CHARACTERISING THE ROLE OF SUBSTANCE P IN ACUTE ISCHAEMIC STROKE

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November 2007

A thesis submitted to the University of Adelaide in fulfilment of the requirements

for the degree of Doctor of Philosophy

CHAPTER 7:

NK_1 RECEPTOR ANTAGONIST FOLLOWING MILD, MODERATE AND SEVERE ISCHAEMIC STROKE

7.1 Introduction

In the previous chapter, we established that the therapeutic window for treatment with an NK_1 receptor antagonist was up to 8 h following stroke onset. However, this was following 2 h of ischaemia, a relatively severe insult, and it is unclear whether antagonism of the SP pathway would have similar beneficial effects following milder insults. Indeed, the relationship between ischaemia duration and behavioural outcome has not been definitively established (Aronowski et al., 1996). However, a large number of studies have assessed functional outcome following stroke (Candelario-Jalil et al., 2004; Ding et al., 2001; Groger et al., 2005; Miyai et al., 1999; Reglodi et al., 2003; Rogers et al., 1997; Roof et al., 2001; Schiemanck et al., 2005), albeit that there is no consensus amongst researchers as to which functional outcome tests are the most appropriate for the evaluation of functional deficits following stroke. Many studies only use a neuroscore as a means of assessing functional outcome. However, this may not provide a complete picture of functional capacity following stroke. The use of a battery of behavioural tests provides a more accurate representation of functional capacity (DeVries et al., 2001; Gladstone et al., 2002; Rogers et al., 1997). Optimally, however, both histological and behavioural end-points are important when assessing the efficacy of potential therapeutic agents (Rogers et al., 1997). As such, the aim of the present study was to examine the histological and functional changes that occur in mild, moderate and severe ischaemia and to ascertain whether the antagonism of the SP pathway was effective in reducing histological abnormalities and functional deficits at all levels of stroke severity.

7.2 Study Design

The duration of ischaemia was adjusted to produce varying degrees of infarction as follows: 1 h in mild group, 1.5 h in the moderate group and 2 h in the severe group. Animals (n=25) were subject to MCAO and reperfusion, as previously described in chapter 2. After animals were randomly assignment to treatment groups they were administered either 25 μmoles/kg NAT or an equal volume of saline vehicle at 8 h following stroke onset. The 8 h time-point was chosen given that it resulted in a significant improvement in functional outcome at a clinically relevant time-point. Drug preparation and administration were as per outlined in chapter 2.

7.2.1 Functional Outcome

Commencing at 24 h post-surgery, the functional outcome of animals was assessed for a 7 d period using the rotarod, bilateral asymmetry test, open field, modified neuroseverity score and angleboard tests. The testing paradigm was as described in detail in chapter 2.

7.2.2 Histological Outcome

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

7.2.3 Statistical Analysis

All parametric data are expressed as mean ± SEM and was analysed using analysis of variance followed by individual student newman-keuls post-hoc tests. The

neuroscore data is expressed as the median and was analysed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test. The level of significance was taken at $p<0.05$.

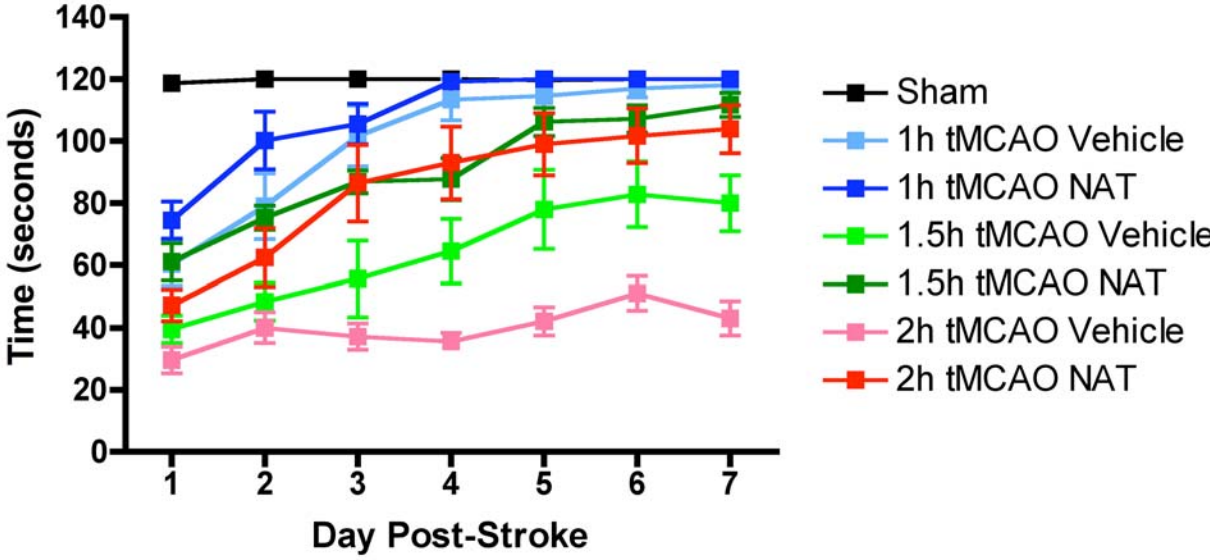
7.3 Results

7.3.1 Functional Outcome

Motor Function: Rotarod

No motor deficits were observed in sham animals, again confirming that the surgical procedure had no effect on motor function. Following 1 h stroke, only very mild motor deficits were observed. Animals in this group were able to rapidly learn the rotarod task, such that both the NAT and vehicle-treated groups were performing at normal functional levels by day 2 and 3 post-stroke respectively. Despite the better performance in the NAT-treated animals at every time-point, there was no significant difference between the vehicle and NAT groups in the mild ischaemia group on any day post-stroke. Following 1.5 h stroke, vehicle-treated animals showed profound motor deficits. Although the rotarod performance in these animals improved over the 7 d assessment period, they did not reach normal functional levels on any day post-stroke ($0.001<p<0.01$ versus shams). Treatment with NAT markedly improved motor function, with animals in this group showing an improved ability to perform on the rotarod with time. Animals in this group recorded rotarod scores significantly better ($0.01<p<0.05$) than the 1.5 h vehicles on days 2, 3, 5 and 7 post-stroke, recording scores comparable to shams by day 5 post-stroke ($p>0.05$). Following 2 h stroke, the vehicle-treated animals demonstrated profound motor deficits. Such deficits persisted ($p<0.001$ versus shams) for the entire assessment period with little improvement in motor performance observed.

Figure 7.1 Mild, moderate and severe ischaemia – NAT at 8 h. Motor function as assessed by the rotarod.
Sham animals had no motor deficits (black). Animals in the 1 h ischaemia group (vehicle or NAT-treated; light blue and dark blue) showed no motor deficits, rapidly learning the task and performing at normal levels by day 2 post-stroke. Animals in the 1.5 h group (light green) had persistent motor deficits, however treatment with NAT (dark green) significantly improved motor performance. Animals in the 2 h vehicle group (pink) had persistent motor deficits and did not reach normal functional levels on any day post-stroke. Treatment with NAT (red) significantly improved rotarod performance, however this group did not reach normal functional levels on any day post-stroke (Sham n=6; 1h Vehicle n=7; 1h NAT n=6; 1.5h Vehicle n=6; 1.5h NAT n=6; 2h Vehicle n=12; 2h NAT n=7).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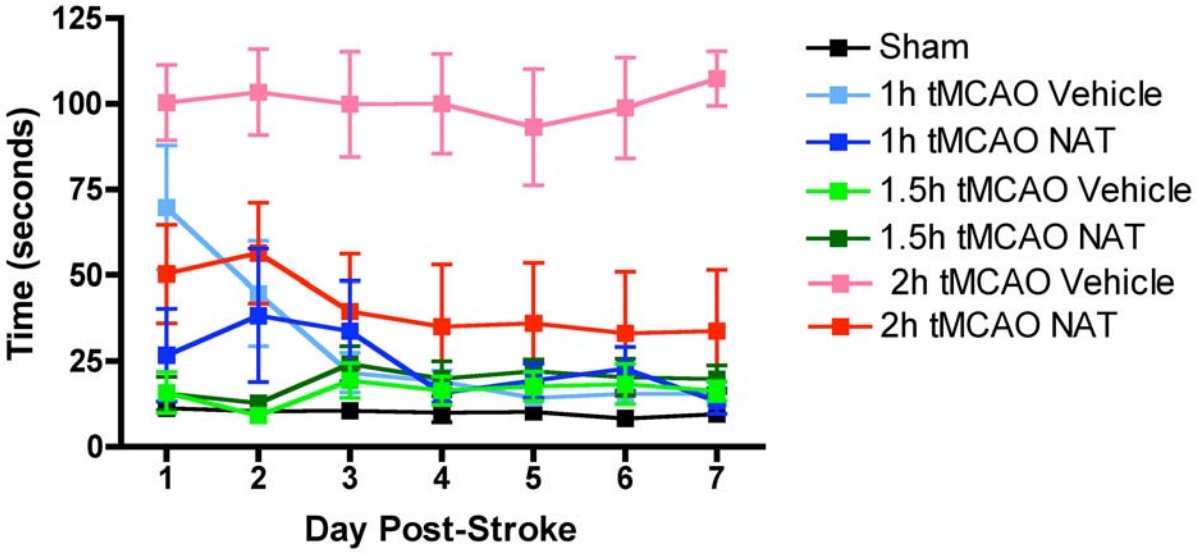
Treatment with NAT resulted in a significant improvement in motor performance. Although animals in this group initially showed motor deficits ($0.001<p<0.05$), by day 3 they were performing at levels significantly better ($p<0.001$) than vehicles, and by day 5 post-stroke no detectable motor deficits were observed ($p>0.05$ versus shams). There was a clear dose-response effect between the length of stroke and rotarod score, indicating that performance on the rotarod test may be an accurate indicator of stroke severity.

Sensory Function: Bilateral Asymmetry Test

No sensory deficits were observed in sham animals, confirming that the surgical procedure had no effect on sensory function. On day 1 following 1 h stroke, vehicle-treated animals demonstrated an impaired ability ($p<0.001$ versus shams) to sense and remove the tape. However, they showed a rapid improvement in sensory function, recording latencies comparable to shams ($p>0.05$) by day 2 post-stroke. Animals treated with NAT had no sensory deficits, performing at normal levels ($p>0.05$ versus shams) on all assessment days post-stroke. Animals in both the 1.5 h vehicle and NAT-treated groups showed no observable sensory deficits on any day post-stroke. Such that, there was no significant difference ($p>0.05$) between these groups and these animals consistently recorded latencies comparable ($p>0.05$) to shams. Following stroke, the 2 h vehicle group demonstrated profound sensory deficits. Animals in this group did not show any recovery of sensory function over the assessment period and recorded latencies significantly worse ($p<0.001$) than shams on all days post-stroke. However, treatment with NAT resulted in a recovery of sensory function, such that, these animals performed significantly better

Figure 7.2 Mild, moderate and severe ischaemia – NAT at 8 h. Sensory function, as assessed by the bilateral asymmetry test.

Sham animals had no sensory deficits (black). Animals in the mild and moderate ischaemia groups all showed normal sensory function by day 3 post-stroke, regardless of treatment type. In contrast, animals in the severe ischaemia group (pink) had persistent sensory deficits that did not improve over the 7 d assessment period. However, NAT treatment (red) significantly improved sensory function (Sham n=6; 1h Vehicle n=7; 1h NAT n=6; 1.5h Vehicle n=6; 1.5h NAT n=6; 2h Vehicle n=12; 2h NAT n=7).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1h tMCAO NAT
1.5h tMCAO Vehicle
1.5h tMCAO NAT
2h tMCAO Vehicle
2h tMCAO NAT

($0.001<p<0.01$) than vehicles on all assessment days, and by day 3 post-stroke recorded latencies comparable to shams ($p>0.05$).

Spontaneous Exploratory Behaviour: Open Field

Normal open field activity levels were observed in sham animals, indicating that the surgical procedure had no effect on spontaneous exploratory behaviour. A decline in spontaneous activity was noted over time, however this is likely to reflect habituation (McIlwain et al., 2001; Paylor et al., 2006; Stohr et al., 1998). Following 1 h stroke, the vehicle- and NAT-treated groups recorded open field scores comparable ($p>0.05$) to shams, despite a trend towards decreased spontaneous exploratory behaviour. There was no significant ($p>0.05$) difference between these groups. The 1.5 h stroke vehicle-treated group showed a trend towards decreased spontaneous exploratory behaviour compared to shams but this was not significant. This pattern of spontaneous exploratory behaviour was unaffected by NAT treatment and there was no significant difference between the groups. In contrast, following 2 h stroke, the vehicle-treated group showed a significant decline ($p<0.01$) in spontaneous exploratory behaviour. However, this was only significant on day 1 post-stroke. Treatment with NAT at 8 h after stroke significantly improved ($0.001<p<0.05$) open field performance, such that the spontaneous exploratory behaviour in this group was comparable to shams ($p>0.05$).

Neurological Function: mNSS

No neurological deficits were observed in sham animals, consistent with the lack of deficits observed in the other tests. Following stroke, the 1 h vehicle group recorded a mNSS ranking of moderate injury (mNSS 5-9). This ranking quickly

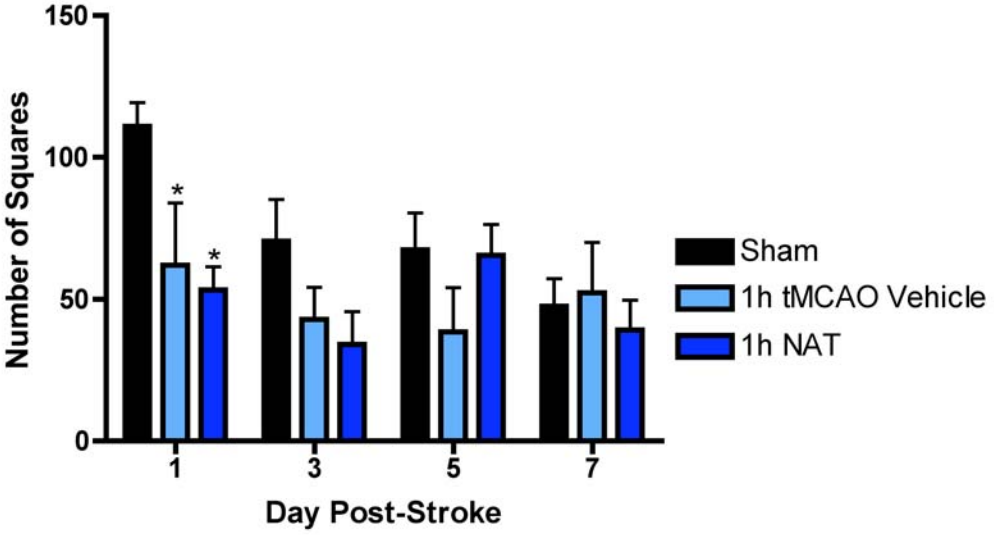
improved to mild injury (mNSS 1-4) by day 2 and by day 4 post-stroke no detectable neurological deficit was observed. NAT treated animals however, recorded a ranking of mild injury on day 1 post-stroke and by day 2 post-stroke no neurological deficits were apparent. However, these differences in mNSS scores were not significant. In contrast, following 1.5 h stroke, the vehicle group showed marked neurological deficits and were ranked as having a moderate injury on day 1 post-stroke. Although a gradual decline in mNSS ranking was observed over the assessment period, these animals still had an mNSS ranking of mild injury by day 7 post-stroke. Treatment with NAT appeared to reduce neurological deficits, however this reduction in mNSS ranking was not significant. Following 2 h stroke, the vehicle group were ranked as having a moderate injury. These neurological deficits were significantly worse ($p<0.001$) than shams and persisted for the 7 d assessment period. Treatment with NAT resulted in a significant improvement ($0.01<p<0.05$) in neurological function, such that by day 5 post-stroke, no detectable neurological deficits were observed. However, overall, little statistical significance was observed for this test, and this is likely to reflect the large within group variability. There was, nonetheless, a dose-response effect noted between severity of stroke and mNSS, as least over the first few days post-stroke.

Hemiparesis: Angleboard

No hemiparesis was observed in sham animals confirming that the surgical procedure had no effect on muscle strength and balance. Following stroke, a decline in angleboard score was observed in the 1 h vehicle group. However, this was only significant ($0.001<p<0.01$) on days 1 and 2 post-stroke. Treatment with NAT at 8 h resulted in a reduction in hemiparesis, as evidenced by improved

Figure 7. 3 Mild, moderate and severe ischaemia – NAT at 8 h. Spontaneous exploratory behaviour, as assessed by the open field.

Sham animals (black) exhibited normal spontaneous exploratory behaviour. No difference in spontaneous exploratory behaviour was observed between the vehicle (light blue) and NAT-treated (dark blue) mild ischaemia group. The number of squares travelled through by these animals was not significantly different to shams. Similarly, no significant difference as observed in the spontaneous exploratory behaviour of the vehicle (light green) and NAT-treated (dark green) animals in the moderate ischaemia group. However, a significant decline in spontaneous exploratory behaviour was observed in the severe ischaemia group (pink). Treatment with NAT (red) increased spontaneous exploratory behaviour to normal levels (*denotes $p<0.05$ versus shams; †denotes $p<0.05$ versus vehicle; Sham n=6; 1h Vehicle n=7; 1h NAT n=6; 1.5h Vehicle n=6; 1.5h NAT n=6; 2h Vehicle n=12; 2h NAT n=7).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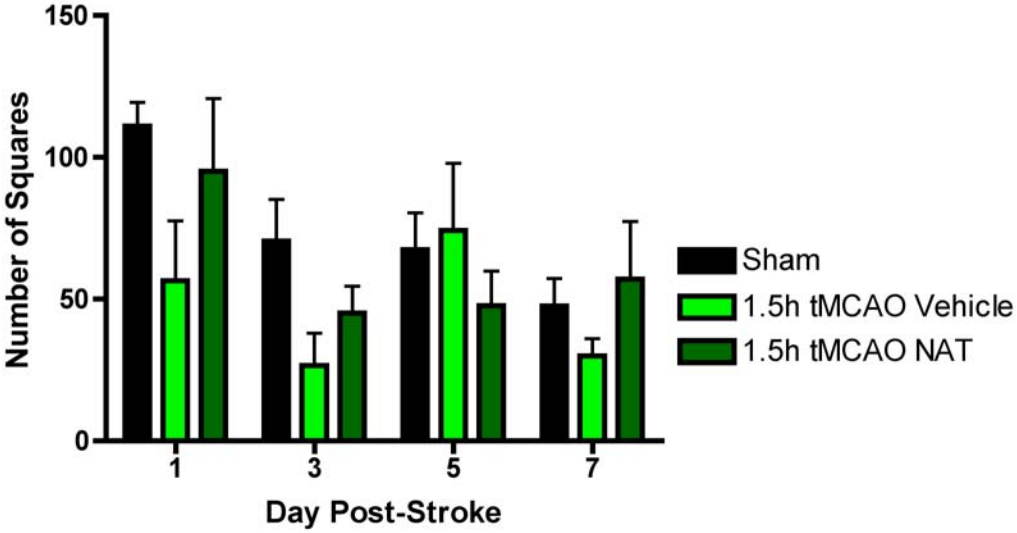
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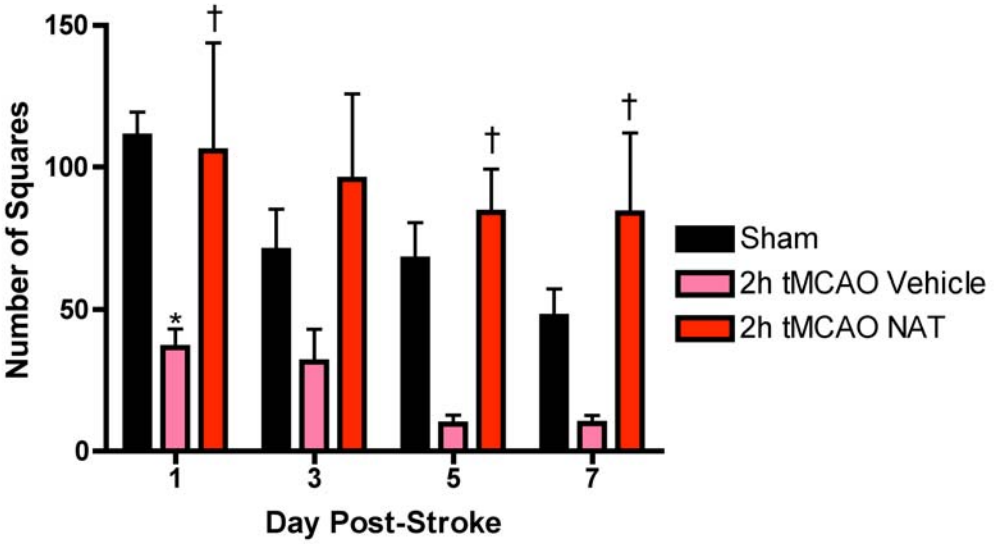
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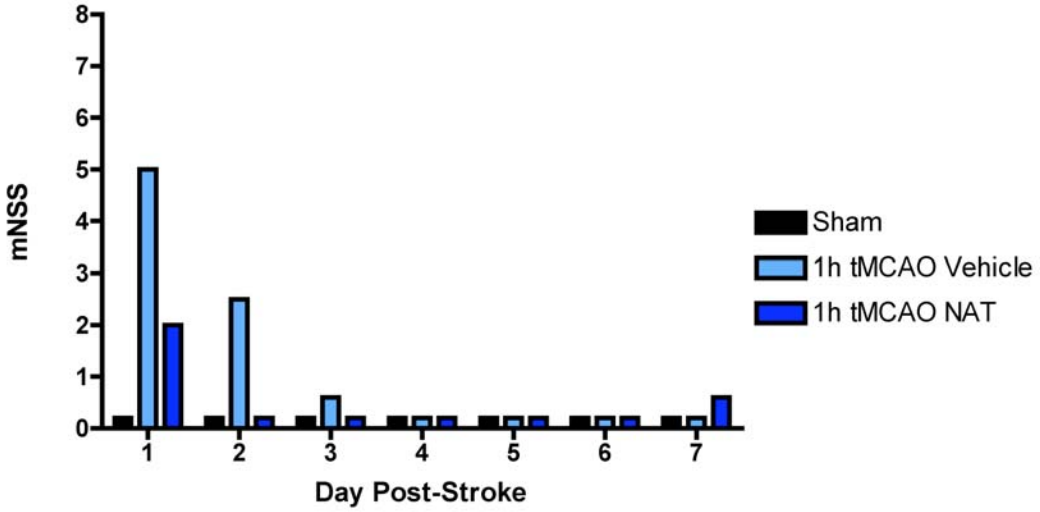
Figure 7.4 Mild, moderate and severe ischaemia – NAT at 8 h. Sensory function, as assessed by the bilateral asymmetry test.

Sham animals had no sensory deficits (black). Animals in the mild and moderate ischaemia groups all showed normal sensory function by day 3 post-stroke, regardless of treatment type. In contrast, animals in the severe ischaemia group (pink) had persistent sensory deficits that did not improve over the 7 d assessment period. However, NAT treatment (red) significantly improved sensory function (Sham n=6; 1h Vehicle n=7; 1h NAT n=6; 1.5h Vehicle n=6; 1.5h NAT n=6; 2h Vehicle n=12; 2h NAT n=7).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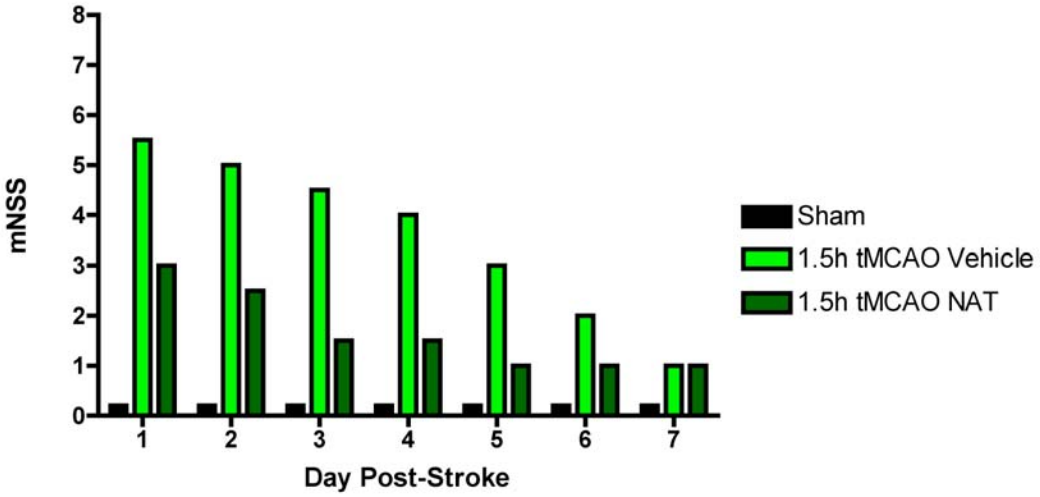
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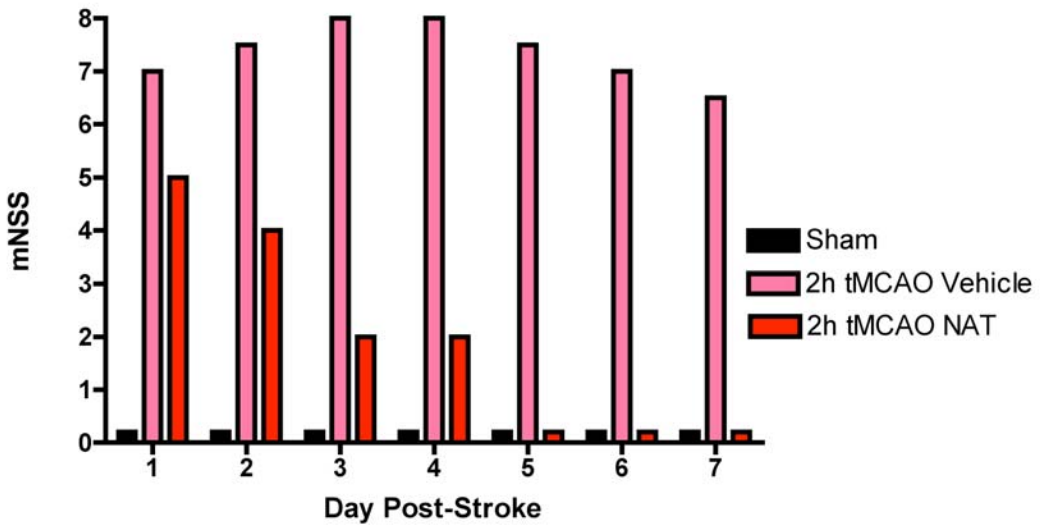
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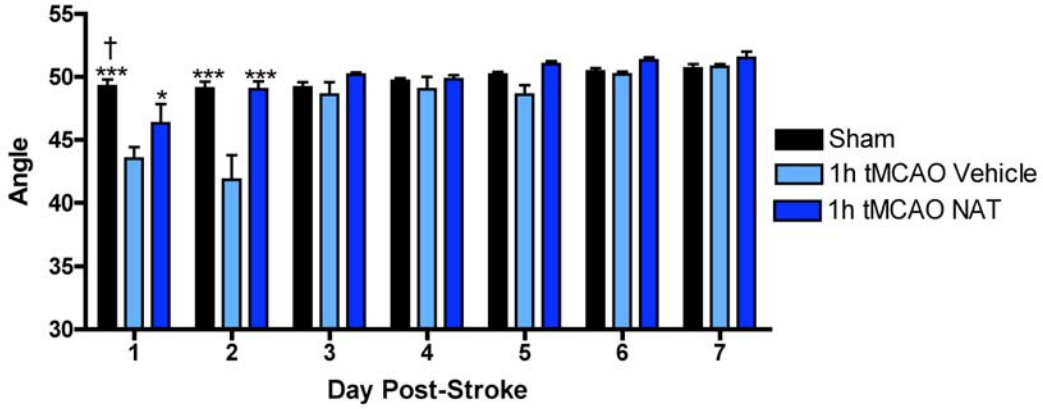
Figure 7.5 Mild, moderate and severe ischaemia – NAT at 8 h. Hemiparesis as assessed by the angleboard.

Sham animals (black) showed no signs of hemiparesis. Hemiparesis was observed in the mild ischaemia group (light blue) following stroke, however it recovered by day 3 post-stroke. Treatment with NAT (dark blue) accelerated this recovery. No hemiparesis was observed in either the vehicle or (light green) NAT (dark green) treated animals in the moderate ischaemia group after stroke. Following severe ischaemia (pink), animals demonstrated profound hemiparesis that persisted for the 7 d assessment period. However, NAT treatment (red) resulted in a reduction in hemiparesis (*denotes $p<0.05$ versus vehicle; **denotes $p<0.01$ versus vehicle; ***denotes $p<0.001$ versus vehicle; †denotes $p<0.05$ verus NAT; Sham n=6; 1h Vehicle n=7; 1h NAT n=6; 1.5h Vehicle n=6; 1.5h NAT n=6; 2h Vehicle n=12; 2h NAT n=7).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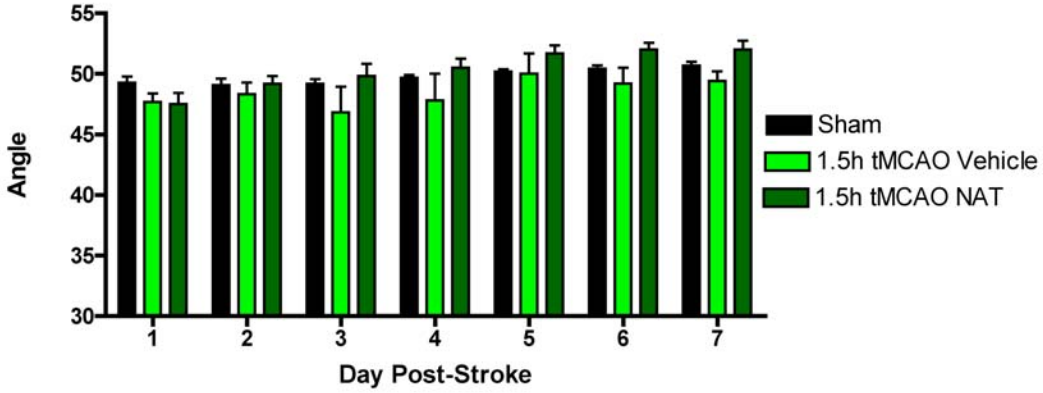
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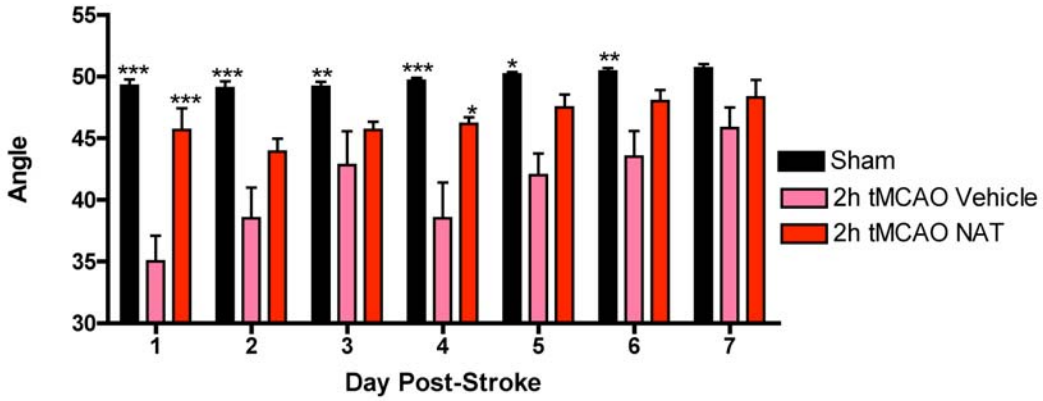
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angleboard scores compared to vehicles on day 2 post-stroke. The angleboard scores of the NAT-treated group were not significantly different from shams on any day post-stroke. Following 1.5 h stroke, the vehicle group showed no hemiparesis, scoring comparable to shams on the angleboard. Similarly, animals treated with NAT showed the same pattern. In contrast, following 2 h stroke, vehicle animals demonstrated profound hemiparesis ($0.001<0<0.05$), as compared to shams on all assessment days. NAT-treated animals showed a reduction in hemiparesis, as reflected by improved angleboard scores over the assessment period, although these animals only scored significantly different from shams on day 2 post-stroke.

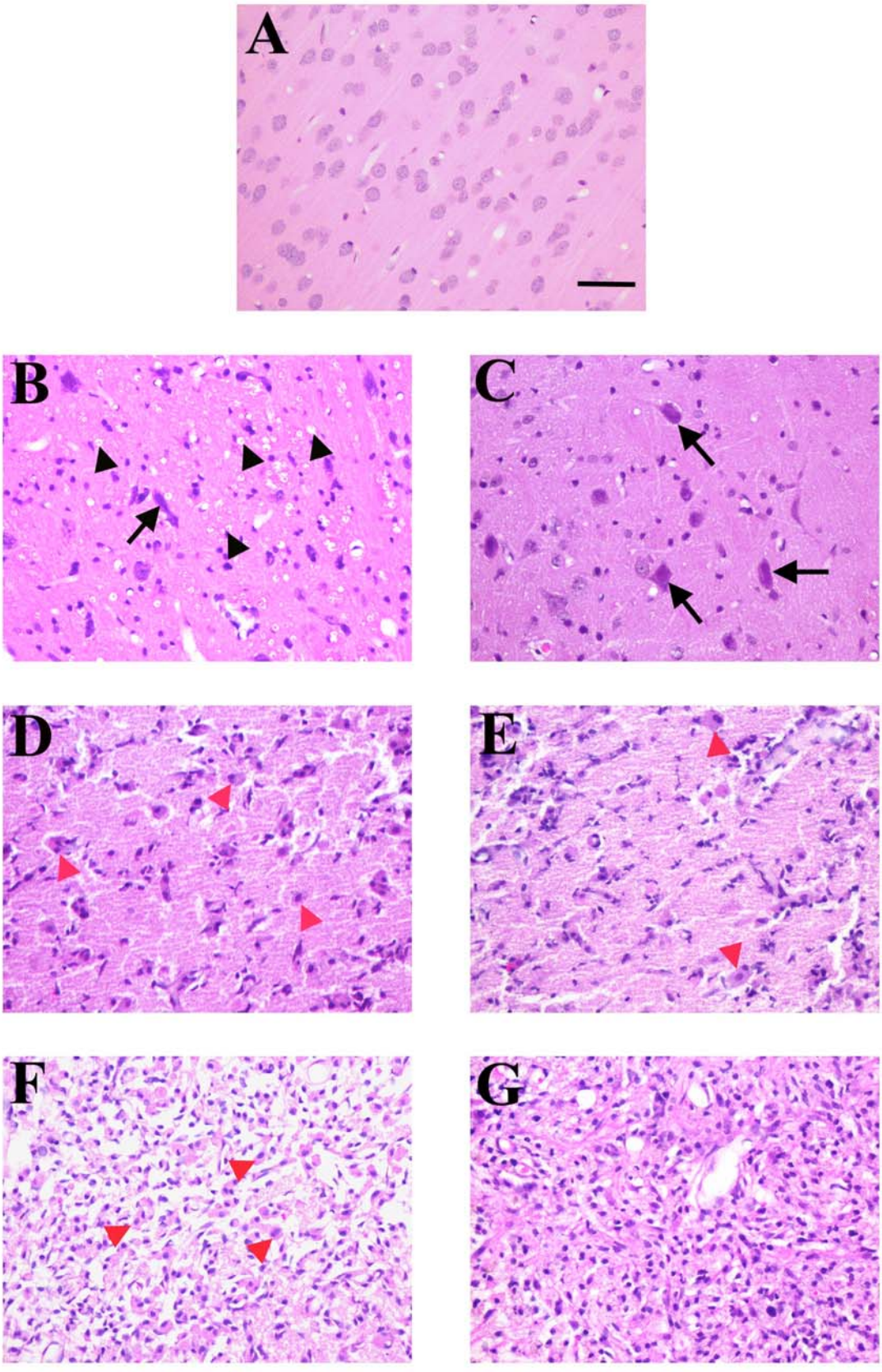
7.3.2 Histological Outcome

General Pathology - H&E

No abnormalities were observed within the cortex of sham animals, confirming that the sham surgery had no effect on neuronal survival. Following mild ischaemia (1 h MCAO) (Figure 7.6), damage to non-neuronal cells, presumably glial cells, and tissue vacuolation was observed within the cortex, in addition to neuronal DCC. Treatment with NAT at 8 h after stroke reduced the amount of cell damage and tissue vacuolation observed, such that only occasional DCC was observed. After moderate ischaemia (1.5 h MCAO) a more marked injury response was apparent with some reactive gliosis observed to occupy the cortex. The infarct was well demarcated from the surrounding tissue and included pancellular necrosis. NAT treatment was unable to afford a marked degree of tissue protection, with the extent of reactive gliosis comparable to the vehicle group. Following severe ischaemia (2 h MCAO), extensive reactive gliosis was observed to occupy the cortex in association with a complete loss of normal cortical architecture.

Figure 7.6 Mild, moderate and severe ischaemia – NAT at 8 h. Cortex at 7 d following stroke. H&E stained sections (Bar = 100 μm).

No abnormalities were observed in sham tissue (A). In the mild ischaemia group (B) following stroke, damage to selective cells was observed, in addition to mild tissue vacuolation (black arrowheads) and DCC (arrows) of neurons. Treatment with NAT (C) produced a reduction in tissue vacuolation neuronal DCC. In the moderate ischaemia (D) group following stroke, reactive gliosis occupied some of the cortex and a loss of normal tissue architecture was observed. Treatment with NAT (E) did not affect cortical tissue. In the severe ischaemia (F) group following stroke, extensive reactive gliosis occupied the cortex with a complete loss of tissue architecture was observed, along with the influx of macrophages (red arrowheads). Treatment with NAT (G) resulted in a modest reduction in the reactive gliosis and some regions of normal parenchyma were observed.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Treatment with NAT was able to somewhat reduce the degree of reactive gliosis, such that regions of normal parenchyma was observed.

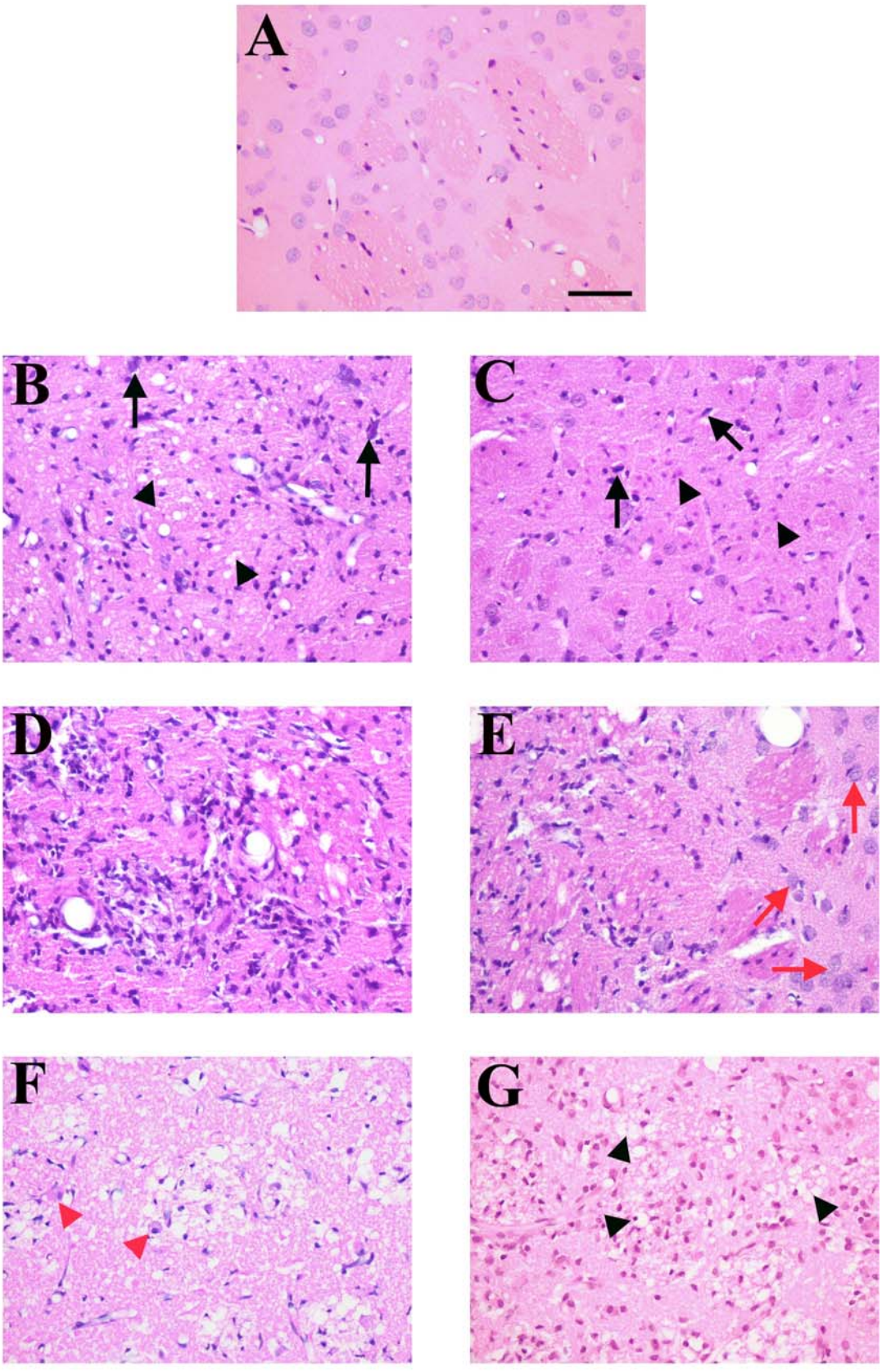
In the white matter no abnormalities in sham animals. Following mild ischaemia (Figure 7.7), some mild vacuolation and cell injury was observed within the white matter. Whilst treatment with NAT at 8 h after stroke was able to reduce the tissue vacuolation, some cell injury was still present. After moderate ischaemia, a more advanced pattern of injury was observed, with tissue vacuolation and reactive gliosis seen to disrupt the white matter tissue architecture. NAT treatment was able to reduce the degree of reactive gliosis and tissue vacuolation within the white matter, however some cell injury was still apparent. Following severe ischaemia, extensive tissue vacuolation, cell loss, reactive gliosis and complete loss of normal tissue architecture were observed. Treatment with NAT was able to reduce the degree of tissue vacuolation, although reactive gliosis was still apparent.

SP response: SP Immunoreactivity

Faint SP immunoreactivity was observed within perivascular tissue of shams. At 7 d following mild ischaemia (Figure 7.8), faint perivascular SP immunoreactivity was also observed, similar to staining levels in sham tissue. This perivascular SP response was unaffected by treatment with NAT at 8 h after stroke, in part because the changes in SP immunoreactivity were so slight that changes were difficult to identify. Following moderate ischaemia, little perivascular SP immunoreactivity was observed and treatment with NAT did not affect this response to ischaemia. In the severe ischaemia group following stroke, no perivascular SP immunoreactivity was observed as reactive gliosis occupied the infarct. Treatment with NAT resulted

Figure 7.7 Mild, moderate and severe ischaemia – NAT at 8 h. White matter at 7 d following stroke. H&E stained sections (Bar = 100 μm).

No abnormalities were observed in sham tissue (A). In the mild ischaemia group (B) following stroke, mild tissue vacuolation (arrowheads) and cell injury (arrows) was observed. Treatment with NAT (C) reduced the amount of vacuolation observed but some cell injury was still apparent. In the moderate ischaemia group (D) following stroke a reactive gliosis was observed within the white matter, accompanied by tissue vacuolation and disruption of tissue architecture. Treatment with NAT (E) reduced the degree of vacuolation and reactive gliosis however some cell injury was still observed. In the severe ischaemia group (F) following stroke, extensive tissue vacuolation, cell loss and a degree of reactive gliosis was observed. Treatment with NAT (G) reduced the degree of tissue vacuolation observed.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in a modest reduction in the degree of tissue injury observed and some perivascular SP immunoreactivity was apparent.

Faint SP immunoreactivity was observed within the cortex of shams, as previously reported (Riberio-da-Silva and Hokfelt, 2000). At 7 d following mild ischaemia (Figure 7.9), an increase in parenchymal but not neuronal, SP immunoreactivity was observed. NAT treatment at 8 h after stroke resulted in a reduction in parenchymal SP immunoreactivity. After moderate ischaemia, an increase in cortical SP immunoreactivity was observed. This increase was not observed in neuronal tissue but other cells within the cortex, most likely macrophages due to their cellular morphology. Regardless, treatment with NAT was able to reduce the amount of cortical SP immunoreactivity. Following severe ischaemia, cortical SP immunoreactivity was not observed due to the extensive reactive gliosis. However, NAT treatment reduced the degree of reactive gliosis and some cortical SP immunoreactivity was observed.

APP response: APP Immunoreactivity

No axonal injury was observed in sham tissue, again confirming that the surgical procedure (without occlusion) had no significant effect. Following mild ischaemia, axonal injury, seen as retraction balls, was observed within the white matter (Figure 7.10). Treatment with NAT at 8 h after stroke reduced the number of retraction balls observed although some axonal injury was still apparent. After moderate ischaemia, axonal injury was observed within the white matter and this was substantially reduced by NAT treatment at 8 h following stroke. At 7 d after severe ischaemia, florid axonal injury and large retraction balls were observed in the white

Figure 7.8 Mild, moderate and severe ischaemia – NAT at 8 h. Perivascular SP response at 7 d following stroke. SP stained sections (Bar = 100 μm).

Faint perivascular SP immunoreactivity was observed in sham tissue (A). In the mild ischaemia group (B) following stroke, perivascular SP immunoreactivity (arrowheads) was comparable to shams. This was unaffected by treatment with NAT (C). In the moderate ischaemia group (D) following stroke, little SP immunoreactivity was observed and this was unaffected by NAT treatment (E). In the severe ischaemia group (F) following stroke, reactive gliosis occupied the infarct and little SP immunoreactivity was observed. Treatment with NAT (G) resulted in a modest preservation of tissue and some perivascular SP immunoreactivity was observed.

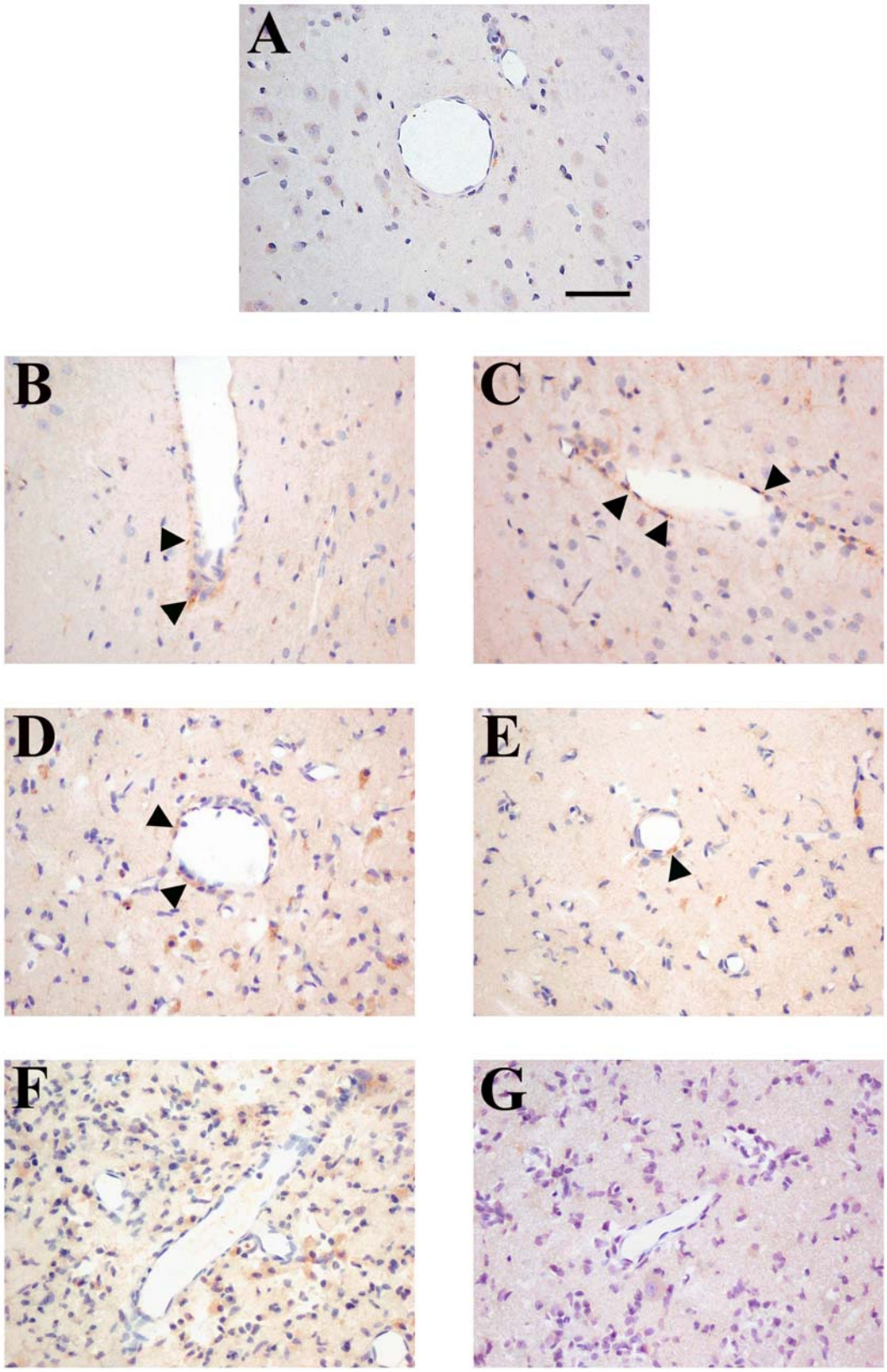
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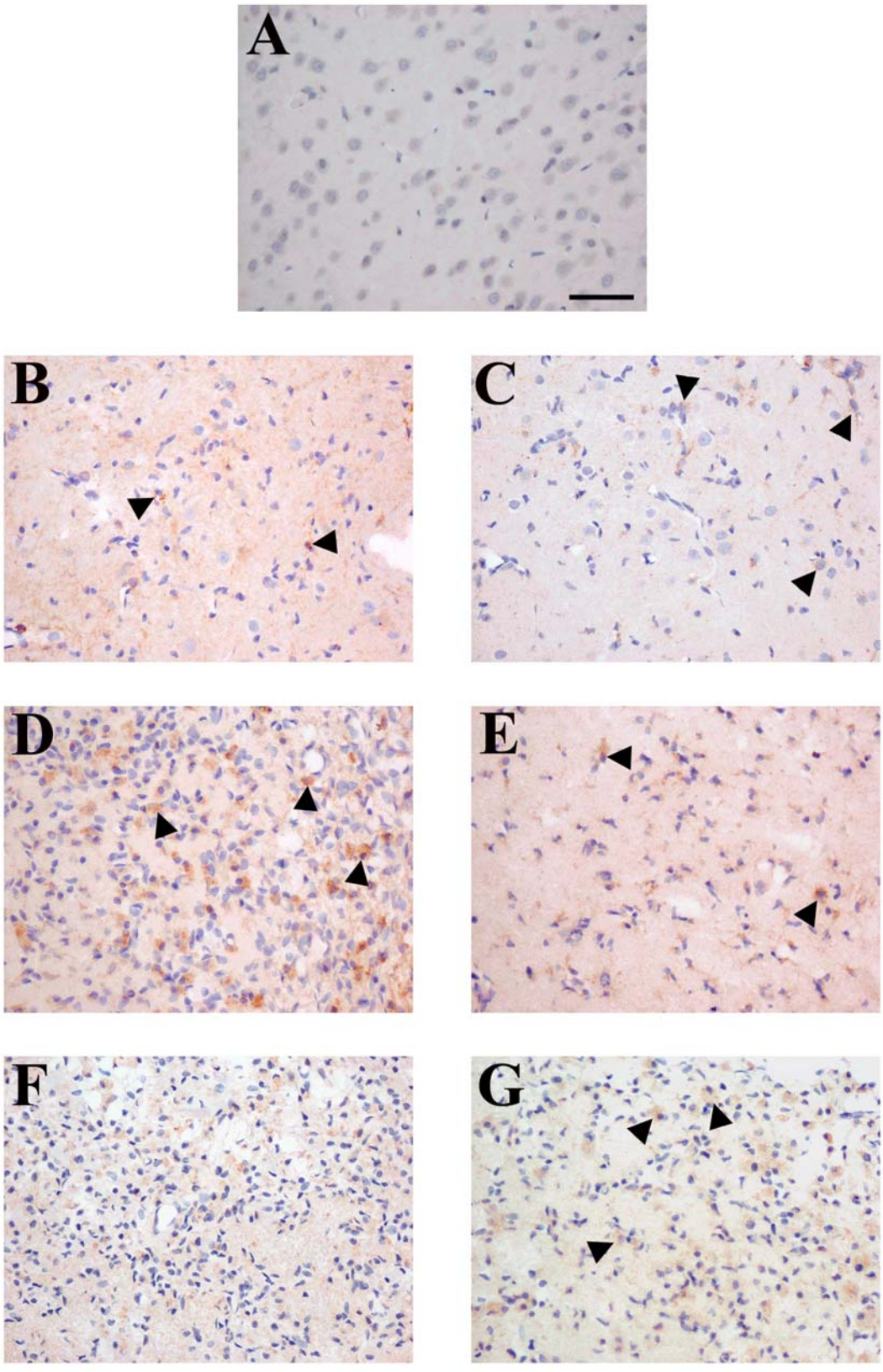
Figure 7.9 Mild, moderate and severe ischaemia – NAT at 8 h. Cortical SP response at 7 d following stroke. H&E stained sections (Bar = 100 μm).

Faint SP immunoreactivity was observed within sham tissue (A). In the mild ischaemia group (B) following stroke, there was in increase in neuronal SP immunoreactivity (arrowheads), this was reduced by treatment with NAT (C). In the moderate ischaemia group (D) following stroke, an increase in cortical SP immunoreactivity was observed and this was decreased with NAT treatment (E). In the severe ischaemia group (F) following stroke, little cortical SP immunoreactivity was observed due to the extensive reactive gliosis. Treatment with NAT reduced the reactive gliosis and some cortical SP immunoreactivity was observed.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matter. Treatment with NAT at 8 h after stroke markedly reduced the degree of axonal injury with fewer retraction balls observed.

Light APP immunoreactivity was observed within the cortex of sham animals (Figure 7.11). At 7 d following mild ischaemia a loss of APP immunoreactivity within cortical neurons was observed and this response was largely unaffected by NAT treatment. Following moderate ischaemia, some neuronal APP immunoreactivity of cortical neurons was observed and a similar pattern was seen in the NAT treatment group. In contrast, following severe ischaemia, APP immunoreactivity within the cortex was markedly increased. This increase was observed within the neurons and the parenchyma. Following treatment with NAT substantial cortical APP immunoreactivity was still observed. In all cases, treatment with NAT had no significant effect on neuronal APP immunoreactivity following stroke.

Degenerating Neurons: FJC

No degenerating neurons were observed in sham tissue, confirming the results with the other stains described above. At 7 d following mild ischaemia, few degenerating neurons were observed scattered within the cortex (Figure 7.12), surrounded by viable neurons. A slight granular appearance of the parenchyma was also observed. Treatment with NAT reduced the number of degenerating neurons observed and the brain parenchyma appeared normal. Following moderate ischaemia, reactive gliosis was observed within the cortex and as a result, few degenerating neurons were apparent. Treatment with NAT did not affect the extent

Figure 7.10 Mild, moderate and severe ischaemia – NAT at 8 h. Axonal injury within the white matter following stroke. APP stained sections (Bar = 100 μm).

No axonal injury was observed within sham tissue (A). In the mild ischaemia group (B) following stroke, axonal injury (arrowheads), seen as retraction balls, was observed within the white matter. Treatment with NAT (C) reduced the number of retraction balls but axonal injury was still apparent. In the moderate ischaemia group (D) following stroke, axonal injury was observed within the white matter, and this was reduced by NAT treatment (E). In the severe ischaemia group (F) following stroke, florid axonal injury was observed. Treatment with NAT markedly reduced the degree of axonal injury observed.

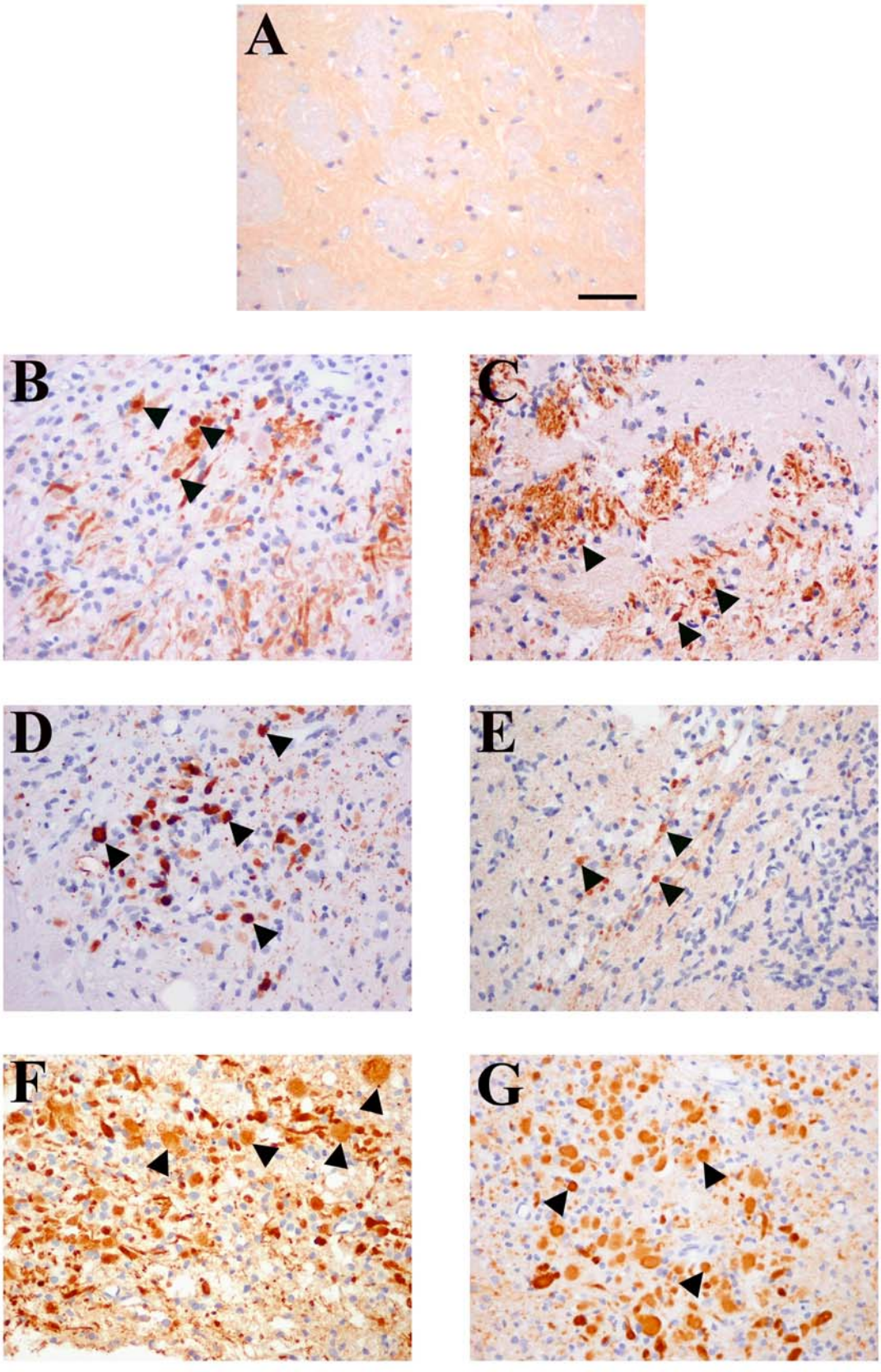
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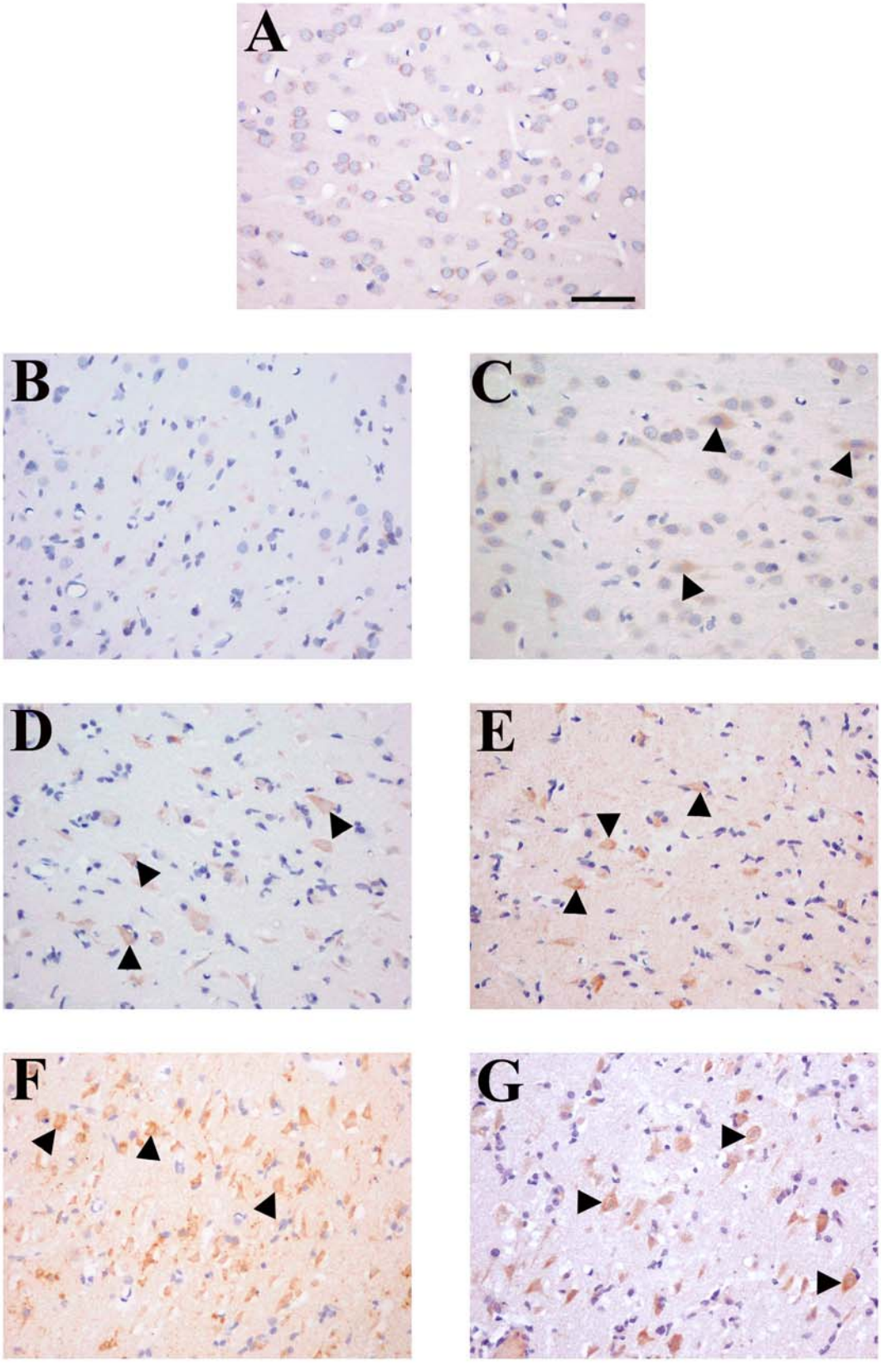
Figure 7.11 Mild, moderate and severe ischaemia – NAT at 8 h. Cortical APP response at 7 d following stroke. APP stained sections (Bar = 100 μm).

Faint APP immunoreactivity was observed within sham tissue (A). In the mild ischaemia group (C) following stroke, little APP immunoreactivity (arrowheads) was observed within cortical neurons and this response was largely unaffected by treatment with NAT (C). Following stroke in the moderate ischaemia group (D), scattered neurons showed APP immunoreactivity and treatment with NAT (E) did not affect this response. In the severe ischaemia group (F) following stroke, increased APP immunoreactivity of cortical neurons was observed and NAT treatment (G) did not affect this response.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of the tissue injury within the cortex. At 7 d following severe ischaemia, extensive reactive gliosis was observed within the cortex and as such, few degenerating neurons were seen. However, NAT treatment produced a modest reduction in the reactive gliosis within the cortex.

Similar results were observed within the white matter (Figure 7.13). At 7 d following mild ischaemia, scattered degenerating cells were observed within the white matter and treatment with NAT at 8 h after stroke resulted in a modest reduction in degenerating cells. Following moderate ischaemia, reactive gliosis was observed within the white matter and few degenerating cells were observed. Treatment with NAT reduced the reactive gliosis and as such, some degenerating cells were observed. Following severe ischaemia, reactive gliosis was extensive and this was reduced by NAT treatment.

Astrocytic response: GFAP Immunoreactivity

Light GFAP immunoreactivity was observed in sham tissue, indicative of resting astrocytes. Following mild ischaemia (Figure 7.14), an increase in GFAP staining within the infarct border zone was observed. A further increase in GFAP immunoreactivity was observed following treatment with NAT. Similarly, at 7 d after moderate ischaemia a marked increase in GFAP staining within the infarct border zone was observed and this was further increased following NAT treatment. Following severe ischaemia, a profound increase in GFAP immunoreactivity was observed within the infarct border zone, characterised by astrocyte hypertrophy and hyperplasia. Once again, NAT treatment at 8 h after stroke further increased the GFAP response to ischaemia.

Figure 7.12 Mild, moderate and severe ischaemia – NAT at 8 h. Degenerating neurons within the cortex at 7 d following stroke. FJC stained sections (Bar = 100 μm).

No degenerating neurons were observed in sham tissue (A). Following mild ischaemia (B), scattered degenerating neurons (red arrowheads) were observed amongst normal; neurons (blue arrowheads). After treatment with NAT (C), a reduced number of degenerating neurons were observed. After moderate ischaemia (D), reactive gliosis was observed within the cortex and few degenerating neurons were seen and this was largely unaffected by NAT treatment (E). Following severe ischaemia (F), extensive reactive gliosis was observed to occupy the cortex and degenerating neurons were not observed. NAT treatment (G) resulted in a modest reduction in reactive gliosis.

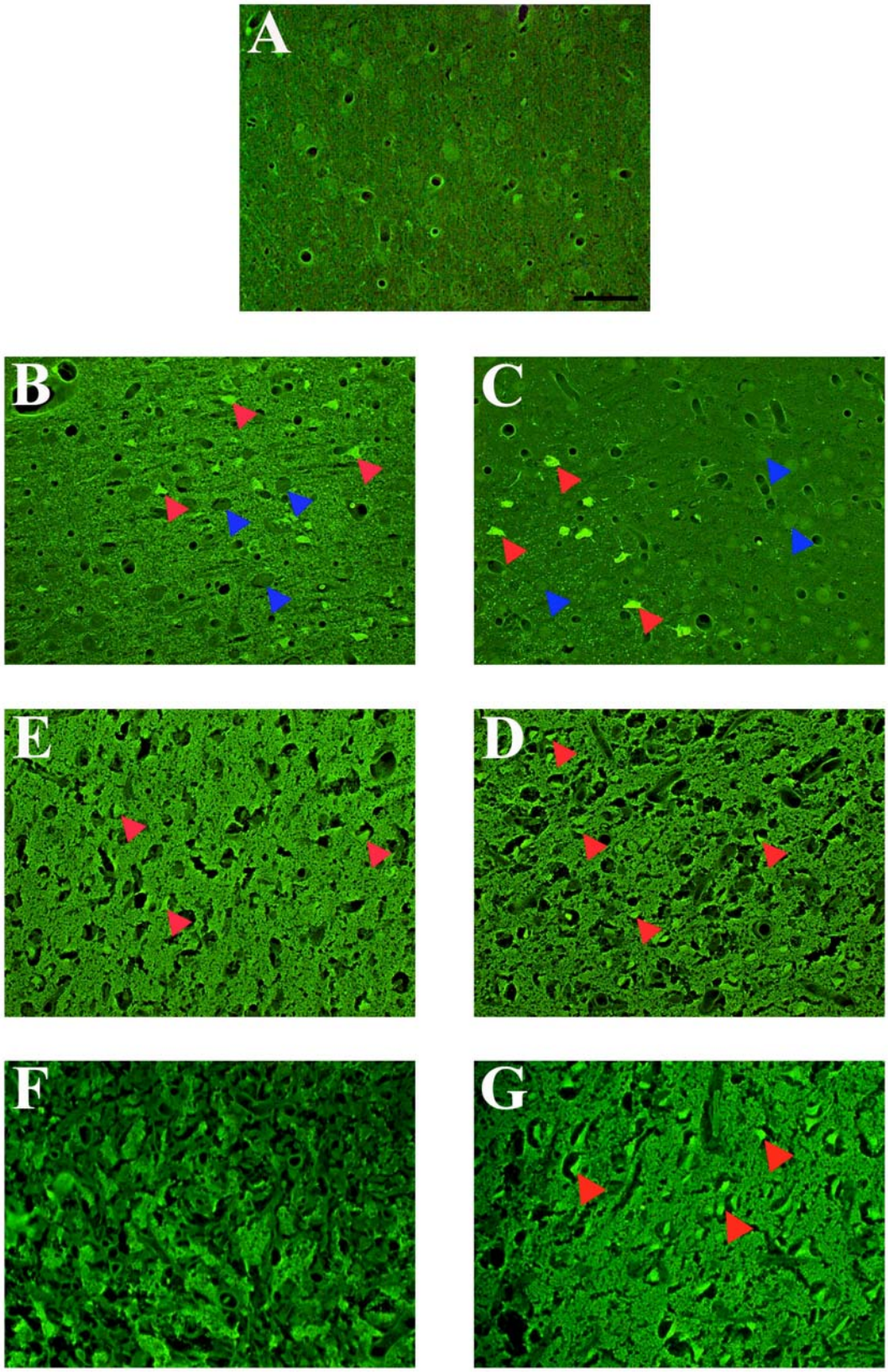
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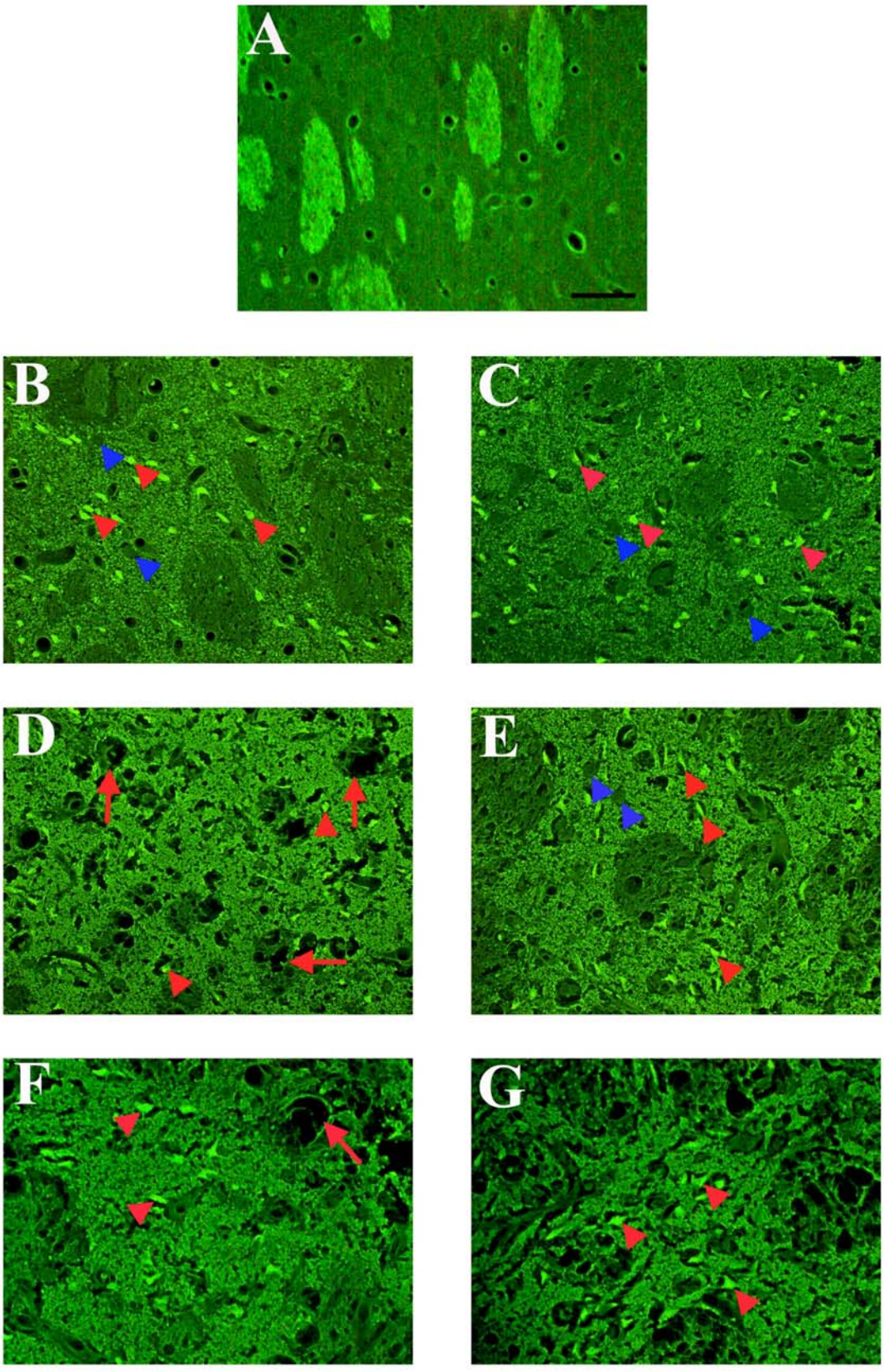
Figure 7.13 Mild, moderate and severe ischaemia – NAT at 8 h. Degenerating neurons within the white matter at 7 d following stroke. FJC stained sections (Bar = 100 μm).

No degenerating neurons were observed in sham tissue (A). Following mild ischaemia (B), scattered degenerating cells (red arrowheads) were observed within the white matter amongst normal neurons (blue arrowheads). The extent of cell injury was reduced by NAT treatment (C). After moderate ischaemia (D), reactive gliosis was observed within the white matter and few degenerating neurons were seen, and this was reduced with NAT treatment (E). Following severe ischaemia (F), extensive reactive gliosis occupied the white matter and this was reduced by NAT treatment (G).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Similar observations were noted with respect to GFAP immunoreactivity around blood vessels following mild, moderate and severe ischaemia. NAT treatment at 8 h after stroke increased the GFAP response to ischaemia with marked GFAP immunoreactivity observed around blood vessels in all treatment groups.

Macrophage/Activated Microglia response: ED-1 Immunohisochemistry

No macrophages/activated microglia were observed in sham tissue, indicating that the surgical procedure did not elicit an inflammatory response. At 7 d following mild ischaemia, a moderate macrophage/activated microglia response was observed (Figure 7.16) within the infarct. NAT treatment at 8 h after mild ischaemia reduced the number of macrophages within the infarct. After moderate ischaemia, a moderate influx of macrophages/activated microglia was observed within the infarct. NAT treatment produced a very modest decrease in the number of ED-1 positive cells infiltrating the infarct. In contrast, following severe ischaemia, a profound macrophage/activated microglia response was observed with ED-1 positive cells occupying the infarct. However, NAT treatment at 8 h after stroke was able to reduce the number of ED-1 positive cells observed within the infarct.

In contrast to the effects of NAT on macrophages/activated microglia within the infarct, treatment with NAT at 8 h after stroke did not affect the perivascular macrophage/activated microglia response to ischaemia. Following ischaemia, regardless of the severity, macrophages/activated microglia were observed in close association with blood vessels (Figure 7.17).

Figure 7.14 Mild, moderate and severe ischaemia – NAT at 8 h. Astrocytic response within the infarct boundary zone at 7 d following stroke. GFAP stained sections (Bar = 100 μm).

Faint GFAP staining was observed within sham tissue (A). In the mild ischaemia group (B) following stroke, an increase in GFAP staining (arrowheads) at the infarct border zone was observed, this response was further exacerbated by NAT treatment (C). Following stroke in the moderate ischaemia group (D), a marked increase in GFAP immunoreactivity within the infarct border zone was observed and this response was largely unaffected by NAT treatment (E). In the severe ischaemia group (F) following stroke, a marked increase in GFAP immunoreactivity within the infarct border zone was observed and this was further exacerbated by NAT treatment (G) at 8 h after stroke.

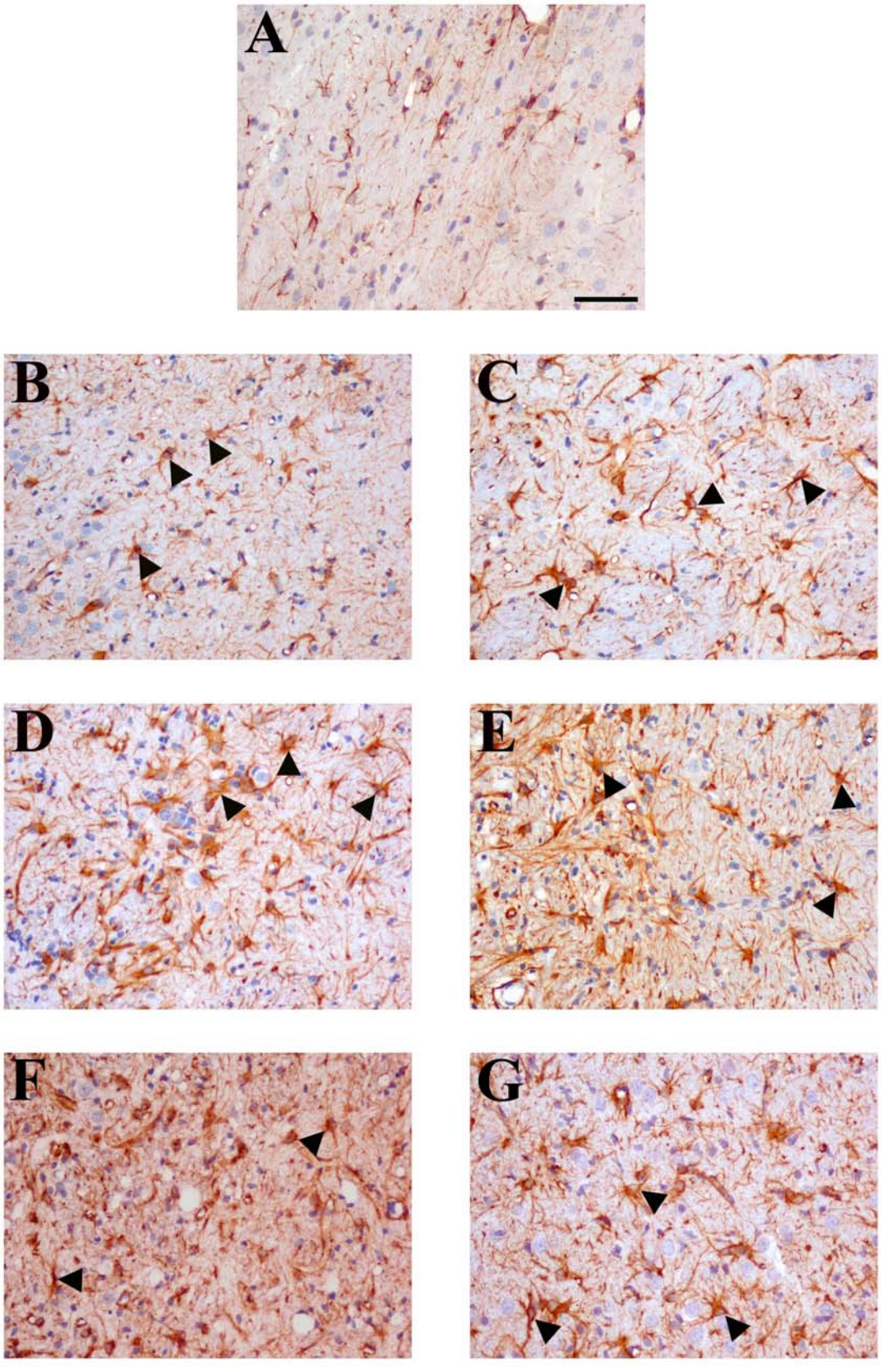
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Figure 7.15 Mild, moderate and severe ischaemia – NAT at 8 h. Perivascular astrocytic response within the infarct boundary zone at 7 d following stroke. GFAP stained sections (Bar = 100 μm).

Light GFAP staining was observed in the perivascular tissue of shams (A). Following stroke in the mild ischaemia group (B), an increase in perivascular GFAP staining (arrowheads) was observed and this was further increased following NAT treatment (C). In the moderate ischaemia group (D) following stroke, an increase in perivascular GFAP staining was also observed and this was exacerbated by treatment with NAT (E). Similarly, in the severe ischaemia group (F) following stroke, increased GFAP staining was observed within perivascular tissue and treatment with NAT (G) at 8 h after stroke further exacerbated this response (arrows).

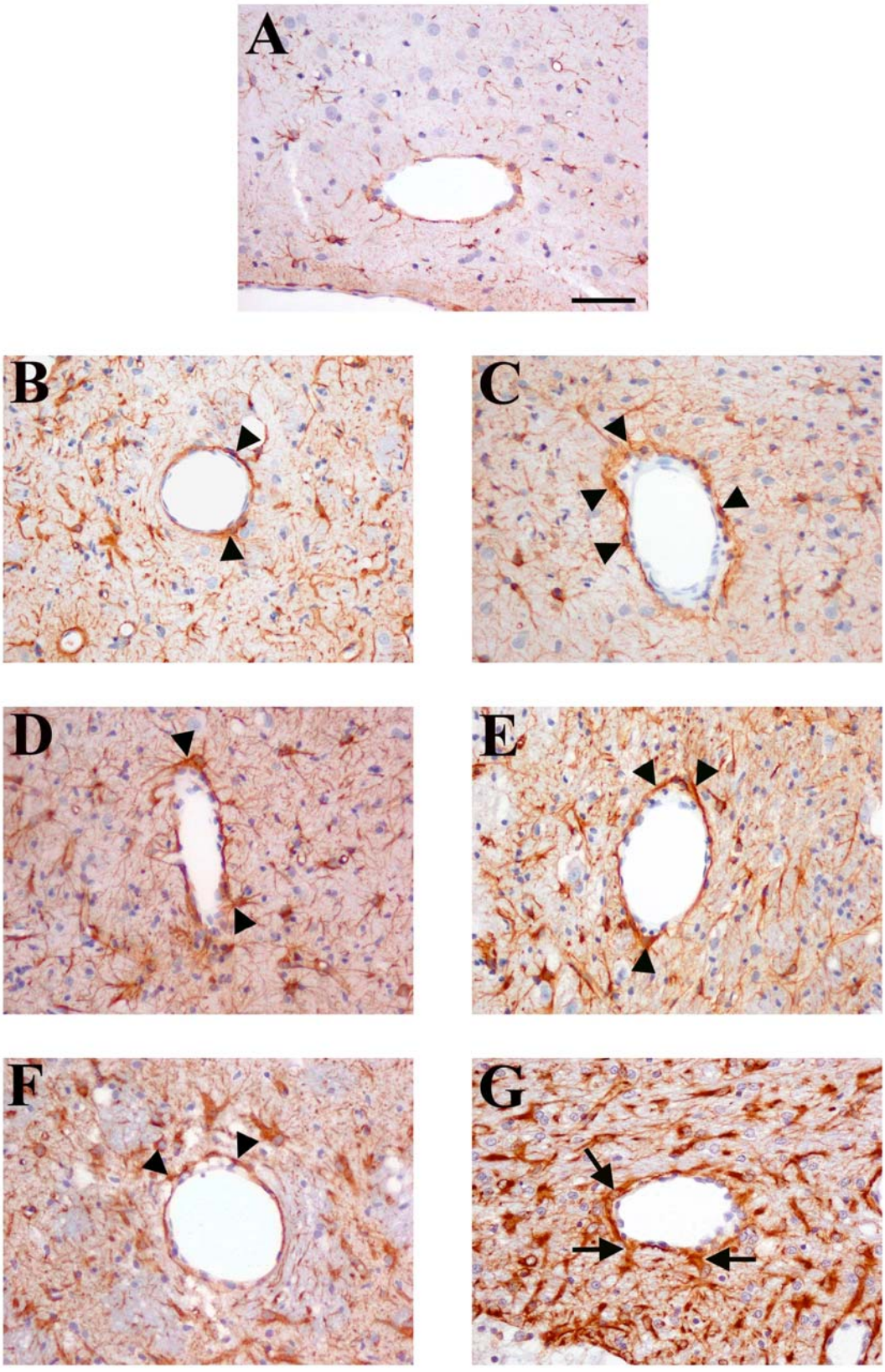
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Figure 7.16 Mild, moderate and severe ischaemia – NAT at 8 h. Macrophage/Activated Microglia response within the infarct at 7 d following stroke. ED-1 stained sections (Bar = 100 μm).

No macrophages were observed within sham tissue (A). Following stroke in the mild ischaemia group (B), an influx of macrophages (arrowheads) was observed. Treatment with NAT (C) resulted in a reduction in the number of macrophages observed within the tissue. In the moderate ischaemia group (D) following stroke, a marked macrophage response was observed, however this was largely unaffected by NAT treatment (E). In the severe ischaemia group (F) following stroke a profound influx of macrophages into the tissue was observed and treatment with NAT (G) produced a reduction in the number of macrophages observed.

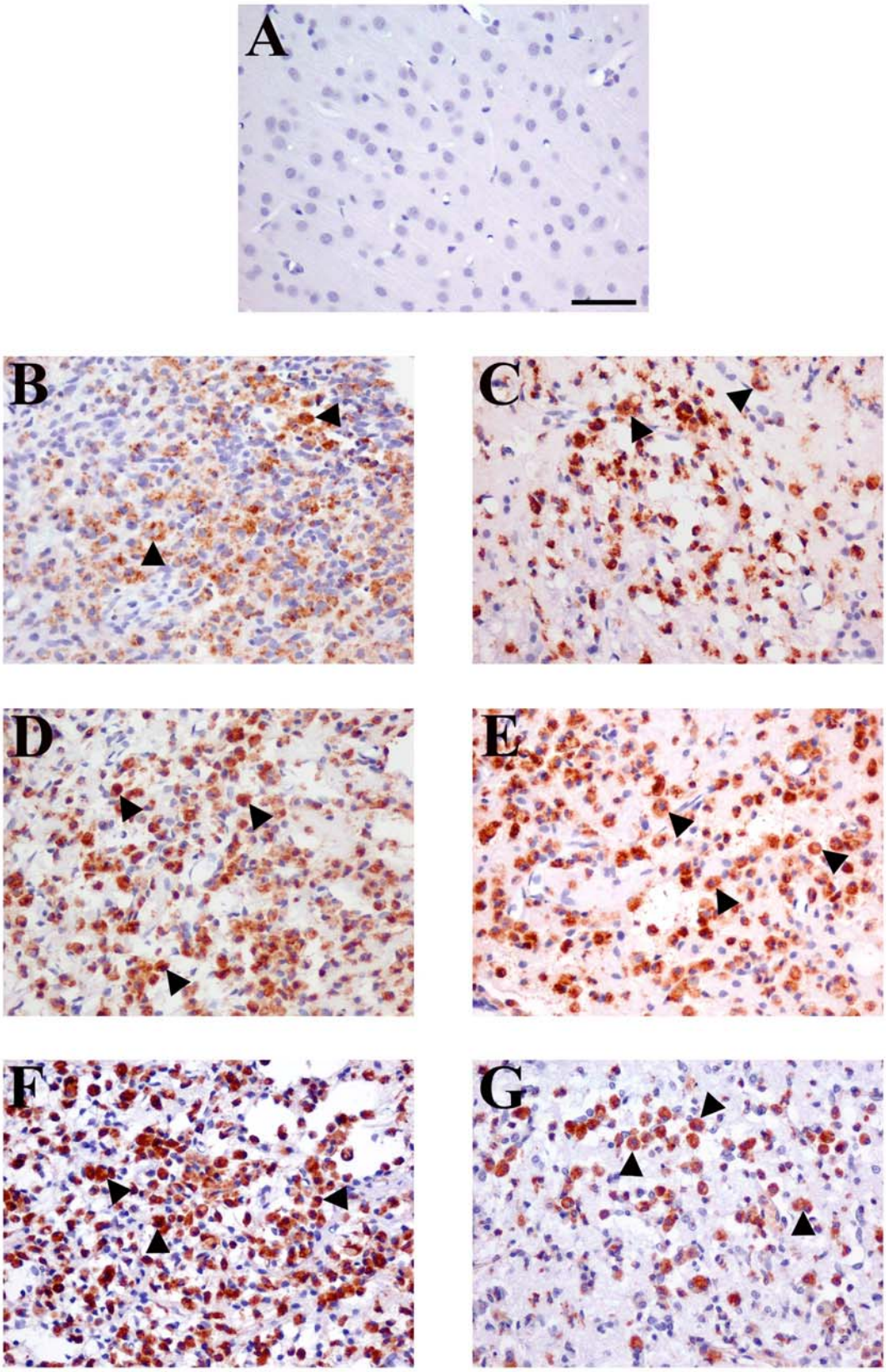
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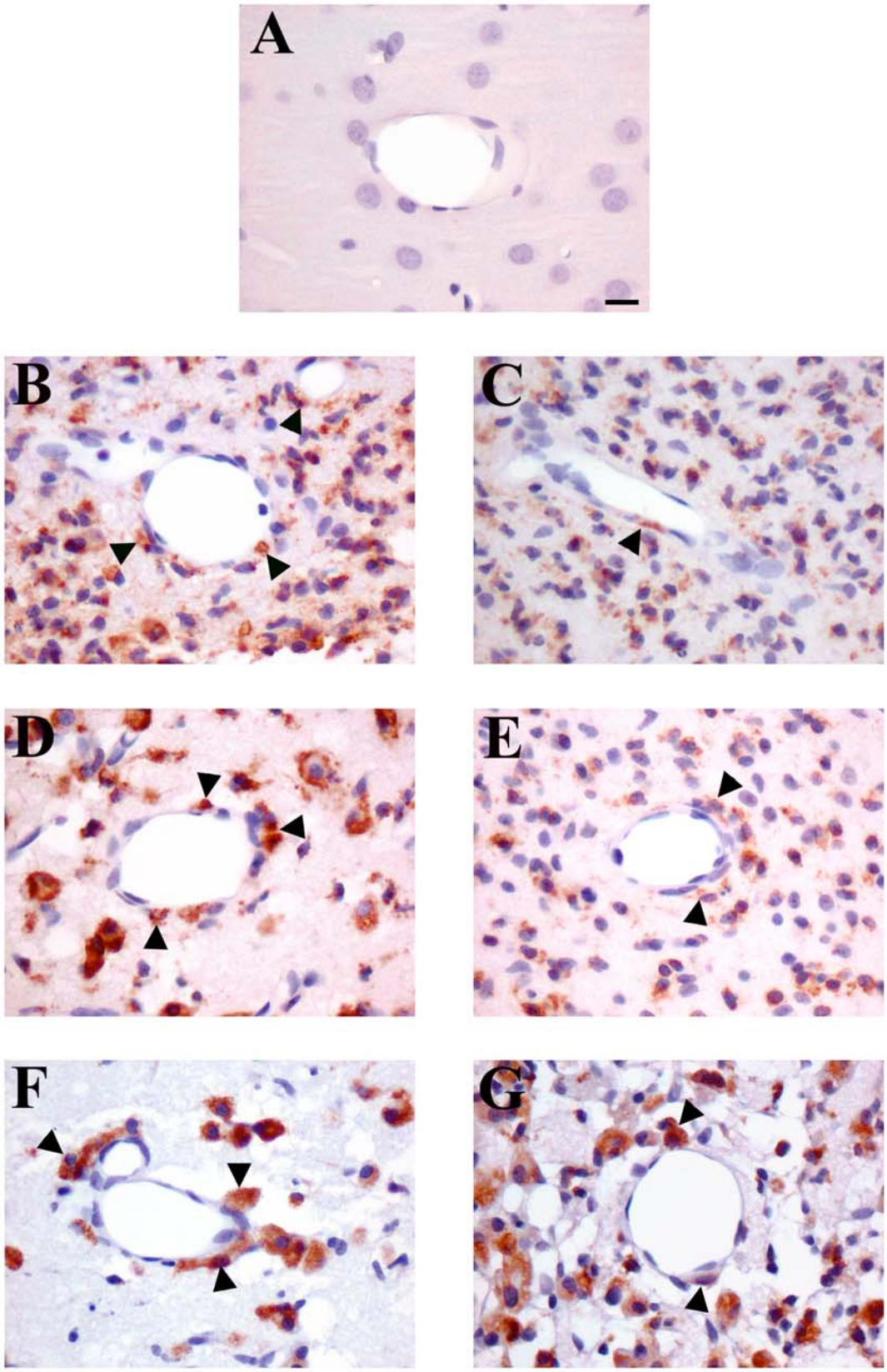
Figure 7.17 Mild, moderate and severe ischaemia – NAT at 8 h. Perivascular Macrophage/Activated Microglia response at 7 d following stroke. ED-1 stained sections (Bar = 100 μm).

No macrophages were observed in association with blood vessels in sham tissue (A). Following stroke in the mild (B), moderate (D) and severe (F) groups macrophages (arrowheads) were observed in close association with blood vessels. This response to ischaemia was largely unaffected by treatment with NAT (C, E, G).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7.4 Discussion

The results of the present study demonstrate that an increasing period of MCAO is associated with increased histological abnormalities and functional deficits. A correlation between the duration of ischaemia and the extent of infarction has previously been reported (Rogers et al., 1997). Moreover, the present study demonstrated that treatment with an NK_1 receptor antagonist administered at 8 h after stroke was able to reduce the extent of the histological abnormalities and functional deficits at all severities of stroke.

It has previously been reported that no significant difference in histological appearance was observed following 30 mins of MCAO as compared to shams (Garcia et al., 1995a). In the present study, only mild abnormalities and selective cell loss were observed following 1 h of ischaemia. As such, it appears that short durations of MCAO produce selective cell injury/loss and only minor functional deficits. In contrast, following moderate or severe ischaemia, extensive infarction was observed characterised by shrunken pyknotic neurons and pancellular necrosis. In these animals, the infarcted region could be clearly delineated from the surrounding tissue. Therefore, 1.5 h of ischaemia appears to be the threshold whereby the grade of injury converts from mild to moderate-severe, as significant functional deficits and histological abnormalities were observed in the 1.5 h and 2 h groups.

Rotarod performance has previously been reported to decrease with increasing period of ischaemia (Rogers et al., 1997). Similarly, in the present study an increased period of occlusion was associated with worsened motor deficits and poor

rotarod performance. The present study therefore represents a characterisation of the functional deficits associated with mild, moderate and severe ischaemia and its' response to intervention with an NK_1 receptor antagonist. We have demonstrated the efficacy of an NK_1 receptor antagonist in different severities of stroke. These represent important findings, as human stroke is a heterogenous condition that encompasses many subtypes and grades. Specifically, we demonstrate that NAT treatment accelerates the recovery from mild stroke and promotes functional recovery following moderate or severe stroke. In all cases, treatment with the NK_1 receptor antagonist produced improvements in functional outcome, with the most profound improvements observed in the severe ischaemia group. These findings suggest that intervention of the SP pathway may be an effective therapeutic intervention for application in the treatment of strokes of varying severity. Such findings are especially encouraging, as NK_1 receptor antagonists are already used clinically as anti-emetics with few side effects. Therefore, the present findings prompt future examination of the possible use of NK_1 receptor antagonists as a therapeutic agent for the treatment of clinical stroke. Future studies should aim to determine whether repeated doses are required to optimise the neuroprotective potential.

Although injury to axons following stroke has been poorly characterised, with the majority of research focused on the neuronal perikarya, early responses of the axon to ischaemia have been reported using APP immunohistochemistry (Valeriari, 2000; Imai, 2001; Yam, 2000; McCracken, 2002). In the present study, axonal injury, as evidenced by the presence of retraction balls on APP staining, was a consistent feature of MCAO, regardless of the duration. Just 1 h of ischaemia was sufficient to

cause axonal injury within the white matter, with the degree of axonal injury increasing with the length of occlusion. As such, the most profound axonal injury was observed within the severe ischaemia group. Indeed, APP has been shown to accumulate within axons in a lesion-dependent manner (Irving et al., 2001), with the volume of ischaemic damage correlating with the amount of APP accumulation (Yam et al., 1998). As such, animals with the greatest APP accumulation were those with the largest volumes of ischaemic damage. This is consistent with the present study where the degree of axonal injury within the subcortical white matter increased in severity with increasing duration of MCAO. Consistent with the findings of chapters 4-6, treatment with NAT was able to profoundly reduce the number of axonal swellings observed within the white matter, irrespective of occlusion duration. Although the mechanism of axonal protection by NAT treatment is unknown, it has been reported that the extent of axonal injury determines the magnitude of motor deficits. Therefore, this may be one mechanism whereby NAT was able to afford protection from the development of functional deficits. Within the cortex at 7 d following stroke, a loss of APP immunoreactivity was generally observed, and this is consistent with previous findings (Irving et al., 2001).

7.5 Conclusions

Administration of NAT at 8 h after stroke is effective in reducing functional deficits associated with moderate and severe cerebral ischaemia. NAT had no effect on outcome measures in the mild ischaemia group and this is likely to reflect the relatively benign injury that was observed. Finally, the degree of damage observed following histological analysis was related to the duration of ischaemia.

Accordingly, given the heterogeneity of clinical stroke, NK_1 antagonists administered up to 8 h following stroke onset may be a novel therapeutic approach to ischaemic stroke management.