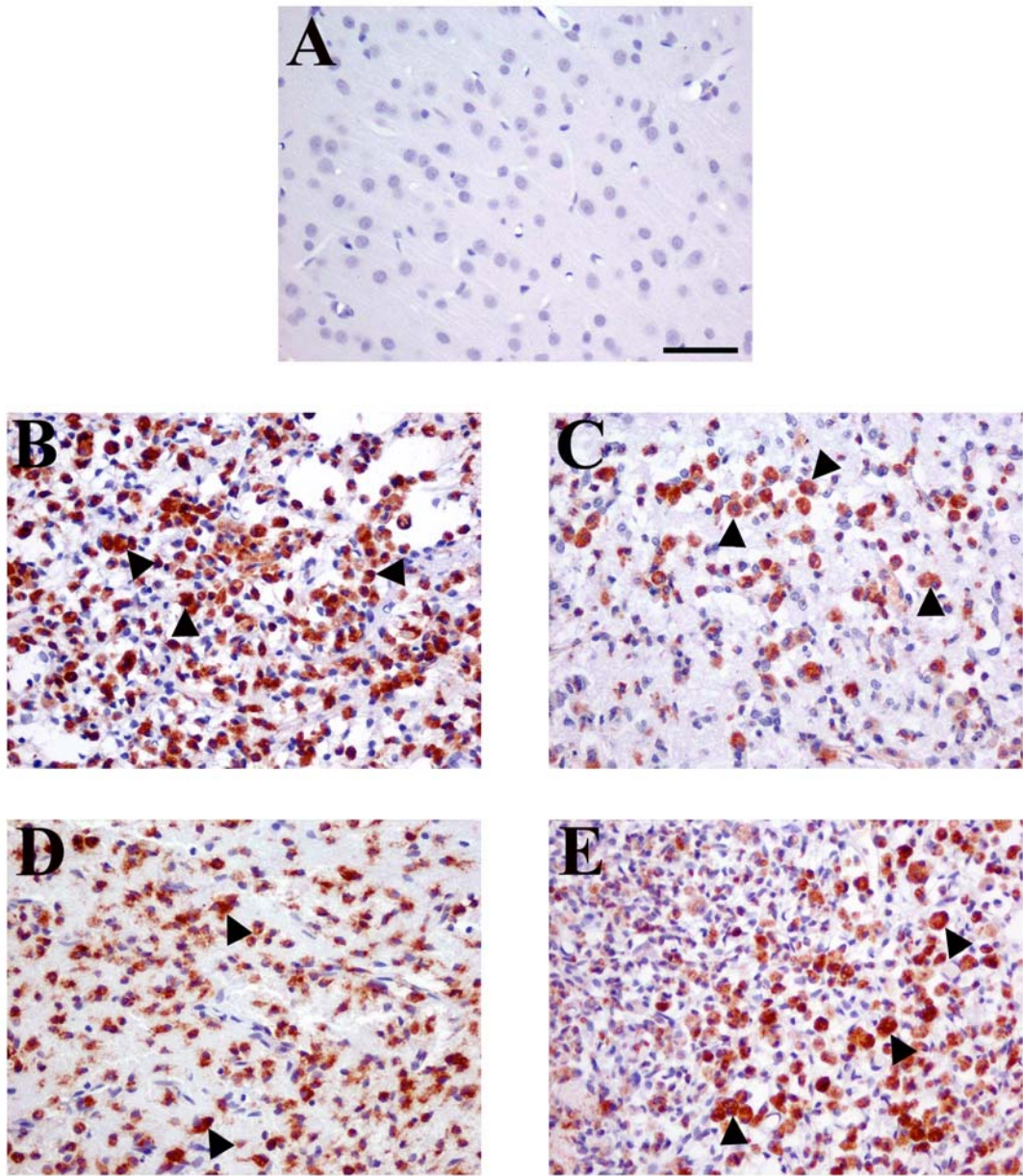
**Figure 6.15 Therapeutic Window – Astrocytic response within the infarct border zone at 7 d post-reperfusion. GFAP stained sections (Bar = 100  $\mu$ m).**

Faint GFAP immunoreactivity was observed in sham tissue (A). At 7 d post-reperfusion (B), an increase in GFAP immunoreactivity (arrowheads) within the infarct border zone was observed. Treatment with NAT at 4 h (C) or 8 h (D) post-stroke onset further exacerbated this response. Treatment with NAT at 12 h post-stroke onset (E) produced GFAP immunoreactivity comparable to vehicles.



**Figure 6.16 Therapeutic Window – Perivascular Macrophage/Activated Microglia response at 7 d post-reperfusion. ED-1 stained sections (Bar = 100  $\mu$ m).**

No macrophages/activated microglia were observed in association with blood vessels in sham tissue (A). At 7 d following 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 at 4 h (C), 8 h (D) or 12 h (E) post-stroke onset.



**Figure 6.17 Therapeutic Window – Macrophage/Activated Microglia response within the infarct at 7 d post-reperfusion. ED-1 stained sections (Bar = 100  $\mu$ m).**

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 at 4 h (C), 8 h (D), or 12 h (E) post-stroke onset resulted in a modest reduction in the number of ED-1 positive cells within the cortex.

By 7 d post-reperfusion, a profound influx of ED-1 positive macrophages/activated microglia was observed to occupy the cortex in vehicle treated animals (Figure 6.17). Treatment with NAT at 4 h, 8 h or 12 h after stroke produced a modest reduction of the number of macrophages/activated microglia observed within the cortex.

## **6.4 Discussion**

In this chapter, we demonstrate that the therapeutic window for the administration of an NK<sub>1</sub> receptor antagonist is up to 8 h following stroke. These studies follow on from previous chapters showing that antagonism of the SP pathway significantly improves functional and histological outcome. Efficacy of NAT up to 8 h following stroke is a significant therapeutic window, especially when viewed in the context of the 3 h window of the currently approved stroke treatment, tPA (Kwiatkowski et al., 1999). Despite thrombolytic therapy within 3 h of symptom onset still being the most effective therapy available for treating ischaemic stroke, the use of tPA in the clinic has received much criticism, mainly due to its' short therapeutic window. As a result, only a small subset of eligible patients, as little as 5-15%, receive thrombolytic therapy (Marler and Goldstein, 2003), and this is mainly due to the time taken for patients to recognise stroke symptoms, get to a hospital and receive a diagnosis and appropriate imaging to exclude haemorrhagic stroke

A number of studies have attempted to elucidate mechanisms to increase the window for thrombolysis and make thrombolysis safer. Indeed, in the previous chapter we demonstrated that adjunctive therapy was effective in reducing the neurotoxicity of tPA. However, whether an NK<sub>1</sub> receptor antagonist can extend the

window for thrombolysis still remains to be elucidated. Given the efficacy of NAT up to 8 h following stroke onset, NAT administration nonetheless represents a more clinically relevant treatment window that shows potential to inhibit deleterious extravascular effects mediated by tPA in any combination therapy. Studies of TBI in our laboratory reveal that a membrane permeable NK<sub>1</sub> receptor antagonist may be administered up to even 12 h following the traumatic event with significant improvements in functional outcome. However, the period of BBB permeability following TBI differs from that observed in stroke and the studies presented in this thesis have used NAT, an impermeable NK<sub>1</sub> receptor antagonist, in all studies. This should not be a barrier in terms of drug efficacy given that following stroke there is profound and sustained breakdown of the BBB, facilitating NAT access to the brain tissue.

There is no shortage of studies investigating the efficacy of neuroprotective agents following stroke. Such agents include glutamate antagonists, Ca<sup>2+</sup> channel antagonists, Na<sup>+</sup> channel blockers, growth factors and free radical scavengers (Lee et al., 2000). Unfortunately, many of these studies have relied on drug administration before or very soon after (within minutes) the onset of ischaemia. Whilst such studies are important to ascertain the contribution of various injury pathways to ischaemic damage, the therapeutic window for administration is not clinically relevant. Accordingly, with over 50 neuroprotective agents evaluated in over 100 clinical trials, it is not surprising that few have proven clinically useful. Hypothermia was found to be effective in improving post-stroke outcome by reducing both infarct volume and functional deficits in the long-term (Colbourne et al, 1999; 2000). However, clinical trials have not been consistently positive, in part

because of the variability in applying the hypothermia protocol. The reactive oxygen species component of reperfusion injury is another common target for neuroprotective agents. Free radical scavengers were found to reduce infarct volume when administered up to 3 h after the onset of experimental ischaemia. However, subsequent clinical trials of a free radical scavenger were terminated due to safety concerns with the drug (Lee et al., 2000). Finally, inhibition of iNOS up to 24 h following experimental stroke significantly reduced lesion size after pMCAO (Nagayama, 1998) however, subsequent clinical trials have failed to reproduce this positive effect. The search for an effective stroke therapy remains an urgent priority, and given that tPA is the only intervention approved for clinical use, it is important that potential neuroprotective agents demonstrate safety for use in conjunction with tPA, and also a clinically relevant therapeutic window.

While NAT administered up to 8 h following stroke onset was efficacious in terms of functional motor outcome, NAT administered up to 12 h following stroke reduced the number of macrophages/activated microglia observed within the infarct. This may, in part, be related to the role SP plays as a chemotactic factor for monocytes (Ruff, 1985). Therefore, a reduced recruitment of monocytes into the brain would manifest as a reduced number of activated macrophages within the tissue. However, such a decreased inflammatory reaction was not accompanied by an improvement in functional motor outcome.

Axonal injury is a characteristic feature of ischaemic injury (Lewis et al., 1996) and can be visualised as retraction balls throughout the white matter axonal tracts. In the present study, florid axonal injury was observed within the white matter at 7 d

following stroke. Treatment with NAT up to 12 h following stroke was able to markedly reduce the degree of axonal injury observed. Whilst the mechanism whereby NAT was able to reduce axonal injury following stroke is unknown, it may partially explain the resolution of functional deficits observed. However, in the case of 12 h treatment, a reduction in axonal injury was observed in the absence of any detectable improvement in functional outcome. A criticism of rodent models of stroke is that they have small lissencephalic brains with a large grey matter/to white matter ratio. Many human strokes occur within the white matter. As such, many neuroprotective agents demonstrating efficacy in rodent models may not convey protection in human stroke. However, in the present study NAT was able to reduce the degree of white matter injury, as evidenced by a reduced number of axonal swellings. Therefore, NAT may potentially be a clinically relevant therapeutic agent with efficacy in both grey and white matter.

An increase in GFAP staining within the infarct boundary zone was observed following treatment with NAT at 4 h or 8 h following stroke. Such an increase in GFAP staining reflects an increase in astrocyte hypertrophy and hyperplasia within this region. This response has been purported to contribute to restoration of BBB integrity following ischaemia. As such, this may, in part, explain the improved outcome observed in these animals. Furthermore, it may also explain the maintenance of BBB integrity observed in the NAT at 4 h treatment group (see chapter 4). The more immature phenotype displayed by these astrocytes, indicated by the 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- $\beta$ , NGF and VEGF, amongst others (Chen and Swanson, 2003).

Taken together, these findings indicate that interference with the SP pathway conveys significant protection from the development of functional deficits following stroke. They represent a thorough evaluation of the efficacy of NAT, through the use of functional and histological end-points.

## **6.5 Conclusions**

Administration of an NK<sub>1</sub> antagonist, NAT, is an effective means of providing protection from neurological and functional deficits whilst also preserving brain tissue survival following ischaemia. Specifically, NAT may be administered up to 8 h following the onset of cerebral ischaemia with significant improvements in histological and functional outcome. Treatment as late as 12 h after injury reduces inflammation and axonal injury, albeit without a significant improvement in functional outcome. Having established the therapeutic window for administration of an NK<sub>1</sub> receptor antagonist, the next logical step was to determine whether it was also effective in different grades of ischaemia. This is the subject of chapter 7.