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Research Report

A substance P antagonist improves outcome when administered 4 h after onset of ischaemic stroke

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ABSTRACT

Previous studies have suggested that substance P (SP) plays a critical role in the development of brain oedema and functional deficits following traumatic brain injury and that SP receptor antagonism may improve outcome. No studies have described such a role in ischemic stroke. The present study characterized the effects of the NK1 tachykinin receptor antagonist, *n*-acetyl-L-tryptophan (NAT), on blood–brain barrier (BBB) breakdown, oedema formation, infarct volume and functional outcome following reversible ischemic stroke in rats. Ischemia was induced using a reversible thread model of middle cerebral artery occlusion where occlusion was maintained for 2 h before reperfusion. Animals received either NAT or equal volume saline vehicle intravenously at 2 h post-reperfusion. Ischaemic stroke resulted in increased perivascular SP immunoreactivity at 24 h. Administration of NAT significantly reduced oedema formation and BBB permeability at 24 h post-ischemia and significantly improved functional outcome as assessed over 7 days. There was no effect on infarct volume. We conclude that inhibition of SP activity with a NK1 tachykinin receptor antagonist is effective in reducing cerebral oedema, BBB permeability and functional deficits following reversible ischemia and may therefore represent a novel therapeutic approach to the treatment of ischaemic stroke.

1. Introduction

Stroke is the third leading cause of death, second leading cause of dementia and the most common cause of disability worldwide (Bakhai, 2004). Whilst a number of injury factors have been associated with the development of neuronal cell death after stroke, cerebral oedema has been strongly associated with poor outcome post-stroke and is a leading cause of death within the first week. At present, treatments for cerebral oedema remain limited and have not advanced significantly in some 50 years. Such treatments are largely inadequate in that they do not target the cause of the swelling, but simply aim to contain the symptoms and accommodate the swelling process.

Although the factors associated with the development of cerebral oedema remain largely unclear, neurogenic inflammation is well known to contribute to tissue swelling in peripheral tissues (Black, 2002; Harrison and Geppetti, 2001). Neurogenic inflammation, which has the typical characteristics of an inflammatory reaction, such as vasodilation and increased vascular permeability, is a neurally elicited reaction arising from

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the stimulation of C-fibers. The C-fibers release both substance P (SP), which is generally believed to be associated with increased microvascular permeability leading to oedema formation, and calcitonin gene-related peptide (CGRP), which is an extremely potent vasodilator (Hokfelt et al., 2000). Cerebral blood vessels are surrounded by a dense supply of sensory C-fibers that contain both CGRP and SP. It is therefore consistent that these neurons may play a role as mediators of the inflammatory process.

SP mediates its peripheral effects through activation of the NK1 tachykinin receptor, with administration of NK1 tachykinin receptor antagonists shown to ameliorate neurogenic inflammation and tissue swelling (Alves et al., 1999). Given that the functions of central NK1 tachykinin receptors are similar to those in the periphery, neurogenic inflammation may also be involved in the genesis of cerebral oedema. Indeed, recent results from our laboratory have shown that neurogenic inflammation plays an integral role in oedema development following traumatic brain injury (Vink et al., 2003; Nimmo et al., 2004; Donkin et al., 2009). As such, the aim of the present study was to determine the effect of inhibition of neurogenic inflammation with an NK1 tachykinin receptor antagonist on BBB permeability, cerebral oedema, infarct volume, histological damage and functional outcome following ischemic stroke.

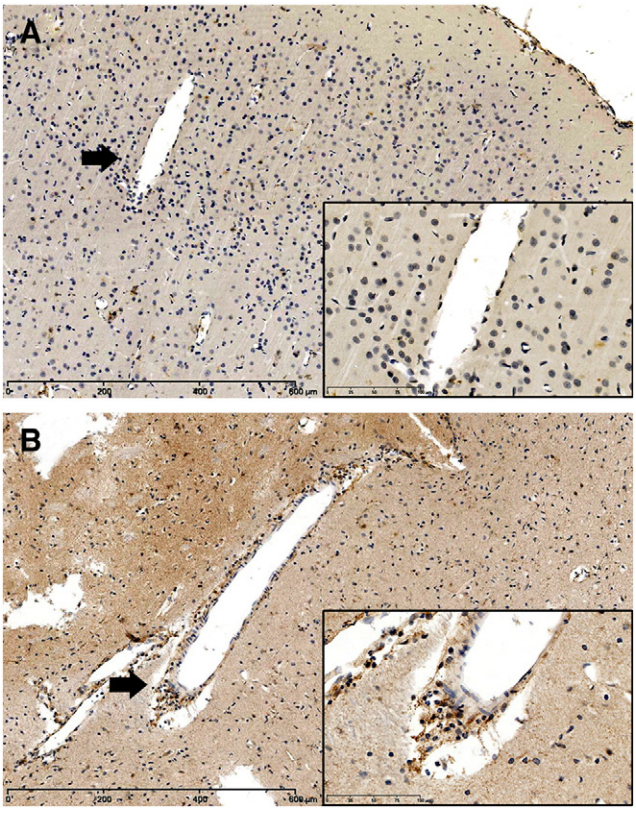

Fig. 1 – Immunohistochemistry for SP 24 h after ischaemic stroke. Faint SP immunoreactivity (brown stain) was observed in sham tissue (A). At 24 h following ischaemic stroke with reperfusion, a profound increase in SP immunoreactivity was observed within penumbral tissue (B). Arrows indicate the region of the magnified insert.

2. Results

2.1. SP immunoreactivity

Profound increases in SP immunoreactivity were observed in brains subject to ischaemic stroke with reperfusion, particularly in the perivascular tissue of the penumbral region (Fig. 1). Semi-quantitation using color deconvolution indicated that the ipsilateral hemisphere had significantly ($p<0.01$) increased DAB staining (23.4±2.3%) compared to the contralateral hemisphere (17.8±1.5%), while shams showed no such difference (20.0±0.1% versus 18.2±1.5%).

2.2. Blood–brain barrier permeability

The permeability of the BBB to EB dye in sham animals was 4.17 ng/mg brain tissue. Following stroke there was a significant ($p<0.001$) increase in the BBB permeability of vehicle animals at 24 h to 5.94 ng/mg brain tissue (Fig. 2A). Administration of NAT significantly ($p<0.001$) reduced the permeability of the BBB to EB to 3.81 ng/mg brain tissue. These results demonstrate that at 24 h following 2 h of MCAO there is significant opening of the BBB and that administration of NAT was able to significantly reduce the extent of barrier opening to levels comparable to shams.

2.3. Brain water content

Hemispheric water content in sham animals was 80.27±0.70% (Fig. 2B). Following stroke, there was a significant ($p<0.001$) increase in water content of the ipsilateral hemisphere of vehicle animals to 83.87±1.79%. Following NAT administration brain water content at 24 h was 81.12±0.80%, levels comparable to shams ($p>0.05$).

2.4. Infarct volume

Following stroke, the infarcted volume occupied approximately 43% of the cortex and 84% of the striatum, equating to 48% of the hemispheric volume (Fig. 3). Treatment with NAT did not significantly change the degree of infarction.

2.5. Functional outcome

Over the 7 day post-ischemia, vehicle treated animals were unable to complete the 2 min rotarod task (Fig. 4A), indicating that they did not reach normal functional levels at any time after stroke. Conversely, NAT-treated animals showed a rapid improvement in their ability to walk on the rotarod device and by day 4 post-stroke had reached normal functional levels. At all time points following ischemia, NAT-treated animals performed significantly ($p<0.001$) better than vehicle-treated animals. Similarly, NAT-treated animals also showed a significantly ($0.001<p<0.05$) improved bilateral asymmetry test latency from day 2 post-stroke (Fig. 4B), consistently

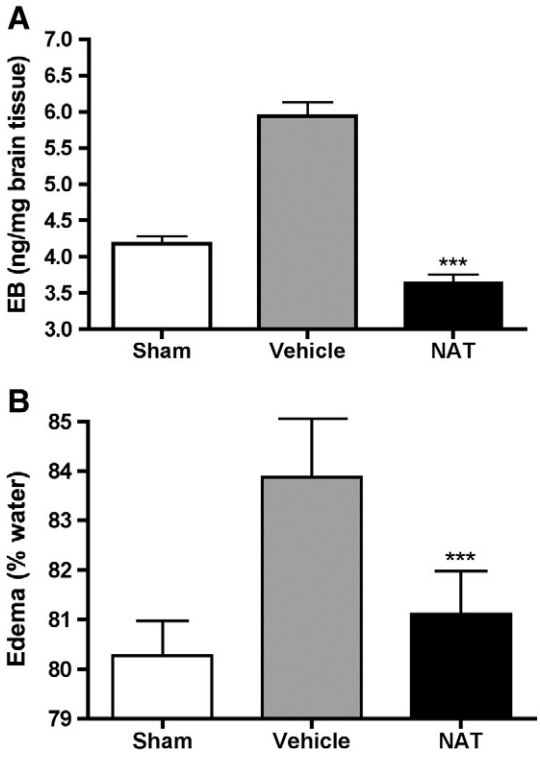

Fig. 2 – Blood–brain barrier permeability and brain water content, as measured at 24 h post-reperfusion. A significant increase in BBB permeability (A) and brain water content (B) was observed at 24 h post-reperfusion. Treatment with NAT restored BBB integrity and normal brain water content. ***$p<0.001$.

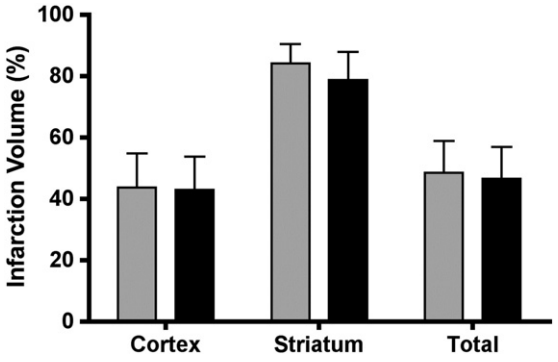

Fig. 3 – Infarct volume measured at 24 h post-reperfusion. No differences in the degree of infarction were observed between the vehicle and NAT treatment groups.

recording a latency lower than vehicle-treated animals. With respect to the neuroscore following ischemia, vehicle-treated animals were ranked as having a moderate injury persisting for the 7 day assessment period. NAT-treated animals showed a mild injury on day 1 and by day 5 had no observable neurological deficits (Fig. 4C). The group differences between the two treatments was highly significant ($p<0.001$ by ANOVA), with individual day differences between NAT and vehicle treatment being significant ($p<0.05$) from day 4 onwards.

3. Discussion

In the present study we have demonstrated that ischemic stroke results in increased SP immunoreactivity, indicative of the presence of neurogenic inflammation. Moreover, inhibition of the SP component of neurogenic inflammation by administration of the NK1 tachykinin receptor antagonist, NAT, at 4 h after induction of stroke, significantly reduced BBB permeability, cerebral oedema and functional deficits, albeit without a significant reduction of infarct volume.

Few research groups have previously investigated the role of SP in cerebral ischemia (Bruno et al., 2003; Stumm et al., 2001; Yu et al., 1997), and none have characterized the role of neurogenic inflammation. Indeed, the study of neuropeptides in acute nervous system injury has been mainly confined to isolated reports in peripheral nerve injury (Malcangio et al., 2000), spinal cord injury (Sharma et al., 1993), brain ischemia (Bruno et al., 2003; Stumm et al., 2001; Yu et al., 1997) and those of our own research group in TBI (Vink et al., 2003; Nimmo et al., 2004, Nimmo and Vink, 2004). In contrast, neuropeptides have been extensively studied in the peripheral nervous system, as well as in asthma, dental pain and osteoarthritis (Hokfelt et al., 2000).

In the present study, increased SP immunoreactivity within penumbral tissue was observed at 24 h following stroke, and was particularly profound in perivascular tissue. Increases in SP levels have previously been reported in other central nervous system (CNS) disorders such as depression (Bondy et al., 2003) and TBI (Donkin et al., 2009). In TBI, the increase in perivascular SP was associated with increased serum levels of SP, an observation also noted in patients with completed stroke or transient ischemic attack (Bruno et al., 2003). Notably, increased SP immunoreactivity has not always been observed following permanent occlusion as opposed to transient occlusion (Fu et al., 2004), suggesting that SP release may be a feature of reperfusion.

The increase in BBB permeability observed at 24 h following stroke supports previous observations of a delayed opening of the BBB after cerebral ischemia (Preston et al., 1993). This opening was observed in the setting of profound cerebral oedema, which suggests that the oedema had a vasogenic contribution. Subsequent administration of the NK_1 tachykinin receptor antagonist profoundly attenuated the BBB permeability and brain water content, suggesting a direct role for SP in both of these events. Studies examining neurogenic inflammation in the skin of NK1 tachykinin receptor knockout mice have shown that they are also unable to produce oedema, even though application of SP produces plasma extravasation and oedema formation in a dose-dependent manner in wild-type mice (Cao et al., 1999). Moreover, we have previously demonstrated in TBI that such neurogenic inflammation results in BBB dysfunction and cerebral oedema formation that can be attenuated by inhibiting the activity of the associated neuropeptides (Nimmo et al., 2004; Donkin et al., 2009). Thus the evidence supports a role for neurogenic inflammation in the development of vasogenic oedema in the CNS (Turner and Vink, 2007).

The findings of the present study have also shown that despite a remarkable recovery of function as assessed by a number of outcome measures, infarct volume did not differ between treatment groups. These findings suggest that infarct

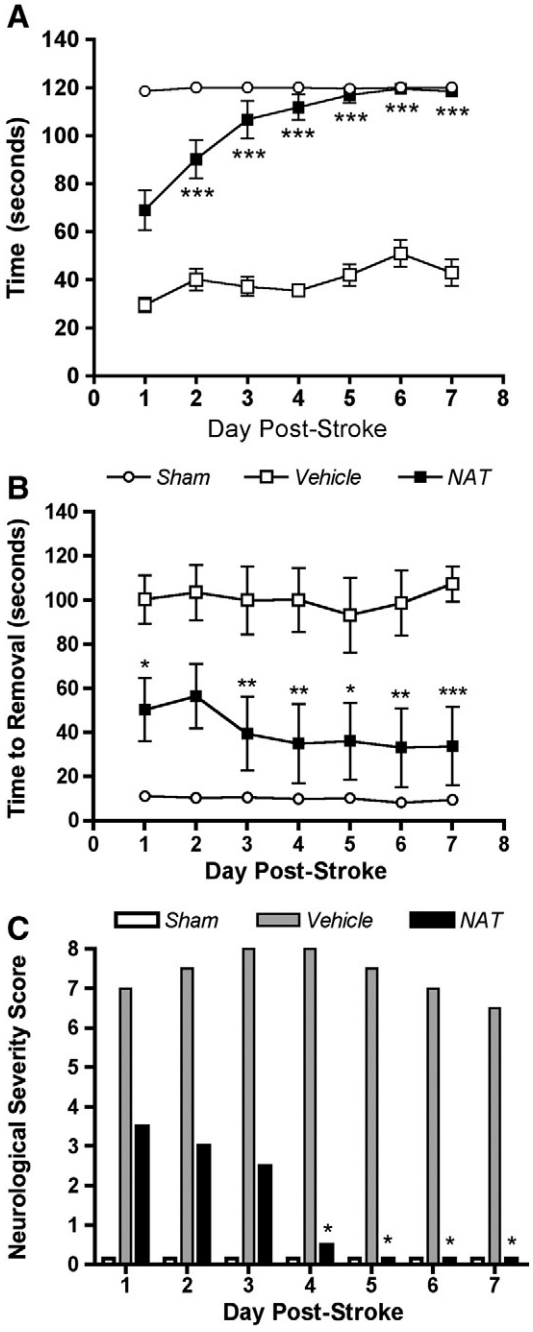

Fig. 4 – Functional outcome as assessed by the rotarod, bilateral asymmetry test and neuroscore for a 7-day period post-stroke. NAT treatment (black squares) significantly improved motor (A), sensory (B) and neurological (C) function, as compared to vehicle treatment (white squares). $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

volume may not be the only predictor of outcome. An increasing number of researchers are reporting improvements in functional outcome without improvements in infarct volume or histological outcome (Grotta et al., 1990; Aronowski et al., 1996; van der Staay et al., 1996; Lecrux et al., 2008). It is thus clear that the relationship between infarct volume and functional outcome is more complex than previously thought. While the exact mechanisms are not clear, improved synaptic plasticity and connectivity may be critical for effective functional improvement. Whether the NK1 tachykinin antagonists improve synaptic connectivity requires further investigation.

NK1 tachykinin receptor antagonists may also confer a neuroprotective effect through a number of alternative mechanisms. For example, receptor binding sites for SP have been shown to increase on glia after neuronal injury (Lin, 1995) suggesting a potential role in the glial response to TBI. Several studies have confirmed that astrocytes become 'reactive' in response to SP following injury and induce mitogenesis and the production of several soluble mediators, such as cytokines, prostaglandins and thromboxane derivatives (Marriott et al., 1991; Palma et al., 1997). SP also induces endothelial cells to produce nitric oxide (NO) (Persson et al., 1991), which has been implicated as an injury factor in stroke, and primes polymorphonuclear cells for oxidative metabolism (superoxide production) (Hafstrom et al., 1998), thus providing a source of reactive oxygen species. NK1 tachykinin receptor antagonists have been shown to reduce pre-necrotic perivascular inflammatory infiltration, as well as circulating histamine, PGE2 and lipid peroxidation products (Kramer et al., 1997). Finally, SP binding to its NK1 tachykinin receptor in the CNS has been shown to directly induce a non-apoptotic form of programmed cell death in hippocampal, striatal and cortical neurons that is independent of caspase activation, but still requiring gene expression (Castro-Obregon et al., 2002).

In conclusion, the present findings suggest that the release of SP may be an early pathological event associated with reperfusion following cerebral ischemia and that administration of an NK1 tachykinin antagonist is highly efficacious in reducing the BBB opening, cerebral oedema and functional deficits that occur following reversible ischemic stroke. Accordingly, antagonism of neurogenic inflammation and more specifically the SP pathway may provide a novel target for interventional pharmacologies aiming to reduce the deleterious effects of ischemia with reperfusion.

4. Experimental procedure

All experimental protocols were approved by the experimental ethics committees of the University of Adelaide and the Institute of Medical and Veterinary Science and conducted according to guidelines established for the use of animals in experimental research as outlined by the Australian National Health and Medical Research Council.

4.1. *Reversible middle cerebral artery occlusion*

Adult male Sprague–Dawley rats ($n=86$; 265–295 g) were fasted overnight before surgery. Anesthesia was induced with isoflurane (3%, 1.5 l/min O_2), animals intubated and then mechanically ventilated (Harvard rodent ventilator) with anesthesia maintained at 1–1.5% isoflurane (1 l/min O_2) throughout. Middle cerebral artery occlusion (MCAO) was performed as described in detail elsewhere (Longa et al., 1989) with normal body temperature being maintained throughout all surgical procedures with the use of a thermostatically controlled heating pad. Briefly, a 4–0 monofilament nylon suture with a tip rounded by heating near a flame and coated with 0.1% poly-L-lysine (Sigma) was

introduced into the lumen of the external carotid artery (ECA) and subsequently advanced into the internal carotid artery (ICA). The suture was advanced 17 mm beyond the ECA/ICA bifurcation to occlude the origin of the middle cerebral artery (MCA). Lignocaine (0.5 ml) was applied to the surgical area and the wound closed with wound clips (9 mm Autoclip, Becton Dickinson). Anesthesia was discontinued, and when animals were able to breathe spontaneously they were extubated and allowed to recover. Reperfusion of the ischemic territory was achieved at 2 h after the onset of ischemia by withdrawal of the suture into the ECA under isoflurane anesthesia. Any animals that did not show circling toward the side of MCA occlusion at 2 h following thread insertion, indicative of successful stroke surgery, were excluded from further study. An additional group of animals ($n=28$) were subject to all surgical procedures used to induce MCAO without advancement of the thread (sham surgery). Physiological variables including arterial blood pH, PCO_2, PO_2, blood pressure, blood glucose and temperature do not change significantly from shams as a result of this procedure.

4.1.1. *Experimental grouping*

Following stroke, animals were randomly assigned to the experimental groups, specifically, functional outcome ($n=6$/group) by rotarod, bilateral asymmetry test and neuroscore, cerebral oedema ($n=7$/group), BBB integrity ($n=5$–7/group), infarct volume ($n=7$/group) and immunohistochemistry for SP ($n=5$/group).

4.1.2. *Drug administration*

At 2 h after the onset of reperfusion, animals either received 25 μmol/kg of the NK1 tachykinin receptor antagonist, *n*-acetyl-L-tryptophan (NAT), or equal volume of saline vehicle, administered intravenously. This dose was chosen on the basis of blood–brain barrier dose/response studies previously published by this laboratory (Donkin et al., 2009).

4.2. *Histological analysis*

At the pre-determined time-point animals were transcardially perfused with heparin, followed by formalin under isoflurane anesthesia. Brains were blocked using a rodent brain matrix (Kopf, USA) and sectioned into consecutive 2 mm coronal slices, embedded in paraffin wax and 5 μm sections cut. Sections were subsequently immunolabelled with SP primary antibody (1:2000 in NHS; polyclonal Santa Cruz Cat. No. SC-9758) for an overnight incubation at room temperature. After washing in PBS, slices where then incubated with an anti-goat IgG-HRP conjugated secondary antibody (1:250 in NHS; Sigma-Aldrich) for a minimum of 30 min at room temperature. Following this, sections were incubated in the tertiary streptavin peroxidase conjugate (SPC) (1:1000 in NHS; Pierce) for at least 1 h at room temperature. Antigen was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB) as a chromogen in a peroxidase reaction (Sigma-Aldrich, Sydney, Australia) and slides counterstained with hematoxylin. Digital images of all sections were acquired using a Nanzoomer Digital Pathology Scanner at a magnification of 40 times (NDP Scan U10074-01, Hamamatsu Photonics K.K., Japan). Slides were viewed with the associated proprietary viewing software and the area of interest exported as a jpeg (virtual dissection). For automated semiquantitation, stains were digitally separated using Rufroik and Johnston's (2001) color deconvolution method. Code to implement this algorithm was obtained from Landini (2007) as an NIH ImageJ macro. The algorithm assumes images are generated by light absorbing dyes. We used the staining vectors included with the macro. Background staining was subtracted using the "rolling ball" method of Castle and Keller (2007). The deconvoluted DAB channel (representing the whole of image antigen content) was semiquantitated (because we do not measure protein) by performing a histogram analysis and summing pixel frequency as a product of pixel intensity and then expressing this as a percentage of the total. This has the effect of weighting the histogram to make more frequent pixels "darker" and so improve the signal to noise ratio.

4.3. *Assessment of blood–brain barrier permeability*

Evan's Blue (EB; Sigma; FW 960.8) extravasation was used to assess BBB integrity as previously described in detail elsewhere (Kaya et al., 2001). Briefly, 2 ml/kg of 4% EB solution was injected intravenously at 23.5 h post-reperfusion. At 24 h post-reperfusion the chest cavity was opened under isoflurane anesthesia and the animal transcardially perfused with saline. Perfusion was discontinued when the perfusate from the right atrium was colorless, this was consistent amongst animals. The brain was removed and the left and right hemispheres dissected. Tissue samples were then weighed and homogenized in phosphate buffered saline and trichloroacetic acid (Sigma). Following centrifugation at $1000\times g$ for 30 min, the absorbance of the supernatant was measured at 610 nm using a UV/Vis spectrophotometer. The level of extravasated EB was determined using a previously obtained EB standard curve.

4.4. *Assessment of brain water content*

The amount of brain water was calculated using the wet weight/dry weight method as previously described (Turner et al., 2006). Briefly, at 24 h post-reperfusion, animals were decapitated under isoflurane anesthesia and their brains were rapidly removed. The left and right hemispheres were dissected and weighed to obtain wet weight (ww). Hemispheres were then dried at 100 °C for 72 h and re-weighed to obtain dry weight (dw). The percentage of brain water was subsequently calculated as a percent of total hemisphere weight as follows:

$$\%\ \text{water} = (\text{ww} - \text{dw}/\text{ww}) \times 100$$

4.5. *Assessment of infarct volume*

At 24 h post-stroke animals were decapitated under anesthesia. Their brains were then rapidly removed and 2,3,5-triphenyltetrazolium chloride (TTC; Sigma) staining, which stains viable mitochondria, was used to determine infarct volume, as described elsewhere (Li et al., 1997). Non-infarcted tissue stains a red/pink in color and infarcted tissue remains a pale cream/white color. Using a brain matrix (Kopf) the brain was cut into 2 mm slices and placed into Tris–saline (Sigma). Brain slices were then incubated in 3% TTC at 37 °C under dark room conditions for 20 min, turning once. Anterior and posterior sides of all brain slices were scanned (Canon). The degree of cortical, striatal and total infarction was then determined by an observer

blinded to the treatment groups and experienced in the evaluation of infarct determination.

4.6. *Assessment of functional outcome*

Animals were assessed daily (in the morning) for a 7 day period, commencing at 24 h post-surgery, by an observer who was blinded to the treatment groups. The motor deficits were assessed using a rotarod device (Hamm et al., 1994), which comprises a metal frame with a rotating assembly of eighteen 1 mm rods. Animals were placed on the device and remained stationary for 10 s. The rotation speed was then increased to a maximum of 30 rpm, with each speed being maintained for 10 s. Animals were required to grip the rods in order to walk on the rotarod. The score recorded was when the animal completed the 2 min trial, fell off completely or gripped the rungs for two revolutions without walking.

The bilateral asymmetry test was used to assess tactile extinction probing sensory neglect following stroke as previously described (Modo et al., 2000). Briefly, two strips of tape (2 cm × 3.5 cm) were applied to the saphaneous part of the forepaws. Time to removal for the left and right forepaws was recorded. Each trial lasted 120 s and animals were given two consecutive trials. The mean of the two trials was taken as the bilateral asymmetry test latency.

A modified neuroscore was used to assess general neurological function (Li et al., 2000). One point was awarded for the inability to perform the task or the lack of a tested reflex. Scores were determined as follows: 10–15, severe injury; 5–9, moderate injury; 1–4, mild injury; 0, no observable injury.

4.7. *Statistical analysis*

All parametric data are expressed as mean and SEM; non-parametric data are expressed as the median. Statistical differences were determined using ANOVA followed by individual Student–Newman–Keuls post-hoc tests (GraphPad Prism Software). The neuroscore data that was analyzed using a two-tailed non-parametric ANOVA followed by a Mann–Whitney *U* test. A p value of 0.05 was considered significant.

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