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COMBINED TISSUE PLASMINOGEN ACTIVATOR AND AN NK1 TACHYKININ RECEPTOR ANTAGONIST: AN EFFECTIVE TREATMENT FOR REPERFUSION INJURY FOLLOWING ACUTE ISCHEMIC STROKE IN RATS

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Abstract—We have recently reported on the efficacy of an NK1 tachykinin receptor antagonist in improving outcome following stroke, including reduced blood–brain barrier (BBB) disruption, reduced cerebral edema and improved functional outcome. The clinically approved stroke treatment, tissue plasminogen activator (tPA), has been associated with an increased risk of hemorrhage and death, if given at later time points. Accordingly, adjunctive therapies have been investigated to reduce the adverse effects of tPA and improve outcome. The aim of the present study was to characterize the effects of a combination of an NK1 tachykinin receptor antagonist with tPA, on BBB permeability and functional outcome following transient ischemic stroke in rats. Stroke was induced in male Sprague–Dawley rats using a reversible thread model of middle cerebral artery occlusion where occlusion was maintained for 2 h, followed by reperfusion. Animals received either 25 mg/kg of N-acetyl-L-tryptophan or 1 mg/kg of tPA, either alone or in combination, or equal volume saline vehicle, intravenously at the time of reperfusion. Functional outcome was assessed by the rotarod, bilateral asymmetry test, modified neuroscore and open field tests. BBB permeability was assessed by Evans Blue extravasation. Combination therapy of an NK1 tachykinin receptor antagonist with tPA significantly reduced BBB permeability, functional deficits and the incidence of intracerebral hemorrhage and death. As such, combined tPA–NK1 tachykinin receptor antagonist treatment may represent a novel therapeutic intervention for the treatment of reperfusion injury in acute ischemic stroke.

Key words: ischemic stroke, NK1 tachykinin receptor antagonist, tissue plasminogen activator, blood–brain barrier, neuropeptides, neurogenic inflammation.

INTRODUCTION

Currently, thrombolysis with tissue plasminogen activator (tPA) within 4.5 h is the only clinically approved therapy for the treatment of ischemic stroke (Zhang et al., 2010). Reperfusion of the ischemic territory is desirable to salvage tissue (Yang and Betz, 1994; Aronowski et al., 1997) but it may also be associated with reperfusion injury and increased tissue damage (Przyklenk and Kloner, 1989). Indeed, a number of clinical studies have documented an increased risk of hemorrhagic complications and death associated with tPA therapy, which has prompted investigation into the safety profile of tPA. In addition, clinical application of tPA therapy is limited with as little as 5% of ischemic stroke patients receiving tPA treatment (Marler and Goldstein, 2003).

In the blood, tPA functions as a fibrinolytic agent, however when it gains access to the brain parenchyma it may mediate events associated with blood–brain barrier (BBB) damage, cerebral edema and cell death (Zhang et al., 2002; Polavarapu et al., 2007). Exogenous tPA may cross both the intact and damaged BBB with potentially neurotoxic effects (Tsirka et al., 1997; Wang et al., 1998; Goto et al., 2007). There is a need for an intervention that may be combined with tPA to reduce neurotoxicity, decrease the risk of hemorrhage and death, reduce reperfusion injury, amplify the neuroprotective effect and potentially increase the therapeutic window. Such adjunctive therapies may provide a means of safely administering tPA, enabling the beneficial thrombolytic effects while disabling the damaging extravascular effects. In this study we sought to examine the effects of tPA beyond fibrinolysis, such as those effects at the BBB.

Our laboratory has recently demonstrated the involvement of the neuropeptide substance P (SP) and neurogenic inflammation in BBB breakdown and resultant vasogenic edema following traumatic brain injury and stroke (Vink et al., 2004; Turner et al., 2006, 2011; Donkin et al., 2007, 2009, 2011; Turner and Vink, 2007; Donkin and Vink, 2011; Turner et al., 2011). We have established that SP release is a feature of ischemic stroke and that it is associated with profound BBB dysfunction, cerebral edema and persistent functional deficits (Turner et al., 2006, 2011; Turner and Vink, 2007). Furthermore, blocking the action of SP with an NK1 tachykinin receptor antagonist significantly reduces this BBB disruption, cerebral edema and functional deficits (Turner et al., 2011). An NK1 tachykinin receptor antagonist may therefore

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Abbreviations: EB, Evans Blue; ECA, external carotid artery; ECASS, European Cooperative Acute Stroke Study; ICA, internal carotid artery; ICH, intracerebral hemorrhage; LDL, low-density lipoprotein; LRP, LDL-related receptor protein; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; *MMP*, matrix metalloproteinase; NAT, N-acetyl-L-tryptophan; SP, substance P; tPA, tissue plasminogen activator; TTC, 2,3,5-triphenyltetrazolium chloride.

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represent a novel adjunctive therapy for combination with tPA, to reduce reperfusion injury and treat ischemic stroke. Accordingly, the aim of the present study was to assess the efficacy of the combination therapy comprising tPA and an NK1 tachykinin receptor antagonist on outcome, particularly BBB permeability and functional outcome, following acute ischemic stroke in rats.

EXPERIMENTAL PROCEDURES

All experimental protocols were approved by the Animal 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.

Adult male Sprague–Dawley rats (n = 107; 365–395 g) were used in the study. Animals were group housed in a conventional rodent room on a 12-h day–night cycle and provided with a standard diet of rodent pellets and water *ad libitum*. After transport, animals were rested for several days before inclusion in any experiment. At the time of the experiment, they were randomly assigned to an experiment and then to naïve, sham surgery, stroke surgery and treatment groups.

Middle cerebral artery occlusion (MCAO)

Animals were fasted overnight before surgery and then anesthetized with isoflurane (1.5–3%; Abbot Australasia), intubated and MCAO was performed, as described in detail elsewhere (Longa et al., 1989), and occlusion maintained for 2 h. 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 Castle Hill, NSW, Australia) was introduced into the lumen of the external carotid artery (ECA), and was subsequently advanced into the internal carotid artery (ICA). The suture was then 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 via withdrawal of the suture into the ECA, under isoflurane anesthesia.

Study design

Animals (n = 107) were randomly assigned to naïve, sham and treatment groups. Following stroke, animals received either equal volume of sterile saline (Baxter Healthcare Regency Park, SA, Australia) vehicle or the drug treatments N-acetyl-L-tryptophan (NAT) (Sigma) (25μmole/kg) (Turner et al., 2011) and tPA (Actilyse, Boehringer Ingelheim) (1 mg/kg), either alone or in combination, administered via the tail vein at the onset of reperfusion. Drugs were prepared and stored as per the manufacturer's instructions and then administered in a blinded fashion. Dose of tPA was determined from previous experimental stroke studies (Wang et al., 1998).

Assessment of blood–brain-barrier permeability

Evans Blue (EB; FW 960.8; Sigma; 2 ml/kg of 4% solution) extravasation was used to assess BBB integrity as previously described in detail elsewhere (Kaya et al., 2001). The assessment of BBB permeability study was divided into two parts: the effects of tPA and NAT, given immediately after one another, on barrier integrity in naïve animals (n = 5–8/group); and the effects of combined tPA + NAT treatment on barrier integrity in stroke animals (n = 3–7/group). In the naïve group, tPA was administered first, followed by NAT, BBB permeability was then assessed 4 h after drug administration. Briefly, 2 ml/kg of 4% EB solution was injected intravenously at 23.5 h post-reperfusion or sham surgery. At 24 h post-reperfusion or sham surgery 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 among animals. The brain was removed and the left and right hemispheres dissected. Tissue samples of the left and right hemispheres were then weighed and homogenized in phosphate-buffered saline (2.5 ml). Undiluted trichloroacetic acid (2.5 ml; Sigma T-0699) was then added to the homogenate and samples vortexed for 2 min before being stored at 4 °C overnight. Following centrifugation at 1000g 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.7

Assessment of functional outcome

Ischemic stroke produces short-term and long-term motor, sensory and neurological dysfunction (Modo et al., 2000; Ding et al., 2002) and accordingly, a battery of tests are required to evaluate post-stroke impairments. Commencing at 24 h post-reperfusion or sham surgery, a subset of animals (n = 6–9/group) were assessed using the rotarod, bilateral asymmetry test and the modified neuroseverity score on days 1–7 post-stroke and on the open field on days 1, 3, 5 and 7. Functional outcome testing was carried out by an observer blinded to the experimental grouping of the animals.

Assessment of motor outcome. 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 revolutions per min, 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.

Assessment of sensory outcome. 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 saphenous 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.

Assessment of neurological outcome. 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. A score of 10–15 indicated severe injury, 5–9: moderate injury, 1–4: mild injury and 0: no observable injury.

Assessment of spontaneous exploratory behavior. The open field test (Giulian and Silverman, 1975) was used to assess spontaneous exploratory behavior, considered to reflect stress and anxiety. The open field comprises a white paneled 1 m × 1 m enclosure with 100 equal 10-cm squares marked on the base. Animals were placed in the center of the enclosure and allowed to explore for 5 min. The number of squares traveled through by the animals was taken as the spontaneous exploratory behavior. Naïve animals explore the entire open field and transverse > 150 squares, whereas stroke animals remain in the perimeter and exhibit large amounts of freezing behavior.

Assessment of infarct volume

At 24 h post-stroke a subset of animals (n = 4–9/group) were decapitated under isoflurane anesthesia (5%). 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 Tujunga, CA, USA) 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 North Ryde, NSW, Australia). 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 (Turner et al., 2011).

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 were analyzed using a two-tailed non-parametric ANOVA followed by a Mann–Whitney U test. A p value of 0.05 was considered significant.

RESULTS

ICH and death

No animals were excluded due to unsuccessful surgery. Although the overall survival rate varied considerably among treatment groups (Table 1), such differences were not statistically significant ($p > 0.05$). However, a significant treatment effect on ICH-related deaths was observed ($p < 0.001$), with a trend toward an increased incidence of ICH in the tPA-treated group compared to all other groups. However, these trends were not statistically significant following post hoc analyses. Specifically, of the 55% of animals in the tPA-treated group that died following stroke, there was evidence of ICH in 77% of these animals.

Table 1. Mortality and incidence of ICH. Survival rates and deaths (shown as percentages) attributable to ICH following stroke in the various treatment groups

Treatment group	Vehicle (n = 12)	tPA (n = 7)	NAT (n = 17)	tPA + NAT (n = 13)
Survival rate at 7 d (%)	50	45	83	61
Deaths from ICH (%)	9	77	5	21

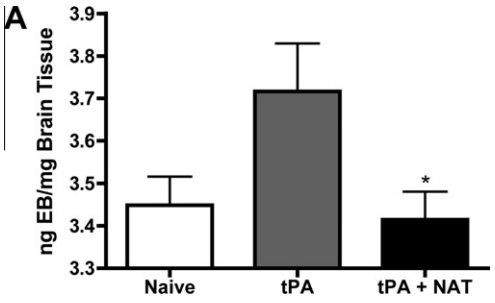

Fig. 1A. BBB permeability following tPA administration in naïve animals. Administration of tPA to naïve animals resulted in EB extravasation into the brain tissue, indicative of BBB opening. Administration of NAT significantly reduced tPA-induced BBB opening ($^{*}p < 0.05$; n = 5–8/gp).

BBB permeability

In naïve animals, administration of tPA resulted in an opening of the BBB (Fig. 1A), which was significantly reduced ($p < 0.05$) by co-administration of the NK1 tachykinin receptor antagonist, NAT. Following stroke, a highly significant increase ($p < 0.001$) in the permeability of the BBB to EB was observed at 24 h post-reperfusion (Fig. 1B). Treatment with tPA alone resulted in a modest reduction in BBB permeability compared to vehicle-treated animals ($p < 0.05$). In contrast, NAT alone ($p < 0.001$) or in combination with tPA ($p < 0.05$),

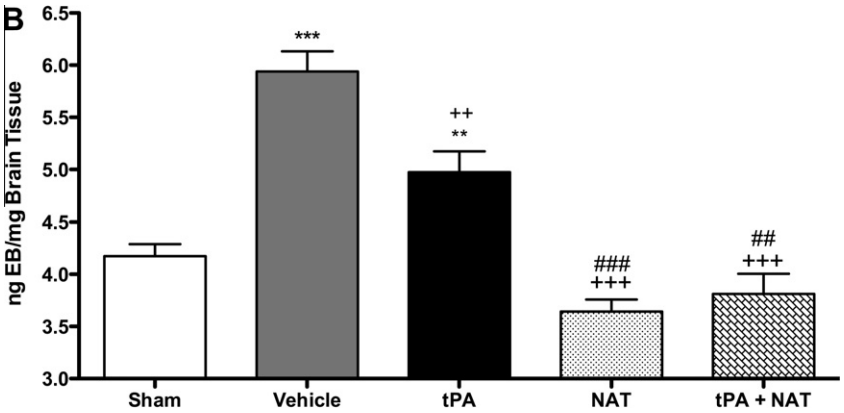

Fig. 1B. BBB permeability at 24 h following MCAO. Significant BBB permeability was observed following stroke. Administration of NAT, tPA or tPA + NAT significantly reduced BBB permeability. However, NAT or tPA + NAT treatment produced the most profound reduction in BBB permeability ($^{**}p < 0.01$ versus sham animals; $^{***}p < 0.001$ versus sham animals; $^{++}p < 0.01$ versus vehicle-treated animals; $^{+++}p < 0.001$ versus vehicle-treated animals; $^{\#\#}p < 0.01$ versus tPA-treated animals; $^{\#\#\#}p < 0.001$ versus tPA-treated animals).

resulted in a highly significant reduction in BBB permeability down to levels comparable to sham animals ($p > 0.05$).

Functional outcome

Motor function – rotarod. Sham animals recorded no rotarod deficit over the 7-d assessment period averaging 120 s at all time points. In contrast, stroke animals treated with saline vehicle demonstrated profound motor deficits (Fig. 2A) that persisted over the entire 7-d assessment period. Administration of tPA resulted in a significant ($p < 0.001$) improvement in motor function as compared to vehicles, however these animals did not reach normal (sham) functional levels by day 7 ($p > 0.05$). In contrast, animals treated with NAT alone ($p < 0.001$) or in combination with tPA ($p < 0.001$), rapidly improved on the rotarod task to reach sham levels by day 4.

Sensory function – bilateral asymmetry test. Sham animals had no observable sensory deficits consistently removing the sticky tape in under 10 s. Vehicle-treated animals after stroke showed profound sensory deficits

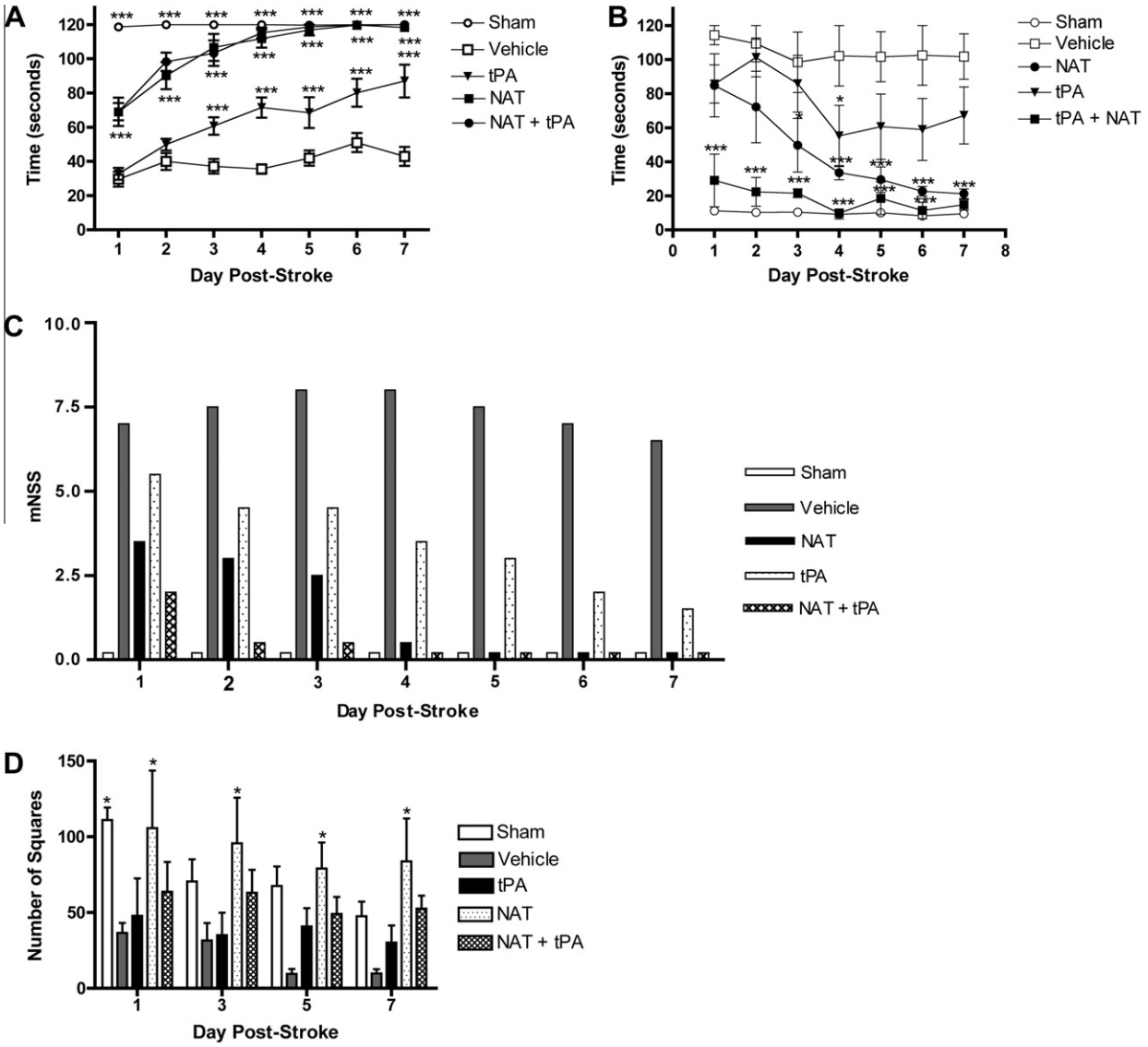

Fig. 2. 7 d Functional outcome performance. (A) Rotarod: Following stroke, vehicle animals showed profound motor deficits that did not recover over the assessment period. Treatment with tPA produced a modest improvement in motor function however, the most profound improvement in motor function was observed in the NAT and tPA + NAT treatment groups ($^{***}p < 0.001$ compared to vehicle-treated animals). (B) Bilateral Asymmetry Test: Following stroke, vehicle-treated animals demonstrated profound and persistent sensory deficits. Treatment with tPA produced a modest reduction in sensory deficits but the most profound reduction was observed in the NAT and tPA + NAT groups ($^{*}p < 0.05$; $^{***}p < 0.001$ compared to vehicle-treated animals). (C) Modified Neurological Severity Score: Following stroke, vehicle-treated animals showed profound neurological deficits that persisted for the 7-d assessment period. All of the drug treatments reduced neurological deficits but the most profound reduction was observed in the NAT and tPA + NAT groups. (D) Open Field: All of the treatments improved spontaneous exploratory behavior to varying degrees following stroke. The most significant improvement was observed in the NAT and tPA + NAT treatment groups ($^{*}p < 0.05$ compared to vehicle-treated animals).

($p < 0.001$), as indicated by a mean latency to remove the tape of greater than 100 s (Fig. 2B). These animals did not recover over the 7-d assessment period. Treatment with NAT (day 3: $p < 0.05$; days 4–7: $p < 0.001$), tPA ($p < 0.05$) or tPA + NAT ($p < 0.001$) produced significant improvements in sensory function as compared to vehicle-treated animals. tPA-treated animals showed a modest recovery ($p < 0.05$) in sensory function over the 7-d assessment period. However, the most marked improvement in sensory function was observed in the NAT ($0.001 < p < 0.05$) and tPA + NAT ($p < 0.001$) treatment groups.

Neurological function – modified neurological severity score. Sham animals showed no neurological deficits over the 7-d assessment period. Following stroke, vehicle-treated animals showed profound neurological deficits as compared to sham animals, that persisted during the 7-d assessment period (Fig. 2C). In comparison, NAT ($p < 0.05$) or tPA + NAT ($p < 0.01$) treatment produced a recovery of neurological function by day 4 post-stroke. Treatment with tPA alone produced a modest reduction in modified neurological severity score but this was not significant ($p > 0.05$).

Spontaneous exploratory behavior – the open field. Sham animals demonstrated a normal level of activity in the open field, although a decline was observed over the 7-d assessment period, most likely reflecting habituation which is well documented in uninjured animals (McIlwain et al., 2001; Paylor et al., 2006). Following stroke, vehicle-treated animals showed a profound decline in spontaneous activity as compared to sham animals (Fig. 2D), which was apparent for the 7-d assessment period. Treatment with NAT significantly ($p < 0.05$) increased open field activity on day 1 compared to vehicle-treated animals with a trend evident for the remainder of the assessment days. Treatment with tPA produced a significantly lower ($p < 0.05$) activity level than shams on day 1 post-stroke before improving over the 7-d assessment period. There was no significant difference ($p > 0.05$) between the spontaneous exploratory behavior in the tPA + NAT-treated group compared to shams and vehicle-treated animals, although a trend toward increased activity was apparent.

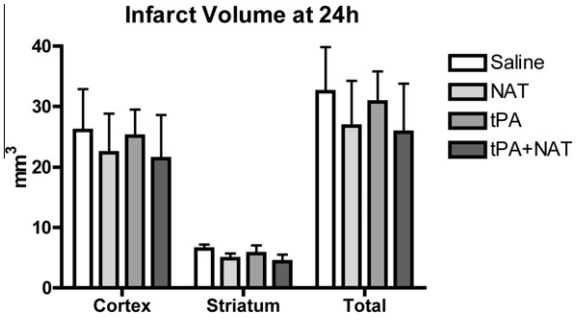

Fig. 3. Infarct volume, as determined by TTC staining. There was no significant difference between the degree of infarction at 24 h in any of the treatment groups with respect to the cortex, striatum and total area of infarction. There was a trend towards a reduction in infarct volume in the tPA + NAT group.

Infarct volume

There was no significant difference between the degree of cortical, striatal or total infarction between the vehicle and any of the treatment groups (Fig. 3), as measured at 24 h post-stroke.

DISCUSSION

The present findings demonstrate that combination therapy comprised of tPA and the NK1 tachykinin receptor antagonist, NAT, is highly effective in ameliorating BBB dysfunction and functional deficits following stroke. Furthermore, NAT administration was able to counteract negative consequences of tPA administration, such as increased BBB permeability and an increased risk of hemorrhage and death.

A role for tPA in BBB dysfunction following ischemia has been widely documented (Pfefferkorn and Rosenberg, 2003; Yepes et al., 2003; Kahles et al., 2005; Kelly et al., 2006; Yang et al., 2007). As early as 1 h post-stroke, blood vessel associated tPA activity can be observed, followed by an increase in vascular permeability at 5 h post-MCA occlusion (Yepes et al., 2003). In the present study, intravenous administration of tPA to naïve animals resulted in opening of the BBB which was completely reversed by treatment with NAT. Indeed, increased BBB permeability has also been observed in uninjured animals following intracrebroventricular administration of tPA (Yepes et al., 2003), with the degree of damage to the BBB correlated with the dose of tPA. In our stroke study, significant BBB permeability was still observed following administration of tPA at the onset of reperfusion, albeit that there was a slight reduction in BBB permeability compared to vehicle-treated animals. Subsequent combination of tPA with NAT reduced permeability to levels comparable to shams. The fact that NAT was able to further reduce the BBB permeability changes induced following tPA administration suggests that SP is involved in increased BBB permeability in reperfusion injury. This is consistent with our previous findings that SP levels were increased within the infarcted hemisphere and were associated with significant BBB breakdown and cerebral edema, both of which were attenuated with the administration of an NK1 tachykinin receptor antagonist (Turner et al., 2011).

A number of mechanisms have been proposed as to how tPA increased BBB permeability. One mechanism whereby tPA may open the BBB is via low-density lipoprotein (LDL)-related receptor protein (LRP) (Makarova et al., 2003; Benchenane et al., 2005; Jin et al., 2010). LRP regulates vascular permeability in the brain under physiological conditions. It acts as a functional receptor for matrix metalloproteinase 9 (MMP-9) (Mantuano et al., 2008) and interactions between LRP and tPA are key regulators in maintaining the integrity of the neurovascular unit (Polavarapu et al., 2007). Indeed, interactions with MMPs has also been proposed as a mechanism whereby tPA induces barrier opening (Horstmann et al., 2003; Pfefferkorn and Rosenberg, 2003; Wang et al., 2004; Kahles et al., 2005; Ning et al., 2006; Yang et al., 2007; Jin et al., 2010). Indeed, an increase in MMP-9 is

observed in human (Montaner et al., 2003; Ning et al., 2006) and experimental (Romanic et al., 1998; Aoki et al., 2002; Sumii and Lo, 2002) stroke and is regarded as a negative prognostic factor (Ning et al., 2006). Such activation of MMPs has a direct effect on the integrity of the extracellular matrix and basal lamina, with integral structural proteins being degraded. Such disruption of the vascular architecture leads to increased BBB permeability, cerebral edema formation and hemorrhagic transformation (Yang et al., 2007; Batra et al., 2010; Tai et al., 2010; Tang et al., 2010; Liu et al., 2012). All of these events further exacerbate the ischemic damage and may lead to extension of the infarct. Thrombolysis in an embolic stroke model was associated with significantly increased MMP-9 expression, accompanied by increased cerebrovascular permeability and vasogenic edema (Lapchak et al., 2000). As such, a link between tPA therapy, MMP-9 expression and hemorrhagic transformation is well documented and may explain how tPA affects barrier integrity following stroke. Therefore, inhibition of MMPs may also potentially improve the safety profile of tPA (Fujimoto et al., 2008).

Although an interaction between SP and MMPs in the brain has not been documented, a role in extracellular matrix metabolism has been reported in other tissues. In the lung, SP upregulates MMP-1 expression (Ramos et al., 2007) and significantly correlates with MMP-12 levels in chronic obstructive pulmonary disease (Xu et al., 2007). SP can also induce the secretion of MMP-2 from human synovial fibroblasts, while increasing overall MMP activity. Insofar as SP may have a similar effect on MMPs within the brain as in peripheral tissues, an involvement of SP in the barrier dysfunction observed following stroke is feasible. A reduction in MMP expression may potentially be a mechanism whereby NAT affords protection at the level of the BBB in the acute phase following stroke. Although beyond the scope of the present study, zymography studies to investigate these potential interactions are warranted.

To date, there are no published reports of an association between SP and tPA within the brain. However, it is tempting to speculate that similar events may occur in the brain as in peripheral tissue. Indeed, neurogenic inflammation has recently been demonstrated to occur in the brain following injury, once thought only to occur in peripheral tissues (Vink et al., 2004; Turner et al., 2006, 2011; Donkin et al., 2007; Turner and Vink, 2007). In lung tissue, infusion of SP induces a dose-dependent increase in tPA release and activity, accompanied by increased blood flow (Newby et al., 1999, 2001). Accordingly, the effect of SP on tPA and the plasminogen system requires further study.

A number of studies have examined the effects of tPA on neurological outcome following experimental stroke (Bowes et al., 1995; Toomey et al., 2002; Zhang et al., 2003). In our study, tPA treatment was generally associated with a modest improvement in outcome, in parallel to that observed in the European Cooperative Acute Stroke Study I (ECASSI) and ECASSII clinical trials (Hacke et al., 1995, 1998). The results may have been influenced by the survival rate of the tPA-treated group in particular, with only 3/7 animals surviving to the end of the 7-d assessment period.

The beneficial effect of tPA above the vehicle group was unexpected and there are several potential explanations. It is possible that the ‘‘worst-case’’ tPA-treated animals died before 24 h and thus the BBB and functional outcome data reflect the ‘‘better-case’’ animals. Alternatively, the results may be explained by the beneficial intravascular effects of tPA. The present study used a thread model of MCAO, where occlusion is achieved through mechanical obstruction of the artery. During the period of occlusion a blood clot forms on the tip of the thread, which may be dislodged during reperfusion. Administration of tPA at this stage would reduce the likelihood of any clots being released during reperfusion, thereby maintaining the patency of the vasculature, with a host of beneficial downstream effects. This may have also had a favorable effect on BBB integrity as reflected by the reduced EB extravasation in the tPA group compared to vehicle animals. In contrast, tPA administration in naïve animals resulted in increased BBB permeability, suggesting that in the absence of clotting tPA was only having negative effects on the vasculature. Although there are no published data on this phenomenon following MCAO by the intraluminal thread model it remains a plausible explanation for the reduction in BBB permeability in the tPA-treated group.

Indeed, a number of studies have reported no improvement in behavioral outcome with tPA treatment, but when it was combined with an adjunctive agent significant improvements in neurological outcome were observed (Bowes et al., 1995; Toomey et al., 2002; Zhang et al., 2003). Taken together, these studies demonstrate that adjunctive treatment with tPA is an effective means of reducing the functional deficits associated with stroke and improving outcome.

We observed an increase in the incidence of ICH in the tPA-treated group, this could be expected in light of reports of increased risk of hemorrhage with tPA therapy (Hacke et al., 1998; Asahi et al., 2000; Sumii and Lo, 2002). In a thromboembolic model of stroke, tPA treatment was associated with a 67% increase in the rate of hemorrhage (Lapchak et al., 2000), which is comparable to the present study. A threefold increase in hemorrhage was independently reported in tPA-treated versus untreated groups (Hacke et al., 1995). However, there are also reports of no effect of tPA treatment on hemorrhage (Toomey et al., 2002). In the present study, such an increase in ICH was associated with an increase in mortality within the tPA-treated group. Adjunctive treatment with an NK1 tachykinin receptor antagonist was able to reduce both the incidence of tPA-associated hemorrhage and mortality. Previous studies have also demonstrated the efficacy of adjunctive therapy in reducing hemorrhage following tPA administration (Lapchak et al., 2000). A close association exists between BBB dysfunction and hemorrhagic transformation (Knight et al., 1998; Latour et al., 2004; Warach and Latour, 2004) and the mechanism whereby tPA + NAT treatment was able to afford protection from ICH and death is most likely due to stabilization of the BBB and the neurovascular unit.

Mortality in the present study was higher than that previously reported in some MCAO studies (Onal et al., 1997; Engelhorn et al., 1998; Schabitz et al., 1999; Maier et al., 2001; Chen et al., 2007). However, experimental mortality can vary considerably with rates ranging from 14% to 58% depending upon a number of factors including survival time, treatment and duration of ischemia, among others (Raghavendra Rao et al., 2001; Boyko et al., 2011; Lee et al., 2012). Most experimental studies assess mortality at more acute time-points, such as 24 h, whereas the current study assessed mortality at 7 days post-stroke. It is well known from the clinical and experimental literature that cerebral edema and its complications frequently lead to death at between 1 and 3 days following stroke (Forsting et al., 1995; Doerfler et al., 1996; Hacke et al., 1996; Heiss et al., 2003). Thus it is more likely that we captured the time-period where cerebral edema was maximal. Indeed, our higher 7-day mortality rate may be a better reflection of clinical malignant MCA stroke where mortality rate is on the order of 60–80% (Hacke et al., 1996).

Despite marked improvements in neurological outcomes we observed no significant effects of NAT or tPA treatment on stroke lesion volume, despite a trend toward a reduction in the tPA + NAT group. Nevertheless, these results suggest that NAT may be having a long term positive effect on outcome and functional recovery post-stroke. Given the profound improvements in functional outcome, an improvement in lesion size was anticipated. Given that NAT is able to cross the disrupted BBB it may have been having a beneficial effect on the brain parenchyma due to the targeting of cells with the NK1 tachykinin receptor, such as astrocytes, thereby assisting stabilization of the neurovascular unit. However, a lack of improvement in lesion volume in light of improved functional outcome has previously been reported in experimental stroke studies (Grotta et al., 1988, 1990; Aronowski et al., 1994, 1996; van der Staay et al., 1996; Turner et al., 2011). Some authors have concluded that there appear to be no neurotoxic effects of tPA in models where reperfusion is guaranteed, such as in the MCAO thread model (Meng et al., 1999). Indeed, infarct volume may not be an accurate predictor of functional capacity. While the present study did not demonstrate a reduction in lesion volume with tPA, previous reports in the literature clearly support that under some conditions tPA has the capacity to affect lesion size and therefore outcome following stroke (Meng et al., 1999; Sood et al., 2008). Similarly, a number of experimental stroke studies have reported neuroprotection following administration of neuroserpin, the endogenous inhibitor of tPA (Yepes et al., 2000; Cinelli et al., 2001; Zhang et al., 2002). Direct injection of neuroserpin into the ischemic area (Yepes et al., 2000) or over-expression of the neuroserpin gene (Cinelli et al., 2001) not only leads to a reduction in tPA activity but also a marked decrease in infarct volume. Such studies confirm the deleterious roles that tPA plays in ischemia and the benefits of timely thrombolysis.

One concern would be that the apparent positive treatment effect is caused by the self-elimination of the ''worst-case'' animals in the tPA-treated group. However, this is unlikely given that elimination of the ''worst-case'' animals in the tPA-treated group would have the opposite effect.

The combination of tPA with NAT is a highly effective therapeutic intervention for the treatment of ischemic stroke. Not only does this intervention allow for the break down of blood clots impeding blood flow, it also reduces BBB permeability and improves functional outcome. The combined treatment approach permits the beneficial actions of tPA while at the same time reducing the negative effects such as increased BBB permeability and incidence of hemorrhage.

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