Assessment of the neuroprotective potential of poly-arginine peptides in stroke models

Diego Milani
The University of Notre Dame Australia

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Assessment of the neuroprotective potential of poly-arginine peptides in stroke models

by

Dr Diego Milani, MD

Thesis presented for the degree of Doctor of Philosophy
The University of Notre Dame Australia
School of Health Sciences
2017
ABSTRACT

Currently, there is no available neuroprotective treatment for reducing acute brain injury following stroke. Recent studies have demonstrated that poly-arginine and arginine-rich peptides (e.g. R18; R = arginine residues) exhibit potent neuroprotective properties in both *in vitro* and *in vivo* stroke models, and therefore have the potential to be developed into a neuroprotective treatment for stroke. Consequently, the aim of this thesis was to further assess the neuroprotective efficacy of several poly-arginine and arginine-rich peptides in permanent and transient middle cerebral artery occlusion (MCAO) stroke models in the rat.

The poly-arginine peptides R12, R15, R18 and the arginine-rich peptide protamine were assessed in a permanent MCAO model when administered intravenously 30 minutes after stroke onset. Treatment with R12, R18 and protamine significantly reduced infarct volume in this model. In a subsequent dose response study (100, 300, 1000nmol/kg) with the lead R18 peptide, when administered intravenously 60 minutes after stroke onset using the permanent MCAO model, infarct volume was reduced by 12 - 24%. Surprisingly, the well characterised neuroprotective peptide TAT-NR2B9c, clinically known as NA-1, which was used as a positive control did not exhibit any significant neuroprotection.

When examining R18 efficacy in a milder transient/reperfusion 90-minute MCAO stroke model, the peptide provided significant neuroprotection. R18 decreased infarct volume by 24 and 35% when administered intravenously 60 minutes post-occlusion at the 300 and 1000nmol/kg doses. In comparison, NA-1 decreased infarct volume by 16 and 26% at the same doses. In addition, R18 had a significantly greater beneficial effect in reducing cerebral oedema, when compared to NA-1 treatment.

Following the positive results obtained with R18 when administered 60 minutes after stroke onset, its therapeutic window was further investigated. The effectiveness of R18 was examined when administered intravenously 2-hours after the onset of permanent MCAO or transient MCAO of 180-minutes duration, or when administered intra-arterially immediately after reperfusion following a 120-minute duration of MCAO. R18 did not significantly reduced infarct volume in these
studies. However, following permanent MCAO R18 significantly reduced cerebral oedema. NA-1 was also ineffective in the transient MCAO studies.

The findings presented in this thesis have further confirmed the neuroprotective properties of several poly-arginine and arginine-rich peptides. Although additional studies are still required to evaluate R18 as a neuroprotective agent in stroke, the peptide represents a promising lead agent with the findings of this thesis laying the foundation for future pre-clinical and clinical studies.
ACKNOWLEDGEMENTS

I would like to thank all the people who have helped me to achieve the challenging task of completion of a PhD.

Firstly, my supervisors, Assoc/Prof Bruno Meloni, Clinical/Prof Neville Knuckey and Dr Ryan Anderton for their guidance, encouragement and assurance during the course of my studies. A special note of thanks to Assoc/Prof Bruno Meloni, my principal supervisor, for his generosity, advice and support during my studies and for matters beyond the laboratory activities.

Thank you to the staff, my colleagues and friends in the Stroke Research Group and WANRI, for their support in helping make the laboratory an enjoyable and stimulating place to be. Particularly, I would like to thank Dr Jane Cross and Mr Vince Clark for their assistance during animal experiments.

To my ever supportive wife Claudia, who always gave me perspective and most of all love.

To my parents, Alessandro and Rita, who ensure I had the educational opportunities which were out of reach for them.
DECLARATION

I hereby declare that:

- This thesis is submitted as part of the requirement for a Doctor of Philosophy degree as a result of my own work and research. All other sources have been indicated and acknowledged.

- Permission has been granted by co-authors for any work that has been co-published to be included in this thesis.

- This thesis has been substantially completed during the course of enrolment and its content have not previously been submitted or accepted for any other degree in this or any other institution.

- I understand that this work may be electronically scanned for the detection of plagiarism.

Signed……………………………………..

Diego Milani

Signed……………………………………..

Supervisor: Bruno Meloni
Approval of final thesis
PUBLICATIONS ARISING FROM THIS THESIS


CONFERENCE ABSTRACTS AND PRESENTATIONS ARISING FROM THIS THESIS


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Australian Postgraduate Award (APA)
The University of Notre Dame Australia;

PhD top-up Scholarship
Western Australian Neuroscience Research Institute (WANRI);

Stroke Society of Australasia New Investigator Award
The 26th Annual Scientific Meeting of the Stroke Society of Australasia;
Melbourne, Australia (2015).

My Research in 3 minutes competition
Winner of 2nd price - Western Australian Neuroscience Research Institute (WANRI);
Perth, Australia (2016).
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Results

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Functional outcome assessment

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Discussion

Conclusion

Methods

Peptides

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<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>ACA</td>
<td>Anterior cerebral artery</td>
</tr>
<tr>
<td>AF</td>
<td>Atrial fibrillation</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ARRIVE</td>
<td>Animal research: reporting of in vivo experiments</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>CCA</td>
<td>Common carotid artery</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon oxide</td>
</tr>
<tr>
<td>CPP</td>
<td>Cell penetrating peptide</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>ECA</td>
<td>External carotid artery</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney 293 cells</td>
</tr>
<tr>
<td>ICA</td>
<td>Internal carotid artery</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>JIP-1</td>
<td>c-Jun N-terminal kinase interacting protein-1</td>
</tr>
<tr>
<td>JNK1</td>
<td>c-Jun N-terminal protein kinase 1</td>
</tr>
<tr>
<td>KA</td>
<td>Kainic acid</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCA</td>
<td>Middle cerebral artery</td>
</tr>
<tr>
<td>MCAO</td>
<td>Middle cerebral artery occlusion</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NR2B</td>
<td>N-methyl-D-aspartate receptor subtype 2B</td>
</tr>
<tr>
<td>O</td>
<td>Oxygen</td>
</tr>
<tr>
<td>PCA</td>
<td>Posterior cerebral artery</td>
</tr>
<tr>
<td>pMCAO</td>
<td>Permanent middle cerebral artery occlusion</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Postsynaptic density protein 95</td>
</tr>
<tr>
<td>Ptm</td>
<td>Protamine</td>
</tr>
<tr>
<td>R</td>
<td>Arginine</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SCU</td>
<td>Stroke care unit</td>
</tr>
<tr>
<td>SD</td>
<td>Sprague Dawley</td>
</tr>
<tr>
<td>STAIR</td>
<td>Stroke therapy academic industry roundtable</td>
</tr>
<tr>
<td>tMCAO</td>
<td>Transient middle cerebral artery occlusion</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td>TAT</td>
<td>Transcriptional activator protein</td>
</tr>
<tr>
<td>TIA</td>
<td>Transient ischemic attack</td>
</tr>
<tr>
<td>TTC</td>
<td>2,3,5-triphenyltetrazolium chloride</td>
</tr>
<tr>
<td>-----</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>W</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>Zn</td>
<td>Zinc</td>
</tr>
</tbody>
</table>
Chapter 1

General introduction
Introduction

Stroke is a devastating condition responsible for increasing morbidity and mortality worldwide, with no clinically available pharmacological neuroprotective treatments. For this reason, there is an urgent need for the development of a safe and widely applicable neuroprotective agent for stroke patients. The development of an effective neuroprotective agent for stroke would have the effect of reducing mortality, improving patient quality of life and lessening the social and economic impact of this devastating condition. Current interventions to minimise ischaemic brain injury are largely limited to pharmacological (tissue plasminogen activator; tPA) and endovascular (thrombectomy) reperfusion therapies, however the availability of a neuroprotective agent that can be administered soon after stroke onset would provide the best opportunity to preserve brain tissue when used alone and/or in combination with reperfusion therapies.

Recent studies in A/Prof Meloni’s laboratory have demonstrated that poly-arginine and arginine-rich peptides have potent neuroprotective properties in *in vitro* injury models that mimic the effects of stroke (Meloni et al., 2014; Meloni et al., 2015ab). In addition, in an *in vivo* study, poly-arginine R9 (R = arginine) has been shown to reduce brain injury (infarct volume) in a permanent middle cerebral artery occlusion (MCAO) model of stroke in the rat (Meloni et al., 2015a). However, in order to determine the full neuroprotective potential of poly-arginine and arginine-rich peptides as neuroprotective treatments for stroke, additional pre-clinical animal studies are required. Consequently, the focus of this project was to undertake additional exploratory studies to assess the neuroprotective efficacy of different poly-arginine and arginine-rich peptides using permanent and transient MCAO models of stroke in the rat.
Stroke

Epidemiology

It is estimated that stroke is responsible for 9% of all deaths worldwide, making it the second most common cause of death after ischaemic heart disease (Donnan et al., 2008). Every year, about 15 million people worldwide suffer a stroke, and of these, nearly six million die, and another five million are left permanently disabled. Stroke is considered to be the second leading cause of disability, after dementia (Leys et al., 2005; Pinkston et al., 2009), where in Australia, affects approximately 60,000 individuals per year (Australian Institute of Health and Welfare, 2009).

In developed countries, while the incidence of stroke is declining, the burden of stroke is paradoxically increasing due to an ageing population and the ongoing epidemics of diabetes, hypertension and obesity (Kim et al. 2015). However, in developing countries, the incidence of stroke is increasing, mainly due to the increasing prevalence of diabetes and obesity. In fact, approximately 80% of all people who have suffered a stroke now live in low and mid-income countries (Truelsen et al., 2007). Hardly surprisingly, approximately 2-4% of global healthcare costs are directed to stroke (Donnan et al., 2008). In addition, as the mortality of stroke decreases, the demand for treatments and social support is destined to rise over the coming years.

Stroke subtypes

Stroke occurs when there is a reduced blood supply to a localised brain region, and for this reason is commonly referred to as focal cerebral ischaemia, especially in the context of research. Stroke can be further subdivided into two subtypes, ischaemic stroke and haemorrhagic stroke. Ischaemic stroke occurs due to a blockage in a brain artery, typically due to thrombosis or embolism, and accounts for about 80% of all cases of stroke. In comparison, haemorrhagic stroke occurs due to a rupture in a brain artery and accounts for 15% of strokes. Haemorrhagic stroke mainly arises due to a rupture of small-vessel aneurysms, with hypertension considered a major contributing factor. A small proportion of haemorrhagic strokes (1-5%) are the result
of a ruptured cerebral aneurysm and bleeding into the subarachnoid space (Donnan et al., 2008).

While not typically classified as a stroke, brain injury due to global cerebral ischaemia occurs when blood flow to all or the majority of the brain is significantly reduced, as occurs following cardiac arrest, severe hypotension and cerebral oedema. Due to its high prevalence and high clinical burden, the experimental studies described in this thesis mainly focus on ischaemic stroke.

Risk factors and aetiology of stroke

Risk factors associated with stroke include demographic factors (age, gender, ethnicity), pathophysiological characteristics (blood pressure, blood cholesterol levels, blood glucose levels, cardiovascular diseases), and behavioural factors (smoking, alcohol consumption, diet, exercise). While some risk factors (Table 1.1) cannot be modified by life style changes and pharmaceutical treatments, others (Table 1.2) are modifiable.

Table 1.1 Non-modifiable risk factors for ischaemic stroke (adapted from Goldstein et al., 2006).

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increasing age</td>
<td>The incidence of stroke increases dramatically after age 65</td>
</tr>
<tr>
<td>Gender</td>
<td>Males show an incidence rate of 1.25 compared to women</td>
</tr>
<tr>
<td>Genetic factors</td>
<td>A positive family history of cerebrovascular disease is considered a risk factor for stroke</td>
</tr>
<tr>
<td>Transient ischaemic attack (TIA)</td>
<td>Patients presenting with a TIA have a risk of stroke estimated at around 4% per year</td>
</tr>
<tr>
<td>Prior stroke</td>
<td>Patients with prior stroke are at higher risk of having a subsequent stroke</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>The probability of stroke in black individuals is nearly twice that of white, while the incidence in hispanics falls between these two groups, irrespective of lifestyle factors</td>
</tr>
</tbody>
</table>
Table 1.2  Modifiable risk factors for ischaemic stroke (adapted from Goldstein et al., 2006).

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carotid atherotromboembolia</td>
<td>Complications of atheroma, such as thrombosis or embolism, are the most common causes of ischaemia and cerebral infarction</td>
</tr>
<tr>
<td>Hypertension</td>
<td>High blood pressure is the most important modifiable risk factor with proven correlation with cardiovascular and cerebrovascular complications</td>
</tr>
<tr>
<td>Atrial fibrillation (AF)</td>
<td>AF causes the blood to pool and clot, increasing the risk of stroke. Other types of heart disease also confer a higher risk of stroke</td>
</tr>
<tr>
<td>Cigarette smoking</td>
<td>The relative risk for stroke is between 1.5 and 3 higher in smokers, the extent of the increased risk depending on the number of cigarettes smoked</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>Individuals with diabetes mellitus are at proven increased risk of developing stroke</td>
</tr>
<tr>
<td>Hyperhomocysteinaemia</td>
<td>Studies have shown a strong association between stroke and high plasma levels of homocysteine</td>
</tr>
<tr>
<td>Alcohol abuse</td>
<td>Alcohol abuse raises blood pressure and increases the risk of stroke</td>
</tr>
<tr>
<td>Dyslipidaemia</td>
<td>Data demonstrate a direct correlation between increased total and LDL cholesterol levels and the incidence of extracranial carotid atherosclerosis</td>
</tr>
<tr>
<td>Obesity and inactivity</td>
<td>Obesity and a lack of physical activity are major risk factors for cardiovascular diseases, including stroke</td>
</tr>
<tr>
<td>Haemostasis and other blood disorders</td>
<td>Studies have demonstrated the role of high levels of fibrinogen and a high red blood cell counts as risk factors for stroke</td>
</tr>
</tbody>
</table>
Clinical aspects

The onset of symptoms following stroke is typically acute, and reflects the region of the brain affected. Initially, symptoms are normally associated with some form of paralysis to one side of the body. Nausea and vomiting are more commonly associated with haemorrhagic stroke, unless the ischaemia affects the vertebrobasilar territory. Mild to moderate headache occurs in about a quarter of ischaemic stroke patients, while severe headaches are common in haemorrhagic stroke patients. Most patients remain conscious during the early stages of stroke. The main clinical features of stroke as defined by the cerebral arterial vessels affected are summarised in Table 1.3.

Table 1.3  Stroke classification per arterial territories involved (adapted from Fisher, 2009).

<table>
<thead>
<tr>
<th>Affect artery</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior cerebral artery (ACA)</td>
<td>Mutism onset, lower limb paresis, hemiparesis, motor aphasia, apraxia, sphincter incontinence, grasping, unilateral sensory deficit, mood disorders, confusional state and hemineglect</td>
</tr>
<tr>
<td>Middle cerebral artery (MCA)</td>
<td>Hemianopia, lower limb paresis, motor aphasia, Broca’s aphasia, Wernicke’s aphraxia, premotor syndrome of Luria, unilateral sensory deficit, confusional state and hemineglect</td>
</tr>
<tr>
<td>Posterior cerebral artery (PCA)</td>
<td>Hemianopia, visual impairment, complex dysfunctions (alexia, colour-blindness, agnosia and impaired visual memory), anopsia, ataxia, Wernicke’s aphasia, apathy, hypoesthesia, pure sensory ictus, confusional state and hemineglect</td>
</tr>
<tr>
<td>Cerebellar artery territories</td>
<td>Nystagmus, vertigo, dysmetria, ataxia, lateropulsion, postural instability and dysarthria, paralysis of the V and VII cranial nerves</td>
</tr>
<tr>
<td>Infarct of the brainstem</td>
<td>Paralysis of the eye movements, hemiparesis, emiatassia, vertigo, paralysis of the III-VI and VII cranial nerves, paralysis of pharynx and larynx, Bernard-Horner syndrome, hemianaesthesia of the face and hemisoma anaesthesia</td>
</tr>
</tbody>
</table>
Pathophysiology of stroke

The human brain comprises 2% of total body weight, but accounts for 20% of total oxygen consumption. As a consequence, the brain is extremely sensitive to the effects of reduced blood perfusion, and requires a constant supply of oxygen in order to generate cellular adenosine triphosphate (ATP), which is used as a cellular fuel to maintain neuronal membrane potential, and preserve calcium, sodium and potassium cellular ionic gradients. To provide the necessary energy required for the maintenance cellular homeostasis, the brain requires about 500 cc of oxygen and 75-100 mg of glucose per minute (Madsen et al., 1993; Jain et al., 2010). When blood flow to a region of the brain is reduced, the survival of the tissue depends on the intensity and duration of ischaemia and the availability of collateral circulation (Frackowiak et al., 1980).

Within a few minutes of the onset of cerebral ischaemia, the central core (brain tissue most severely affected by ischaemia) of the ischaemic region is exposed to the most dramatic reduction in blood flow and consequently undergoes acute brain injury. Surrounding the central core is the penumbra, which consists of a zone of ischaemic tissue less severely affected by the reduction in blood supply. Brain injury within the penumbra occurs over several hours, and represents tissue that is potentially salvageable after stroke onset with treatments such as endovascular and neuroprotective therapies (Ginsberg et al., 1997; Figure 1.1).
The pathophysiology of ischaemic brain injury is complex and involves numerous processes, including energy failure, loss of cell ion homeostasis, excitotoxicity, increased intracellular calcium levels, free radical-mediated toxicity, generation of arachidonic acid products, acidosis, cytokine-mediated cytotoxicity, complement activation, disruption of the blood-brain barrier (BBB), activation of glial cells, leukocyte infiltration and inflammation (Won et al., 2002; Woodruff et al., 2011). These are interrelated and co-ordinated events, which contribute to acute and delayed brain injury.

The initial stages of the ischaemic cascade include oxygen-glucose deprivation and ATP production failure. The lack of ATP synthesis causes the loss of ionic homeostasis resulting in neuronal cell depolarisation, and the release and reduced uptake of the neurotransmitter glutamate. High extracellular concentrations of glutamate trigger excitotoxicity due to the over stimulation of N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainic acid (KA) receptor activation, causing excessive intracellular calcium influx.

**Figure 1.1** Brain showing the central ischaemic core surrounded by penumbra. Since the onset of stroke, the progression of core and penumbra is a progressive process.
(Novelli et al., 1988; Nicholls and Attwell, 1990; Goldberg and Choi, 1993; McCulloch et al., 1993). When the lack of ATP production is sustained, acute cell death and brain injury occurs, while if the ATP synthesis is only transiently or mildly inhibited, delayed brain injury results.

In acute cell death, the raised levels of intracellular calcium ions lead to secondary effects such as the activation of calcium sensitive catabolic enzymes (phospholipases, endonucleases and calpains), a decrease in protein synthesis (Kleihues et al., 1975; Burda et al., 1994; DeGracia et al., 1996), alteration in mitochondrial function (Rehncrona et al., 1979; Almeida et al., 1995) and organelle and cell membrane breakdown. Consequently cell begins to swell and lyse leading to death.

In delayed cell death, the raised levels of intracellular calcium cause milder effects associated with lipid peroxidation (Bromont et al., 1989; Haba et al., 1991), oxidative stress, free radical production (Kader et al., 1993; Chan, 2001), mitochondrial dysfunction and altered cell signalling. In addition, multiple cell death promoting signalling pathways are activated (e.g. MAPK, C-Jun, TNF, MMPs, NFKB, caspases, ERK1/2) (Bouwmeester et al., 2004). Moreover, there are post-translational and translocational protein changes (Rosemberg et al., 1996; Gillardon et al., 1997). The combination of all of these events ultimately leads to cell death through mechanisms associated with apoptosis, necroptosis, autophagy and necrosis (Lipton, 1999; Brott and Bogousslavsky, 2000; Degterev et al., 2005).

In addition, cerebral ischaemia and cellular injury can stimulate an inflammatory response and blood brain barrier disruption (Dirnagl et al., 1999), which can further amplify the initial ischaemic injury pathways (Lee et al., 2000) (Figure 1.2).
Figure 1.2  Summary of the major events associated with the ischaemic cascade and neuronal cell death. Excitotoxicity and calcium overload cause the induction of several ionic, biochemical and cellular cascades.

Ischaemic brain injury and neuroprotection – Important concepts

With respect to the ischaemic stroke injury cascade, several important concepts are relevant when considering the development of an effective neuroprotective agent: 1) most, if not all, of the pathological events activated during the cascade are potential targets for intervention to limit brain injury; 2) the earlier the ischaemic cascade is inhibited, the more likely it is that injury will be reduced; 3) targeting multiple aspects of the ischaemic cascade will provide the best opportunity to limit damage; and 4) at some point, the extent of ischaemic brain tissue becomes so severe that damage is irreversible, even with the successful reperfusion of the ischaemic tissue.
In addition to the considerations outlined above, the ideal neuroprotective agent should be safe to administer, and preferably suitable for application in the field, as time is critical in order to achieve maximum neuroprotection. Along with the ability to reduce brain damage without reperfusion, the neuroprotective agent should also be capable of prolonging the time window for salvageable penumbral tissue, and thereby allowing additional time for the application of reperfusion therapies (i.e. tPA and/or thrombectomy).

**Current acute treatments for stroke**

While stroke continues to have a devastating impact, several acute interventions have been shown to improve patient outcomes (Donnan et al., 2008). These include rapid admission to a specialised stroke unit, tPA thrombolysis, thrombectomy, hemicranectomy and low dose aspirin therapy. The benefits and limitations of these various acute interventions are discussed below.

*Admission to a specialised stroke unit*

Admission to a specialised multi-disciplinary stroke care unit (SCU) provides the best opportunity for patients to receive optimal post-stroke care. Within a SCU, patients receive blood pressure control, early mobilisation and effective venous thromboembolism prevention, treatments proven to reduce mortality and sensorimotor deficits by 20% (Langhorne et al., 1993). Admission to a SCU also reduces mortality and improves outcomes by reducing the incidence of infection after stroke. For example, the proper management of dysphagia and risk of aspiration with the use of naso-gastric tubes, coupled with the utilisation of urinary catheters can reduce the risk of lung and urinary tract infections, respectively.

* tPA thrombolysis and thrombectomy

Currently, the most effective available treatment for ischaemic stroke is reperfusion therapy using tPA, alone or in combination with thrombectomy (Berkhemer et al., 2015; Campbell et al., 2015; Jovin et al, 2015). Intravenous tPA therapy aims to dissolve clots formed in cerebral vessels in order to restore the blood flow to the
affected brain region. It is estimated that successful or partial reperfusion occurs in about 20-50% of patients, and that haemorrhagic transformation can occur in up to 7% of patients. In addition, even though tPA can reduce neurological disability, the treatment does not appear to improve the mortality rate associated with stroke (Hacke et al., 2004).

Thrombectomy is an endovascular procedure performed under angiographic guidance and utilises a clot-retrieving catheter in order to extract a blood clot from an occluded vessel to allow restoration of blood flow. In 2015, five clinical trials (MR CREAN, ESCAPE, EXTEND-IA, SWIFT PRIME and REVASCAT) confirmed the efficacy of endovascular thrombectomy for improving stroke outcomes when compared with patients receiving tPA therapy only (Goyal et al., 2016). Data from the thrombectomy trials have demonstrated the benefit of the procedure for internal carotid and proximal middle cerebral artery occlusion, while uncertainties remain regarding its effectiveness for more distal occlusions (Campbell et al., 2016). Importantly, following thrombectomy, the rate of intracranial haemorrhage is no higher than with tPA therapy alone (Goyal et al., 2016).

Despite the effectiveness of reperfusion therapies, a number of limitations and disadvantages have been documented. Both tPA and thrombectomy must be performed as soon as possible post-stroke, because outcomes worsen the longer the period of ischaemia, such that the therapeutic window for treatment is recognised to be 3–4.5 hours post-stroke. It has been estimated that for every 30 minutes delay from the time of stroke onset to reperfusion, the ability to achieve full sensorimotor recovery decreases by 10% (Khatri et al., 2014). The narrow therapeutic window for reperfusion therapy is further compounded by the time delays in getting patients to medical facilities and the need for a brain scan (e.g. CT or MRI) to confirm a patient has suffered a stroke, and to identify stroke subtype (e.g. ischaemic or haemorrhagic). In addition, some hospitals, especially those in remote and rural areas and in developing countries, may not have the necessary expertise to administer reperfusion therapies. This is especially true for thrombectomy, which requires highly specialised staff and equipment to perform the procedure, which may not always be available. For these reasons, it is currently estimated that only between 5-15% of eligible stroke patients receive tPA and/or thrombectomy (Donnan et al.,
However, efforts are being made to increase the number of patients eligible for tPA/thrombectomy treatment by improving the early detection of cerebral salvageable tissue by the use of magnetic resonance imaging (MRI) and head CT imaging. In addition, it is possible that the development of an effective neuroprotective agent may have the potential to extend the therapeutic time window of reperfusion therapies by preserving or “freezing” penumbral tissue for prolonged periods.

**Hemicraniectomy**

Hemicraniectomy is a surgical procedure performed to reduce the effects of severe intracranial hypertension unresponsive to other medical treatments following large cerebral infarction and space-occupying brain oedema. This is the most devastating form of ischaemic stroke and occurs in a very small percentage of patients, between 2 and 5 days after stroke (Frank, 1995) with a reported mortality rate of 80% (Hacke et al., 1996). The surgical procedure involves removing a large section of skull to reduce intracranial pressure and prevent brain herniation and subsequent brainstem compression.

**Acetylsalicylic acid (Aspirin)**

Aspirin is an antiplatelet agent used clinically to prevent the development of blood clots. It is mainly used as a stroke therapy in patients that have not received tPA thrombolytic therapy, and is usually administered 1 to 2 days after the initial stroke (Sandercock et al., 2014). When assessed in two clinical trials (IST, CAST), it appears that the use of aspirin reduces the risk of a subsequent stroke and therefore its use has mainly focused on stroke prevention. Some of the advantages of aspirin are its low cost, ease of administration and proven safety.
Other neuroprotective approaches under consideration

Hypothermia

Hypothermia has been demonstrated to decrease infarct size and improve outcomes in numerous in vivo animal stroke studies (Meloni et al., 2013; Campbell et al., 2013), and to improve outcomes in some clinical trials following cardiac arrest and neonatal hypoxia-ischaemia (Wu and Grotta, 2013), but not consistently in ischaemic stroke. Several clinical studies have established that therapeutic cooling is technically feasible (Lyden et al., 2005) and safe (Hemmen et al., 2010) in awake stroke patients. However, despite the proven neuroprotective effects in animal studies, some issues such as the difficulty of achieving and maintaining a low body temperature, the management of patient discomfort and shivering, and the ability to provide adequate sedation indicate that hypothermia still presents some challenges in a clinical setting. In addition, it is likely that patients may have to be admitted to an intensive care unit (ICU) during hypothermia maintenance requiring measures to be taken to prevent potential harmful side-effects such as an increased risk of infections, coagulopathies, decreased gastrointestinal motility and electrolyte disturbances. While some hypothermia stroke clinical trials still are underway, no study as yet has demonstrated proven efficacy in stroke patients.

Cellular therapies

In recent decades, as the result of widespread interest in the potential of stem cells to replace damaged tissue in stroke and other neuro-damaging disorders, the application of stem cells as a new therapeutic agent for stroke has been explored. In several animal stroke studies, the transplantation of stem cells (e.g. human mesenchymal stromal cells) has shown to improve outcomes and/or reduce cerebral infarction (Yan et al., 2016; Lees et al., 2012). However, despite showing promising results in terms of outcome improvements, only a few clinical studies with a low number of participants have so far tested the effects of stem cells therapy after stroke in humans (Chen et al., 2016). Therefore, further and more comprehensive randomised trials are needed to investigate the efficacy of stem cells therapy in a clinical setting. In addition, because of ethical issues, concerns about tumour formation from
transplanted cells and limited availability of immune-compatible engraftable cells (Bang et al., 2016), there remain considerable hurdles that need to be overcome before stem cells can be used as a stroke therapy in human trials.

**Pharmacological therapies**

Some recent compounds that have been considered as neuroprotective therapies for stroke include the peptides TAT-NR2B9c (also known as NA-1) and JNKL-TAT (also known as XG-102), as well as ginsenoside, uric acid, edaravone, fingolimod, minocycline, natalizumab and glyburide. These compounds have been designed or used to minimise various neurodamaging events associated with the ischaemic cascade, including excitotoxicity, damaging cell death pathways, oxidative stress, inflammation and vasogenic oedema. Since the focus of this PhD study is examining a peptide treatment as a potential neuroprotective therapy for stroke, the discussion below focuses on the TAT-NR2B9c and JNKL-1-TAT peptides.

**TAT-NR2B9c neuroprotective peptide**

The NR2B9c peptide is composed by 9 amino acids (KLSSIESDV). It is derived from the intracellular terminal carboxyl region of the N-methyl-D-aspartate (NMDA) receptor NR2B subunit protein. The NR2B9c peptide was developed to inhibit an intracellular domain of the NR2B protein binding to the cytoplasmic signalling/adaptor protein postsynaptic density-95 (PSD-95), which also binds neuronal nitric oxide synthase (nNOS), and in doing so generates nitric oxide. However, after excitotoxic NMDA receptor activation, the PSD-95-NR2B interaction results in the over-stimulation of the nNOS and the excessive production of nitric oxide (NO) and cell death. For *in vitro* cell culture and *in vivo* animal neuroprotection studies, the NR2B9c peptide is fused to the arginine-rich cell penetrating peptide, TAT, to allow the peptide to enter cells and cross the blood brain barrier.

Several studies both *in vitro* and *in vivo* have confirmed that the TAT-NR2B9c peptide possesses neuroprotective properties. Table 2 in Chapter 2 provides a summary of the animal studies that have assessed the neuroprotective actions of
TAT-NR2B9c. Importantly, one animal study assessing the effects of TAT-NR2B9c peptide in mild and severe transient middle cerebral artery occlusion (MCAO) in the macaque showed that the peptide is effective in reducing the brain infarct size even when administered 3 hours after stroke onset in a 3.5 hour transient MCAO model (Cook et al., 2012).

In addition, in a clinical study involving the intravenous administration of TAT-NR2B9c at the end of endovascular procedures for the repair of intracranial aneurysms, the peptide, based on MRI scans, was shown to reduce the number, but not the volume, of ischaemic lesions compared to placebo (Hill et al., 2012). The TAT-NR2B9c peptide, which has been proven to be safe and well-tolerated, is currently being assessed in a Phase III clinical trial (ESCAPE-NA1) for neuroprotective efficacy in ischaemic stroke. Enrolment for the trial is based on selecting patients with a small infarct core and good collateral circulation who undergo endovascular therapies.

**JNKI-1-TAT neuroprotective peptide**

JNKI-1 is a peptide composed by 20 amino acids (RPKRPTTLNLFPQVPRSQDT) derived from the JNK binding domain of the c-Jun N-terminal kinase interacting protein-1 (JIP-1). JIP-1 is a component of the scaffold protein responsible for the interaction and activation of JNK. JNK is a c-Jun N-terminal protein kinase and one of the three identified families of mitogen activated protein (MAP) kinase, involved in the final steps of a stress-activated signalling pathway that leads to c-Jun activation and other post-translational protein changes associated with cell death and/or apoptosis. As the JNKI-1 peptide is a competitive inhibitor of the JNK interaction with JIP-1, the inhibition of JNK activation is thought to be the mechanism by which the peptide exerts its neuroprotection. Several studies both in vitro and in vivo have shown that the JNKI-1-TAT peptide possesses neuroprotective properties. For a summary of the results of animal studies that have assessed the neuroprotective actions of JNKI-1-TAT, see Table 3 in Chapter 2.
Poly-arginine and arginine-rich peptides and neuroprotection

As mentioned above, this project aims to extend previous findings that initially identified that poly-arginine and arginine-rich peptides possess intrinsic neuroprotective properties. Neuroprotective poly-arginine and arginine-rich peptides (hereafter referred to as arginine-rich peptides otherwise unless indicated) typically range in size from 6 to 30 amino acids, and are positively charged due to the presence of arginine residues. In addition, they have the capacity to cross the plasma membrane and enter cells, and for this reason, are also known as cell penetrating peptides (Munyendo et al., 2012). In addition, arginine-rich peptides also have the capacity to cross the blood brain barrier and enter the brain.

The method of intracellular transduction by arginine-rich cell penetrating peptides can vary between different peptides and cell types, but three main mechanisms are used: 1) direct penetration; 2) endocytosis; and 3) inverted micelle formation. Endosomal mechanisms are thought to be the most common method used by arginine-rich peptides for cell entry. Due to the membrane traversing properties of arginine-rich cell penetrating peptides, they have been extensively used for the intracellular delivery of agents in experimental in vitro and animal studies, as well as in clinical applications (Hill et al., 2012; Meloni et al., 2015b).

Furthermore, due to the ability of arginine-rich peptides to cross the blood brain barrier, they are commonly used for the delivery of potential therapeutics, including neuroprotective agents (e.g. peptides and proteins) into the brain. Potential therapeutic substances fused to arginine-rich peptides have been assessed in neuronal cell culture and animal models that mimic neurodegenerative disorders, including stroke, epilepsy, Parkinson’s and Alzheimer’s disease (Lai et al., 2005; Liu et al., 2006; Arthur et al., 2007; Colombo et al., 2007, Nagel et al., 2008; Xu et al., 2008; Vaslin et al., 2009; Meade et al., 2009; Craig et al., 2011). By far the most commonly used arginine-rich cell penetrating peptide is the TAT peptide (YGRKKRRQRRR). For a summary of studies that have used TAT and other arginine-rich peptides as carrier molecules to deliver putative neuroprotective peptides into the brain for the treatment of experimental stroke and related conditions, see Chapter 2.
During neuroprotective studies using the TAT peptide as a carrier for the delivery of putative neuroprotective peptides into cultured cells or into the brain, a modest neuroprotective effect was observed by some for TAT peptide as a control (Xu et al., 2008; Vaslin et al., 2009), including a study from A/Prof Meloni’s laboratory (Meade et al., 2010). Subsequent studies by Meloni and colleagues demonstrated that other arginine-rich cell penetrating peptides (e.g. R9, penetratin) also display neuroprotective properties, however neuroprotective potency was several magnitudes greater than that observed for the TAT peptide (Meloni et al., 2014). Furthermore, in a later study by Meloni et al (2015a) examining the neuroprotective properties of poly-arginine and arginine-rich peptides, it was demonstrated that neuroprotective efficacy increased with increasing arginine content and that the peptide positive charge was critical for neuroprotection. In addition, Meloni et al (2015a) demonstrated that the poly-arginine peptide R9 reduces infarct volume when administered after permanent middle cerebral artery occlusion stroke in the rat. Based of these findings, Meloni et al (2015a) proposed that the neuroprotective action of TAT-fused “putative” neuroprotective peptides (e.g. TAT-NR2B9c, JNKI-1-TAT) is largely, if not exclusively, due to the TAT carrier peptide per se and/or the cargo peptide enhancing the effects of TAT.

Poly-arginine-rich peptides and neuroprotection

While the neuroprotective mechanisms associated with arginine-rich peptides are still being investigated, some confirmed and potential mechanisms have been identified.

*Inhibition of calcium influx following glutamate excitotoxicity*

Results by Meloni and colleagues using Fura-2 AM calcium imaging after glutamic acid exposure in neuronal cultures showed that poly-arginine and arginine-rich peptides (TAT, R9, R12, R15, BEN1079, PYC36-TAT, NR2B9c-TAT, JNKI-1-TAT, protamine) reduce intracellular calcium levels (Meloni et al., 2015ab). This mechanism of action is in line with the other studies demonstrating that arginine-rich peptides, including TAT-fused “putative” neuroprotective peptides (NR2B\textsubscript{CT}-TAT, CBD3-TAT, R9-CBD3, R6) reduce glutamate excitotoxic calcium influx in cortical
neurons (Tu et al., 2010; Brittain et al., 2011; Moutal et al., 2015) and reduce NMDA evoked receptor currents in NR1-NR2 NMDA receptor-expressing oocytes (Ferrer-Montiel et al., 1998). In addition, several studies have demonstrated that TAT-fused peptides and arginine-rich cell penetrating peptides can reduce the expression of cell surface ion channels and receptors in neurons (Fan et al., 2009; Sinai et al., 2010; Zhang et al., 2010; Brittain et al., 2011; Brustovetsky et al., 2014; García-Caballero et al., 2014) and other cells (Fotin-Mleczek et al., 2005). More recently, Meloni and colleagues have confirmed that the poly-arginine peptide, R12 and the TAT-NR2B9c have the capacity to reduce cell surface levels of the NMDA receptor subunit protein, NR2B in cultured cortical neurons (MacDougall et al., 2016).

Maintenance of mitochondrial function

Growing evidence indicates that arginine-rich peptides exert beneficial effects on mitochondria (Rigobello et al., 1995). For example, studies have demonstrated that arginine-rich peptides may limit complex I activity and reactive oxygen species production (Zhao et al., 2004; Batandier et al., 2006), inhibit the opening of the mitochondrial permeability transition pore (Guigas et al., 2004), protect cristae architecture (Szeto et al., 2011) and prevent cytochrome c release (Birk et al., 2015).

It was demonstrated in human embryonic kidney cells (HEK293) that poly-arginine peptides localise to mitochondria, and reduce rates of mitochondrial respiration, membrane potential and levels of reactive oxygen species (Marshal et al., 2015). Furthermore, Marshall et al (2015) have recently confirmed the neuroprotective properties of poly-arginine peptides in an in vivo NMDA-induced retinal ganglion cell excitotoxicity model and provided evidence that the peptides reduce neuronal mitochondrial oxidative stress. Also of interest are recent studies surrounding the arginine-rich Borna disease viral mitochondrial-targeting protein, X. The full-length protein X, as well as an X-derived peptide fused to a cell penetrating peptide have been shown to display neuronal, axonal, and mitochondrial protective properties (Szelechowski et al., 2014; Ferre et al., 2016).
Inhibition of proteolytic enzymes

Another mechanism whereby arginine-rich peptides may exert a neuroprotective effect is through their ability to inhibit the calcium-dependent protein convertase enzyme, furin, which processes precursor proteins into their biologically active products (Cameron et al., 2000; Kacprzak et al., 2004; Fugere et al., 2007), an effect that may be beneficial following brain ischaemia. Furin is a ubiquitously expressed convertase involved in the enzymatic activation of matrix metalloproteinases (MMPs). In the ischemic brain (Yokota et al., 2001; Yang et al., 2007), furin is up-regulated and can activate MMP2, MMP3 and MMP14 (also known as MT1-MMP) (Tian et al., 2011). Furthermore, MMP3 can activate MMP1, MMP7 and MMP9, and MMP14 can activate MMP2 and MMP2 can activate MMP9 (Yang and Rosemberg, 2015; Turner and Sharp, 2016). Significantly, following stroke MMP2, MMP3, MMP9 and MMP14 are associated with blood brain barrier disruption, cerebral oedema, haemorrhage, leukocyte infiltration and progressive inflammatory reactions (Hosomi et al., 2005; Yang et al., 2007; Yang and Rosemberg, 2015; Turner and Sharp, 2016; Hafez et al., 2016), while MMP inhibition reduces blood brain barrier degradation, cerebral oedema and tissue injury (Yang et al., 2007; Jin et al., 2010; Yang and Rosemberg, 2015; Hafez et al., 2016; Turner and Sharp, 2016).

Aims of the thesis

The aim of this project is to assess the neuroprotective efficacy of different poly-arginine peptides and the arginine-rich peptide protamine in different rat stroke models, the goal being to provide evidence that justifies and leads the way to additional pre-clinical studies evaluating arginine-rich peptides as a potential treatment for stroke.

The specific aims of the project are:

Aim 1: To assess the neuroprotective efficacy of poly-arginine peptides (R12, R15, R18 and protamine) using a permanent MCAO rat stroke model.

Hypothesis – Poly-arginine and protamine peptides are neuroprotective following permanent MCAO stroke.
**Aim 2:** To determine the therapeutic time window and dose responsiveness of an effective peptide (as identified in Aim 1) using a permanent MCAO stroke model.
Hypothesis – An effective peptide as identified in Aim 1 will have a therapeutic window of between 1 to 6 hours after stroke onset.

**Aim 3:** To determine the therapeutic time window and dose responsiveness of an effective peptide (as identified in Aim 1) in a transient MCAO stroke model.
Hypothesis – An effective peptide as identified in Aim 1 will also be effective in a transient MCAO model.

**Aim 4:** To assess the neuroprotective efficacy of an effective peptide (as identified in Aim 1) when administered post-reperfusion in a transient MCAO stroke model.
Hypothesis – Peptide administration post-reperfusion following transient MCAO model will be effective.
References


Chapter 2

Neuroprotective peptides fused to arginine-rich cell penetrating peptides: neuroprotective mechanism likely mediated by peptide endocytic properties

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Neuroprotective peptides fused to arginine-rich cell penetrating peptides: neuroprotective mechanism likely mediated by peptide endocytic properties

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Neuroprotective peptides fused to arginine-rich cell penetrating peptides: Neuroprotective mechanism likely mediated by peptide endocytic properties


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- TAT peptide

A B S T R A C T
Several recent studies have demonstrated that TAT and other arginine-rich cell penetrating peptides (CPPs) have intrinsic neuroprotective properties in their own right. Examples, we have demonstrated that in addition to TAT, poly-arginine peptides (R8 to R18; containing 8–18 arginine residues) as well as some other arginine-rich peptides are neuroprotective in vitro (in neurons exposed to glutamic acid excitotoxicity and oxygen glucose deprivation) and in the case of R9 in vivo (after permanent middle cerebral artery occlusion in the rat). Based on several lines of evidence, we propose that this neuroprotection is related to the peptide’s endocytosis-inducing properties, with peptide charge and arginine residues being critical factors. Specifically, we propose that during peptide endocytosis neuronal cell surface structures such as ion channels and transporters are internalised, thereby reducing calcium influx associated with excitotoxicity and other receptor-mediated neurodamaging signalling pathways. We also hypothesise that a peptide cargo can act synergistically with TAT and other arginine-rich CPPs due to potentiation of the CPPs endocytic traits rather than by the cargo-peptide acting directly on its supposedly intended intracellular target. In this review, we systematically consider a number of studies that have used CPPs to deliver neuroprotective peptides to the central nervous system (CNS) following stroke and other neurological disorders. Consequently, we critically review evidence that supports our hypothesis that neuroprotection is mediated by carrier peptide endocytosis. In conclusion, we believe that there are strong grounds to regard arginine-rich peptides as a new class of neuroprotective molecules for the treatment of a range of neurological disorders.

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Abbreviations: AIP, autacamtide-2–related inhibitory peptide; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid; APOE, Apolipoprotein E; APLP, amyloid precursor-like protein; APP, amyloid precursor protein; CaMKII, calcium/calmodulin-dependent protein kinase II; CaM-XIII, calcium/calmodulin-dependent protein kinase II inhibitor; Cav2.2, voltage-gated, N-type calcium channel; Cav2.3, voltage-gated, R-type calcium channel; Cav3.3, voltage-gated, T-type calcium channel; CBD, calcium channel-binding domain; cGMP, cyclic guanosine monophosphate; CCR5, calcitonin gene related peptide; CNX, central nervous system, 6-cyano-7-nitroquinoxaline-2,3-dione; CPP, cell penetrating peptide; CRMP, collapsing response mediator protein; DAPK1, death-associated protein kinase 1 protein; DM, DNA-binding motif; D1R–D2R, dopamine D1–D2 receptor; DRG, dorsal root ganglion; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; FGR, fibroblast growth factor receptor; GluR6, glutamate receptor 6; HSF, heparan sulphate proteoglycan; HIV-TAT, human immunodeficiency virus-type 1 trans-activator of transcription; Insig-1, insulin-induced gene 1; JNK, c-Jun N-terminal kinase; JIP-1, c-Jun N-terminal kinase interacting protein-1; KCa3, Kaposi fibroblast growth factor; mGluR1, metabotropic glutamate receptor 1; MCAO, middle cerebral artery occlusion; ND2.1, NADH dehydrogenase subunit 2; NADPH, nicotinamide adenine dinucleotide phosphate; NCX, sodium calcium exchanger; NMDA, N-methyl-D-aspartate; NO, nitric oxide; nNOS, neuronal nitric oxide synthase; NR2B, NMDA receptor subunit 2B; OGD, oxygen glucose deprivation; PDZ, PSD-95, and Drosophila disc large tumor suppressor, and zonula occludens–1 protein; PDK, Phosphatidylinositol-4,5-bisphosphate 3-kinase; PGC, protein kinase C; PNS, peripheral nervous system; PSD-95, protein postsynaptic density-95; PTPs, protein tyrosine phosphatase; PTX, protein transduction domain; SCAP, SREBP cleavage-activating protein; SCI, spinal cord injury; sEPSC, spontaneous excitatory post-synaptic currents; siRNA, small interfering RNA; SREBP-1, sterol regulatory element-binding protein-1; TAT, trans-activator of transcription; TNF, tumor necrosis factor receptor; VGCC, voltage-gated calcium channel; VR1, vanilloid receptor 1.

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1. Introduction

1.1. Neuroprotective peptides and cell penetrating peptides

In recent years there has been an increased interest in the use of specifically designed peptides targeting cyto-damaging or cyto-protective pathways as neuroprotective agents. There are several reasons why this interest arose, including: i) peptide sequences critical for neurodamaging or neuroprotective intracellular protein–protein interactions can be easily identified and used as competitive inhibitors of target proteins (e.g., JNKI-1 peptide); ii) small peptides (2–40 amino acids) can be synthesised relatively cheaply using commercial sources; and iii) the development of cell penetrating peptides (CPPs), also referred to as protein or peptide transduction domains (PTDs), has provided a way to deliver peptides and other cargos (incl. proteins, nucleic acids and drugs) into cells and across the blood–brain barrier.

The discovery of CPPs has led to studies on the ability of a number of peptides and proteins to act as neuroprotection agents, as well as providing a means to explore the role of protein/protein interactions in brain function in health and disease (viz. neurological and non-neurological disorders). The main focus of this review is the use of arginine-rich CPPs (mainly TAT) for the delivery of neuroprotective peptides (<40 amino acids) particularly in cerebral ischaemia and stroke. The recent observation that CPPs have intrinsically neuroprotective properties on their own right has led us to question the conclusions of other studies. Here, we critically reappraise previous studies that have used putative neuroprotective peptides fused to CPPs as agents in cerebral ischaemia and other models of CNS injury, and examine the mechanism whereby arginine rich–peptides exert their neuroprotective effects. Importantly, we highlight that many past studies on neuroprotective peptides that have used cationic CPPs for CNS delivery may need to be reinterpreted in the light of the intrinsic neuroprotective effects of the carrier-peptide.

1.2. Cell penetrating peptides

Cell penetrating peptides (CPPs) are small peptides (typically 5–25 amino acids) that are commonly used to facilitate the delivery of normally non-permeable cargo molecules such as other peptides, proteins, nucleic acids or drugs into cells, and across the blood–brain barrier. The development of CPPs as drug vehicles was sparked by the discovery of the PTD within the human immunodeficiency virus-type 1 trans-activator of transcription (HIV-TAT) protein (Frankel & Pabo, 1988; Green & Loewenstein, 1988). The active transporting peptide sequence within the HIV-TAT protein was isolated (TATα-37: GRKKRRQRRR) and is now referred to as the TAT peptide or TAT (Becker–Hapak et al., 2001). Subsequently, over 100 CPPs have been identified (Milletti, 2012).

By far the most commonly used CPP peptide is TAT, especially to deliver various cargo molecules to the brain, including neuroprotective peptides and proteins. Other CPPs include penetratin (also known as antennapedia), poly-arginine peptides (R8 to R12; where R refers to arginine residues), Pep-1 and transportan. The amino acid sequences for these peptides, as well as of some less commonly used CPPs, are shown in Table 1. TAT, poly-arginine and penetratin are cationic arginine-rich CPPs.

1.3. Arginine-rich cell penetrating peptides and intrinsic neuroprotection

Potential neuroprotective peptides fused to CPPs have been assessed in cultured neurons and animal models that mimick neural injury mechanisms seen in a variety of disorders, including cerebral ischaemia, spinal cord injury, traumatic brain injury, epilepsy, Parkinson’s disease and Alzheimer’s disease (Arthur et al., 2007; Colombo et al., 2007; Lai et al., 2005; Liu et al., 2006; Meade et al., 2009; Nagel et al., 2008). However, several years ago, we and others demonstrated that TAT possesses intrinsic neuroprotective properties both in vitro in neurons exposed to excitotoxicity and oxygen–glucose deprivation (OGD) and in vivo following cerebral ischaemia in P12 rats after intraventricular injection (Craig et al., 2011; Meade et al., 2010a; Vaslin et al., 2009b; Xu et al., 2008). We subsequently showed that poly-arginine-9 (R9), penetratin and Pep-1 also display neuroprotective actions in vitro on excitotoxic and/or OGD models (Meloni et al., 2014). Furthermore, our data showed that R9 and penetratin were 17- and 4.6-fold respectively more neuroprotective than TAT (Meloni et al., 2014).

The higher potency of R9 relative to TAT and penetratin led us to explore the in vitro neuroprotective potency of other poly-arginine peptides (R1, R3, R6–R15 and R18), as well as, other arginine-rich peptides (Meloni et al., 2015). These studies confirmed that poly-arginine and

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Table 1
Examples of commonly used cell penetrating peptides.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Amino acids: MW (Da)</th>
<th>Net charge at pH 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAT</td>
<td>GRKKRRQRR</td>
<td>10: 1397</td>
<td>8</td>
</tr>
<tr>
<td>TAT-D</td>
<td>rrqrkfrq</td>
<td>10: 1397</td>
<td>8</td>
</tr>
<tr>
<td>Penetratin</td>
<td>RQIKWGFQRRMKWKK</td>
<td>16: 2245</td>
<td>7</td>
</tr>
<tr>
<td>Pep-1</td>
<td>KETWWEYWWTESWQKRRKKV</td>
<td>21: 2848</td>
<td>3</td>
</tr>
<tr>
<td>HSV-1 VP22</td>
<td>DAAATRGRSASRPPRASRPVRRV</td>
<td>33: 3548</td>
<td>6</td>
</tr>
<tr>
<td>Transportan</td>
<td>GWTLNSAYLGCNNLAKALAKKL</td>
<td>27: 2841</td>
<td>4</td>
</tr>
<tr>
<td>kFGFb</td>
<td>AAVALLPVLALLAP</td>
<td>16: 1516</td>
<td>0</td>
</tr>
<tr>
<td>MAP</td>
<td>KLAALAKLAKLAKLA</td>
<td>18: 1877</td>
<td>5</td>
</tr>
<tr>
<td>MPC</td>
<td>GALFLGWLACGSTMGPKKRRKV</td>
<td>24: 2445</td>
<td>5</td>
</tr>
</tbody>
</table>

Notes:

* Sequences are in standard single letter code with l-isof orm amino acid residues represented in uppercase and d-isof orm amino acid residues (sequences in retro-inversed form) represented in lowercase.

* Penetratin is also known as antennapedia peptide and kFGF (Kaposi fibroblast growth factor) is also known as MTS (membrane translocating sequence).


arginine-rich peptides as a group are highly neuroprotective, with efficacy increasing with increasing arginine content, peaking at R15 (Meloni et al., 2015). We also showed that arginine-rich peptides have the capacity to reduce glutamic acid-induced neuronal calcium influx and are neuroprotective with a single treatment several hours after glutamic acid or OGD exposure. Furthermore, neuroprotective efficacy was shown to be directly related to peptide positive net charge conferred by the positively charged arginine (R) and lysine (K) amino acids residues, which could be blocked by fusion with a negatively charged glutamic acid (E9) poly-peptide (e.g. R9/E9 peptide) or by incubation with the highly negatively charged molecule heparin. The latter finding strongly suggests that peptides bind to negatively charged cell surface molecules such as heparin sulphate proteoglycans (HSPGs), chondroitin sulphate proteoglycans (CSPGs) or sialic acid residues present in glycosphingolipids to initiate and stimulate peptide endocytosis (Favretto et al., 2014; Kim et al., 2012; Ravindran et al., 2013; Wallbrecher et al., 2014) a process crucial for neuroprotection (Meloni et al., 2015). In this context, others have demonstrated that the nature of the peptide interaction with HSPGs determines CPPs endocytic properties (Wallbrecher et al., 2014).

With respect to endocytosis, studies have demonstrated that peptide charge conferred by arginine and lysine residues (note: arginine and lysine are the only two strongly positively charged amino acids, with histidine being only weakly positively charged, whereas glutamic acid and aspartic acid are the only two negatively charged amino acids) facilitate HSPG binding, and that mainly arginine residues trigger the endocytic process (Amand et al., 2012; Wallbrecher et al., 2014; Yang et al., 2014). Consistent with our proposed endocytic neuroprotective mechanism, we have demonstrated that poly-lysine (K10) is only weakly neuroprotective in a cortical neuronal glutamic acid excitotoxicity model (Amand et al., 2012; Wallbrecher et al., 2014; Yang et al., 2014). Our hypothesis also links endocytosis to a diverse range of arginine-rich peptides (including TAT-fused peptides), all of which are likely to have endocytic inducing properties.

1.4. Proposed neuroprotective mechanism of action used by arginine-rich peptides

Based on our recent findings we hypothesised that arginine-rich peptides exert their neuroprotective effects by inducing the endocytic internalisation of cell surface ion channels, thereby reducing the damaging effects of excitotoxicity (see Fig. 2). This is a novel hypothesis that essentially identifies arginine-rich peptides as a new class of neuroprotective molecule. There are several lines of evidence based on our findings and those of others that support our endocytosis hypothesis. Arginine-rich peptides, including so-called “neuroprotective peptides” fused to TAT have been shown to: i) reduce neuronal calcium influx (Meloni et al., 2015) and interfere with ion channel function (NMDA receptor: Ferrer-Montiel et al., 1988; Tu et al., 2010; Sinai et al., 2010; Brittain et al., 2011b; Brustovetsky et al., 2014, VR1: Planells-Cases et al., 2000, CaV2.2: Brittain et al., 2011a, 2011b; Feldman & Khanna, 2013; Brustovetsky et al., 2014; sodium calcium exchanger [NCX], CaV3.3: García-Caballero et al., 2014); ii) cause internalisation of neuronal ion channels (Brustovetsky et al., 2014; Sinai et al., 2010); and iii) require endocytosis as a prerequisite for neuroprotection (Meloni et al., 2015; Vaslin et al., 2011). Interestingly, other TAT-fused peptides have also been shown to interfere with the function of neuronal receptors (D1R–D2R; Pei et al., 2010; PTPr: Lang et al., 2015). In this context, it is important to note that endocytosis is a known mechanism used by cells to internalise cell surface receptors (Höller & Dikic, 2004; Marchese, 2014; Maxfield & McGraw, 2004).

Neuroprotective efficacy, at least for poly-arginine peptides (Meloni et al., 2015), appears to correlate with peptide transduction efficacy (Mitchell et al., 2000), a process known to occur by endocytosis (Appelbaum et al., 2012; Bechara et al., 2013; El-Sayed et al., 2009). Furthermore, it is important to note that the rapid and transient (lasting up to 4 h with peptide pre-treatment) nature of the neuroprotection induced by poly-arginine peptides (Meloni et al., 2015) corresponds closely to the timeframes of endocytosis and endosomal receptor re-cycling (Gundelfinger et al., 2003; Maxfield & McGraw, 2004; Yashunsky et al., 2009). Importantly, it is known that TAT, penetratin and R9 can induce the internalisation of EGFR and TNFR in HeLa cells (Fotin-Mlezek et al., 2005). Our hypothesis also links endocytosis as a common neuroprotective mechanism of action for a diverse range of arginine-rich peptides (including TAT-fused peptides), all of which are likely to have endocytic inducing properties.

This neuroprotective mechanism that we propose is also consistent with the link between neuronal cell surface–HSPGs (Litwack et al., 1994) and endocytic activity (Vaslin et al., 2009a), which are known to promote endosomal uptake of cationic CPPs (Nakase et al., 2007; Vaslin et al., 2009a, 2011). It is also possible that other negatively charged cell surface receptors such as CSPGs and glycosphingolipids can promote cationic CPP endocytosis and neuroprotection. As mentioned above, positively charged poly-arginine and arginine-rich peptides are known to bind negatively charged HSPGs to initiate endocytosis. It is important to note that any neuroprotective peptide fused to

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**Fig. 1.** Glutamic acid excitotoxicity model: peptide dose response experiments. Peptides present in neuronal cultures for 10 min before and during (half concentration) 5-min glutamic acid exposure. Peptides: R15, R10/A5: ARRARRARRARRRA and R10/W5: WRRRWRRRWWWRRRW. Neuronal viability measured 24 h following glutamic acid exposure. Concentration of peptide in μM. MTS data were expressed as percentage neuronal viability with no insult control taken as 100% viability and glutamic acid control taken as 5% (mean ± SE; n = 4; *P < 0.05). For additional methodological details see Meloni et al. (2015).
Proposed model of arginine-rich CPP induced endocytic internalisation of neuronal cell surface receptors

Fig. 2. Diagrammatic representation of proposed model of arginine-rich CPPs inducing internalisation of neuronal cell surface structures. Note: model applies to neuronal synaptic and extra-synaptic plasma membranes and potentially the plasma membrane of astrocytes, pericytes, brain endothelial cells, oligodendrocytes and microglia. NMDAR: N-methyl-D-aspartate receptors; AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors; NCX: sodium calcium exchanger; VGCC: voltage-gated calcium channels (e.g. CaV2.2, CaV3.3); ASIC: acid-sensing ion channels; TRPM2/7: Transient receptor potential cation channels 2 and 7; mGluR: metabotropic glutamate receptors; VR1: vanilloid receptor 1 or transient receptor potential cation channel subfamily V member 1; TNFR: tumour necrosis factor receptors; FASR: FAS receptor; EAAT: excitatory amino-acid transporters; AQP4: Aquaporin 4; Trk: tropomyosin-receptor-kinase receptors.

Adapted from Maxfield and McGraw (2004).

2. Examination of studies using CPP-fused to neuroprotective peptides in neuronal injury models

To date, over a dozen of neuroprotective peptides fused to CPPs have been described (Tables 2–4). Three of the most intensely studied peptides developed as potential neuroprotective agents for stroke/cerebral ischaemia are NR2B9c, JNKI-1 and CBD3 (Tables 2–4). This review in particular critically examines the use of these three peptides in a neurological setting, and provides evidence suggesting that the critical neuroprotective and functional structural elements of these peptides are arginine and lysine residues in the carrier and cargo-peptides. The remainder of the review focuses on a range of other peptides to further highlight possible biological effects mediated by the TAT carrier peptide.

2.1. NR2B9c and Tat-NR2B9cpeptide (also known as NA-1)

NR2B9c is a 9 amino acid peptide (KLSSIESDV1479–1484) derived from the intracellular terminal carboxyl region of the N-methyl-D-aspartate (NMDA) receptor NR2B subunit protein (Aarts et al., 2002). This region of the NR2B subunit was selected for peptide design because of its high binding affinity to the cytoplasmic signalling/adaptor protein postsynaptic density-95 (PSD-95) via one of its three PDZ domains (PDZ: PSD-95, and Drosophila disc large tumor suppressor, and zonula occludens-1 protein: three proteins that share this signalling domain). PSD-95 couples the NR2B subunit to intracellular signalling proteins and enzymes, subsequent to NMDA receptor activation. For example, following receptor activation by the neurotransmitter glutamate, PSD-95 binds to the NR2B subunit and the enzyme neuronal nitric oxide synthase (nNOS), resulting in the production of nitric oxide (NO), which under normal conditions serves as a signalling molecule in neuronal, glia and vascular cells. As a consequence, the NR2B9c peptide has the potential to act as a competitive inhibitor of PSD-95 binding to the NR2B subunit, and in doing so blocks down-stream signalling associated with the interaction of these two proteins.

In cerebral ischaemia, one neuro-damaging event linked to NMDA receptor over-activation and PSD-95 signalling is nNOS over-stimulation and the excessive production of NO. In addition to the direct intracellular toxic effects of NO, a major target for this free radical is stress-activated protein kinase p38, which is known to be involved in ischaemic brain injury (Barone et al., 2001). The inhibition of NO over-production is thought to be the basis of the neuroprotective action of the TAT fused NR2B9c peptide (TAT-NR2B9c).
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2.1.1. In vitro studies

In initial in vitro neurophysiological (using hippocampal slices or cortical neuronal cultures for measuring synaptic activity, calcium influx and cGMP production) and NMDA induced injury (using cortical neuronal cultures) studies, TAT-NR2B9c was used at a concentration of 0.05 μM (Aarts et al., 2002). A concentration of 0.05 μM is considerably lower than the concentration required to achieve neuroprotection for other TAT-fused peptides and arginine-rich peptides (Meloni et al., 2014, 2015). It is therefore hardly surprising that when tested at 0.05 μM, TAT-NR2B9c did not affect synaptic responses in brain slices or NMDA induced calcium influx in cortical neurons. By contrast, it is surprising that at 0.05 μM TAT-NR2B9c was effective in reducing cortical neuronal death and cGMP levels (increases after nNOS activation) following NMDA exposure (20–100 μM) after either a 1-hour pre-exposure or continuous post-insult exposure. One possible explanation to account for the neuroprotective efficacy of TAT-NR2B9c at 0.05 μM following NMDA exposure may relate to the lower level of excitotoxicity induced by the model. The model incorporates 6-cyano-7-nitroquinolinic acid (CNQX) and nimodipine in the culture medium during NMDA exposure to prevent secondary activation of AMPA (α-aminooxy-3-hydroxy-5-methyl-4-isoxazolopropionic acid) receptors and voltage-gated calcium channels, respectively. In a subsequent study using a MDNA model in the presence of the voltage-gated calcium channel blocker nifedipine, TAT-NR2B9c at 2 μM was shown to be neuroprotective following exposure to 20–60 μM NMDA, but not 100 μM (Soriano et al., 2008). Interestingly, although individually the TAT-NR2B9c (2 μM) nor JNKI-1-TATD (2 μM) peptides were effective following 100 μM NMDA exposure, together they had a synergistic positive effect (Soriano et al., 2008).

In their initial study of TAT-NR2B9c, Aarts et al. (2002) used a variety of controls including: i) a mutated TAT-NR2B9c peptide (KLSSIEADA; TAT-NR2B9c-AA) incapable of binding to PSD-95 (negative control); ii) a TAT-fused truncated PSD-95 protein (pTAT-PDZ1-2) containing PDZ domains 1 and 2 that is known to competitively block PSD-95 binding to the NR2B subunit and: iii) a pTAT-GK protein containing the PSD-95 guanylate kinase-like domain that does not interfere with PSD-95 binding to the NR2B subunit. At 0.05 μM, none of the controls blocked neuronal calcium influx following NMDA exposure (in the presence of CNQX and nimodipine), while only the pTAT-PDZ1-2 protein (like the TAT-NR2B9c peptide) was neuroprotective following NMDA exposure. Unlike the TAT-NR2B9c peptide, the TAT-NR2B9c-AA peptide did not inhibit cGMP formation.

In a recent study (Chen et al., 2015), TAT-NR2B9c was shown to inhibit neuronal NMDA induced superoxide production by blocking the activation of the membrane-bound NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) complex. It was proposed that the TAT-NR2B9c peptide by inhibiting the PSD-95/NR2B9c interaction blocked PSD-95 adaptor protein APPL1 (adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1) coupling with PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase), thereby inhibiting PKCζ (protein kinase C) to phosphorylate the NADPH oxidase subunit protein p47phox, which is required for activation of the complex. Interestingly, the TAT-fused arginine-rich NADPH oxidase complex inhibitor peptide gp91ds-TAT (CSIRRQL-TAT-NH2; net charge with NH2 group +12) inhibited neuronal NMDA induced superoxide production to a greater extent than TAT-NR2B9c, while the TAT-fused non arginine-rich scrambled peptide (CSFNSYELGSLCY-TAT) did not. The Chen et al. (2015) study also showed that TAT-NR2B9c did not inhibit NMDA induced neuronal calcium influx.

In our laboratory, we have shown that TAT-NR2B9c is not neuroprotective in cultured neurons exposed to glutamic acid induced excitotoxicity, even at high concentrations (20 μM), while modest...
neuroprotection is evident in a milder NMDA excitotoxicity model (Meloni et al., 2015). In addition, we have also shown that at 5 μM, TAT-NR2B9c can reduce neuronal calcium influx following glutamic acid exposure, but not to the same extent as a poly-arginine peptide (Fig. 3 and Meloni et al., 2015).

2.1.2. In vivo studies

Studies utilising the TAT-NR2B9c peptide in different in vivo injury models are presented in Table 2. Animal studies have generally yielded positive outcomes with TAT-NR2B9c being shown to be neuroprotective at doses ranging from 30 to 3000 nmol/kg. Neuroprotection was seen in the macaque when TAT-NR2B9c treatment was commenced 3 h after stroke onset, in a 3.5 hour transient middle cerebral artery occlusion (MCAO) model. While some positive results have been obtained in permanent MCAO models, several studies have reported a lack of neuroprotection even at a dose of 3000 nmol/kg. In the few studies that have investigated the TAT-NR2B9c-AA negative control, no neuroprotection was evident (See Table 2).

Of particular interest is a study in humans using TAT-NR2B9c (renamed NA-1 in the study) following endovascular treatment to repair ruptured or unrepaired intracranial aneurysms (ENACT: Evaluating Neuroprotection in Aneurysm Coiling Therapy) (Hill et al., 2012). The ENACT trial confirmed that TAT-NR2B9c is safe, and showed that while two-thirds of the trial participants (peptide; n = 92/saline; n = 93) had small ischaemic stroke lesions detectable by MRI, patients treated with peptide had significantly fewer brain infarcts.

2.1.3. Is the neuroprotective action of TAT-NR2B9c mediated by TAT or the NR2B9c peptide?

Based on recent observations on the neuroprotective properties of cationic CPPs, we view it as likely that TAT itself is the active neuroprotective component in TAT-NR2B9c. Like TAT alone, TAT-NR2B9c has only modest neuroprotective efficacy in vitro, which can easily be overcome by increasing the severity of excitotoxicity (Martel et al., 2009). The fact that the level of neuroprotection is only modest for TAT-NR2B9c is hardly surprising as the NR2B9c peptide contains one positively charged (lysine; K) and two negatively charged amino acids.
et al., 2015), the NR2B9c peptide is unlikely to potentiate the neuroprotective properties of TAT by way of cationic charge. However, the NR2B9c peptide is larger in size, which may contribute to peptide stabilizing effects in vivo. The importance of peptide charge in peptide neuroprotection (Meloni et al., 2015; Soriano et al., 2008).

Bold data indicate peptides used in studies.

Table 4

<table>
<thead>
<tr>
<th>Peptide name &amp; sequence</th>
<th>Injury model</th>
<th>Route &amp; treatment schedule</th>
<th>Dose</th>
<th>Neuroprotection or reduced pain</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAT-CBD3: TAT-ARSRLAE-LGVPVRGL, TAT-CRMP-2: TAT-GVPRGLYDGVCVE or R9-CBD3: R9-ARSRLAES-LGVPVRGL</td>
<td>Mouse: pMCAO</td>
<td>ICV: before MCAO</td>
<td>10 mg; TAT-CRMP2</td>
<td>Yes (6 h endpoint)</td>
<td>Bu et al., 2011</td>
</tr>
<tr>
<td>Rat (SD)</td>
<td>SC: before formalin</td>
<td>20 μl: 3, 30 or 100 μM</td>
<td>Yes. No 3 μM</td>
<td>Brittain et al., 2011a</td>
<td></td>
</tr>
<tr>
<td>Pain; SC formalin in hind-paw</td>
<td>Pain; capsaicin in eye</td>
<td>Eye: before capsaicin</td>
<td>40 μl: 3, 30 or 100 μM</td>
<td>Yes. No 3 μM</td>
<td>Brittain et al., 2011b</td>
</tr>
<tr>
<td>Pain; IP ddC</td>
<td>Mouse: TBI</td>
<td>IP: 5 min after TBI</td>
<td>6400 nmol/kg</td>
<td>Yes</td>
<td>Brittain et al., 2011a</td>
</tr>
<tr>
<td>Rat (SD): tMCAO; 120 min</td>
<td>IV: 1 h before MCAO or 15 min before reperfusion</td>
<td>6400 nmol/kg</td>
<td>Yes</td>
<td>Brittain et al., 2011b</td>
<td></td>
</tr>
<tr>
<td>Rat (SD): Pain; IP ddT</td>
<td>IP: after ddT</td>
<td>3300 or 9600 nmol/kg: (TAT-CBD3) 3300 nmol/kg: TAT-CBD3-A6K</td>
<td>Yes</td>
<td>Piekarz et al., 2012</td>
<td></td>
</tr>
<tr>
<td>Rat (SD)</td>
<td>Dural surface: before</td>
<td>Capsaicin</td>
<td>50 μl; 10 μM: TAT-CBD3-G14F</td>
<td>Yes</td>
<td>Ripsch et al., 2012</td>
</tr>
<tr>
<td>Migraine; capsaicin nasal mucosa</td>
<td>Pain; IP ddT</td>
<td>IP: 7 days after ddT</td>
<td>3300 nmol/kg: TAT-CBD3-G14F</td>
<td>Yes</td>
<td>Ripsch et al., 2012</td>
</tr>
<tr>
<td>Rat (SD): Pain: reverse transcriptase inhibitor or tibial nerve injury</td>
<td>Details not provide</td>
<td>Details not provide</td>
<td>Yes, R9-CBD3 &gt; TAT-CBD3-A6K &gt; TAT-CBD3</td>
<td>Feldman &amp; Khanna, 2013 (review article)</td>
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<tr>
<td>Rat (SD)</td>
<td>IP: after ddC</td>
<td>33, 330 or 3300 nmol/kg: R9-CBD3</td>
<td>Yes, 3300 nmol/kg</td>
<td>Ju et al., 2013</td>
<td></td>
</tr>
<tr>
<td>Pain; IP ddC</td>
<td>IP: after nerve injury</td>
<td>33, 330 or 3300 nmol/kg: R9-CBD3</td>
<td>Yes, all doses</td>
<td>Ju et al., 2013</td>
<td></td>
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<tr>
<td>Pain; tibial nerve injury</td>
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</tr>
</tbody>
</table>

(a glutamic acid; E and aspartic acid; D), resulting in a peptide net charge (+7; hereafter net charge is at pH 7) lower than TAT (+8). Given the importance of peptide charge in peptide neuroprotection (Meloni et al., 2015), the NR2B9c peptide is unlikely to potentiate the neuroprotective properties of TAT by way of cationic charge. However, the NR2B9c peptide is larger in size, which may contribute to peptide stability and thereby improve peptide efficacy to a small degree. We believe that TAT-NR2B9c has modest neuronal cell endocytic penetrating properties compared to poly-arginine peptides, (e.g. R12 and R15) and therefore is likely to have a modest capacity to reduce the level of surface receptors (e.g. NMDA and VGCC). This would explain why TAT-NR2B9c has a lower capacity to inhibit glutamate induced calcium influx compared to poly-arginine peptides (Fig. 3 and Meloni et al., 2015) and why neuroprotection provided by the peptide is easily overcome by increasing the severity of excitotoxicity (Martel et al., 2009; Meloni et al., 2015; Soriano et al., 2008).

Given the above, it appears likely that the modest neuroprotective actions of TAT-NR2B9c simply reflect the modest level of neuroprotection achievable with TAT. This may also explain why other NDMA receptor activated pathways not associated with NR2B/PSD-95 signalling, such as,
JNK and CREB are not inhibited by TAT-NR2B9c (Soriano et al., 2008). Furthermore, the TAT-fused mutant peptide TAT-NR2B9c-AA is not neuroprotective both in vitro and in vivo. The lack of neuroprotection of TAT-NR2B9c-AA is consistent with a study demonstrating that alanine can significantly impede CPP-HSPG binding, a critical step in the process of endocytosis (Yang et al., 2014).

While it cannot be precluded that one element in the neuroprotective actions of TAT-NR2B9c is mediated by its inhibition of the PSD-95/NR2B9c interaction within the cytoplasm, we view this as highly unlikely as this would require the intact release of the peptide from endosomes following cellular uptake. As discussed previously, CPP endosomal escape is known to be a highly inefficient process, and endosomal fusion to lysosomes leads to the degradation of peptides and proteins (Maxfield & McGraw, 2004). To this end, no evidence for the endosomal release of TAT-NR2B9c, or other CPP-fused neuroprotective peptides (TAT-JNKI-1, TAT-CBD3), has ever been directly demonstrated as is the case for all of the cargo peptides fused to CPPs discussed in this review.

Another issue relevant to this discussion is that TAT-NR2B9c mainly targets synaptically located NMDA receptors where there is an abundant pool of PSD-95 protein. It is generally accepted that extra-synaptic NMDA receptor activation is cell death promoting, whereas synaptic NMDA receptor activation is pro-survival (Hardingham & Bading, 2010). There is the possibility that TAT-NR2B9c may act to some degree by blocking synthetically induced neuronal NO production, which could in theory have a negative impact on neuronal survival, as it could lead to the inhibition of NO-induced blood vessel vasodilatation (Garthwaite, 2008; Lourenço et al., 2014), thereby potentially exacerbating the reduction in blood flow seen in stroke.

It has been demonstrated that a TAT-NR2B9c peptide with a serine (S) to threonine (T) substitution (KLSSIEVDYV/TAT-NR2B9c_gp) is as effective as TAT-NR2B9c as a neuroprotective agent following permanent MCAO (Sun et al., 2008). The proposed mechanism of action of TAT-NR2B9c involves its specific binding to a peptide sequence within the PSD-95 protein, thereby blocking NR2B/PSD-95 engagement (Aarts et al., 2002). Given the high specificity of protein–protein interactions, it is difficult to reconcile this putative mechanism with the fact that the TAT-NR2B9c_gp mutated peptide is capable of inhibiting the NR2B/PSD-95 interaction, at least to the same level as the TAT-NR2B9c peptide.

Recently, it was reported that TAT-NR2B9c reduces synthetically induced neuronal superoxide production (Chen et al., 2015) by disrupting a signalling cascade initiated by NR2B/PSD-95 coupling and activation of the NADPH oxidase membrane complex. However, it is more likely that the TAT-NR2B9c peptide through its endocytosis-inducing properties acts by reducing the levels of neuronal NMDA receptors and/or the NADPH oxidase membrane complex, thereby also reducing superoxide production.

### 2.2. JNKI-1 peptide and JNKI-TAT (also known as XG-102)

JNKI-1 is a 20 amino acid peptide (RPKRPTTLNLPQVPRSQDT157-167) derived from the signalling adaptor protein c-Jun N-terminal kinase interacting protein-1 (JIP-1) (Borsello et al., 2003). The region of JIP-1 that provides the basis for JNKI-1 peptide design is the JNK binding domain. Consequently, the JNKI-1 peptide has the ability to competitively inhibit JNK interaction with JIP-1, thereby block JNK activation (phosphorylation) and JNK downstream signalling (Borsello et al., 2003).

JNK is a protein kinase involved in the final steps of a stress-activated signalling pathway that leads to cell death. JNK is highly expressed in neurons and is activated in the brain in pathological states associated with excitotoxicity, including trauma, epilepsy and stroke (Borsello & Bonny, 2004). Activated JNK can affect cell death pathways by altering protein post-translational structure or by stimulating the expression of pro-death proteins. The inhibition of JNK activation is thought to be the mechanism by which the TAT-JNKI-1 peptide exerts its neuroprotective effects.

### 2.2.1. In vitro studies

The first in vitro study (Borsello et al., 2003) explored the neuroprotective effects of TAT-JNKI-1 in both the l- and d-isomers (i.e. peptide synthesised with protease resistant α-amino acids, retro-inversely). In cortical neuronal cultures exposed to NMDA excitotoxicity, peptides at a concentration of 2 μM were highly neuroprotective after 12 h, but only the d-isomer protected neurons for an extended period (24–48 h). TAT-JNKI-1 treatment in the NMDA model also inhibited phosphorylation of the JNK target protein c-Jun. TAT and JNKI-1 as well as a TAT fused mutant JNKI-1 peptide with three alanine substitutions (RPKRPTAANAFQVPRSQD-TAT) were not neuroprotective in cells exposed to NMDA excitotoxicity (Borsello et al., 2003).

A smaller version of JNKI-1 (RPKRPTTLNLPQVPRSQDT157-167) fused to TAT (TAT-TJJIP) has also been assessed for neuroprotection in cultured neurons exposed to glutamic acid excitotoxicity (Arthur et al., 2007). At 2 μM TAT-TJJIP was highly neuroprotective at 24 h post-insult, even in the presence of the mRNA transcription inhibitor actinomycin D. This latter observation suggests that TAT-TJJIP neuroprotection does not involve the inhibition of expression of pro-death proteins. In addition, TAT-TJJIP reduced neuronal cytosolic calcium levels following glutamic acid exposure, suggesting that the mechanism of neuroprotection may involve improved mitochondrial function and calcium storage capacity, rather than the direct blocking of intracellular influx.

The in vitro neuroprotective findings reported for JNKI-1-TATD are consistent with findings from our laboratory using this peptide in neuronal excitotoxicity models (Craig et al., 2011; Meade et al., 2010a, b).

### 2.2.2. In vivo studies

Studies using the JNKI-1-TATD peptide in different in vivo animal ischaemic injury models are presented in Table 3. As with the NR2B9c peptide, most animal studies using JNKI-1-TATD have yielded positive results. Studies in younger rats (P7–P12) have produced the best results in terms of neuroprotective efficacy and therapeutic time windows. The effective dose ranges appears to be between 0.076 to 760 nmol/kg via the IV route and 7.6 to 2800 nmol/kg when administered via the IP route. Studies yielding negative results have usually been ones that have used more severe models of ischaemic injury (permanent MCAO) or pre- or late-treatment peptide administration time-points.

### 2.2.3. Is the neuroprotective action of TAT-JNKI-1 mediated by TAT or the JNKI-1/TIJIP peptides?

We view it as most likely that the arginine and lysine residues in the JNKI-1 and TJIP peptides potentiate the neuroprotective actions of TAT. The TAT-JNKI-1 and TAT-TJIP peptides are highly cationic with a net charge of +11. In addition, while it has been shown that the TAT-JNKI-1 peptide does not block NMDA induced calcium influx (Centeno et al., 2007), our laboratory has shown that this peptide is capable of reducing neuronal calcium influx following glutamic acid exposure (Meloni et al., 2015). Other studies have shown that the TAT-TJIP peptide reduces neuronal intracellular calcium influx following glutamic acid exposure via a mechanism that is proposed to be associated with an increased capacity of mitochondria to store incoming calcium (Arthur et al., 2007). Given that the TAT fused JNKI-1/TJIP peptides have the ability to reduce neuronal excitotoxic intracellular calcium levels, it is hardly surprising that associated downstream pathological processes, such as activation of calpain, JNK and c-Jun have consistently been shown to be blocked by this peptide (Bessero et al., 2010; Borsello et al., 2003; Meade et al., 2010a). It has never been explained, however, how the TAT-JNKI-1 peptide has the capacity to block the activation of the calcium-sensitive protease, calpain following an excitotoxic insult (Meade et al., 2010a) if the peptide has no effect on neuronal calcium influx (Centeno et al., 2007).

As discussed above, the presence alanine residues are known to impede CPP endocytosis. Consequently, the lack of neuroprotection seen with a TAT fused mutant JNKI-1 peptide containing three alanine substitutions (RPKRPTAANAFQVPRSQD-TAT) in cultured neurons exposed...
to NMDA excitotoxicity may well be due to the reduced endocytic traits of this peptide. In this context, the studies by Vasin et al. (2009a, 2011) suggest that neuronal endocytic uptake of the TAT-JNKI-1 peptide is an essential prerequisite for neuroprotection, a view reinforced by studies in our laboratory (Meloni et al., 2015). However, as discussed elsewhere, our view is that the endocytic process is the key to the neuroprotective mechanism and that the interaction of the TAT-JNKI-1 peptide with its cytoplasmic target (JIP scaffold protein) is unlikely to play a major role due to poor endosomal peptide escape and/or endosomal/lysosomal peptide degradation.

2.3. CBD3 and TAT-CBD3 peptides

CBD3 is a 15 amino acid peptide (ARSKRAELRGVPRLG_498–512) derived from the calcium-channel-binding domain (CBD) of collapsing response mediator protein 2 (CRMP2). The direct binding interaction of CRMP2’s CBD with the first intracellular loop (L1) and the distal carboxyl terminus (Ct-dis) of the alpha-1 subunit protein CaV2.2, of the N-type voltage-gated calcium channel was the factor that led to the identification and isolation of the CBD peptide (Brittain et al., 2011b). The peptide was originally developed to suppress inflammatory and behavioral hypersensitivity associated with CaV2.2 activity.

Along with CRMP2’s other four family members (viz. CRMP1 and CRMP3–5), CRMP2 is predominantly expressed in the nervous system during development where its microtubule interacting functions play important roles in axonal formation and nerve terminal growth cone collapse (Charrier et al., 2003). CRMPs are also involved in regulating the function of ion channels and following brain trauma their degradation contributes to the neurodegenerative process (Zhang et al., 2007). There is also evidence that CRMP2 is involved in endocytosis, as siRNA mediated knockdown in neurons has been shown to inhibit endocytosis of the trans-membrane cell adhesion protein L1 (Nishimura et al., 2003).

It is thought that the CBD3 peptide competitively inhibits the interaction of CRMP2 with the intracellular domains of CaV2.2 and other ion channels (e.g. CaV2.3), as well as glutamate receptors (e.g. NMDA receptor), and calcium transporters (e.g. NCX3), in doing so altering their plasma membrane location and/or function and thereby their calcium influx properties. Consequently, the TAT-CBD3 peptide’s neuroprotective mechanism of action is commonly thought to be related to its ability to suppress the excitotoxic influx of calcium.

2.3.1. In vitro studies

TAT-CBD3 was first shown to reduce calcium currents by around 60% in cultured dorsal root ganglion (DRG) neurons via a process that was not blocked further by a CaV2.2 inhibitor (Brittain et al., 2011b). Other in vitro studies using DRG and cortical neurons, spinal cord slices and cortical slices showed that TAT-CBD3 also reduced several electrophysiological processes (e.g. sEPSC; spontaneous excitatory postsynaptic currents and CGRP release; calcitonin gene-related peptide) associated with CaV2.2 activation (Brittain et al., 2011a,b). Interestingly, in rat spinal cord slices following stimulation of the vanilloid receptor subtype 1 (VR1; also known as TRPV1) with capsaicin, TAT-CBD3 reduced CGRP release. This was not considered to be the result of direct inhibition of VR1 because of evidence that TAT-CBD3 has no effect on VR1 recordings in DRG neurons.

In a subsequent study, a modified TAT-CBD3 peptide with an alanine to lysine amino acid substitution (TAT-CBD3A6K) reduced T- and R-type voltage-dependent calcium currents in DRG neurons (Piekarz et al., 2012). Replacing the TAT peptide with poly-arginine-9 (R9) produced an even greater effect than for TAT-CBD and TAT-CBD3A6K in terms of inhibition of neuronal calcium influx and neuropathic pain (Feldman & Khanna, 2013; Ju et al., 2013).

In terms of neuroprotection, TAT-CBD3 has been demonstrated to reduce glutamate- and NMDA-induced calcium influx in cortical neurons as well as reducing cortical neuronal death following glutamate exposure (Brittain et al., 2011a). The peptide also induced the internalisation of the NMDA subunit protein NR2B in the dendritic spines (but not cell somas) of cortical neurons, and inhibited NMDA receptor currents in hippocampal neurons (Brittain et al., 2011a). siRNA down-regulation of CRMP2 in cortical neurons reduced the ability of TAT-CBD3 to inhibit NMDA induced calcium influx in cortical neurons, which the authors concluded supported the idea that TAT-CBD3 antagonises a function of CRMP2 (Brittain et al., 2011a). It is of interest therefore that in a latter study by this group (Brittain et al., 2012), it was shown that when the TAT-CBD3 peptide was applied intracellurally, it failed to block hippocampal neuronal NMDA receptor calcium currents.

A further study has also shown that TAT-CBD3 inhibits glutamate- and NMDA-induced calcium influx in cultured hippocampal neurons (Brustovetsky et al., 2014), as well as disrupting a CRMP2–NMDA receptor complex interaction, however it did not appear to induce internalisation of the NR2B protein. The same study demonstrated that TAT-CBD3 also inhibits NCX-mediated calcium influx, and that CRMP2 can interact with NCX3 but not with NCX1. Surprisingly, TAT-CBD3 strengthened the CRMP2–NCX3 interaction rather than inhibiting it and also induced NCX3 internalisation in hippocampal neurons. siRNA down-regulation of CRMP2 in hippocampal neurons blocked the ability of TAT-CBD3 to cause the NCX protein to be internalised.

In many experiments a TAT fused non-arginine containing scrambled peptide (TAT-scramble; TAT-WEAKEMYFEALVIE; net charge +5) with no amino acid content relationship to the CBD3 peptide was used as a negative control (Brittain et al., 2011a,b; Brustovetsky et al., 2014). In experiments using the TAT-scrambled peptide, negative results similar to the vehicle control were obtained.

2.3.2. In vivo studies

Studies utilising the TAT-CBD3 peptide in different animal models of injury/disease are presented in Table 4. The CBD3 peptide was identified as a potential therapeutic for suppressing inflammatory and neuropathic hypersensitivity associated with activation of the calcium channel CaV2.2 (Brittain et al., 2011b). Initial in vivo studies established that TAT-CBD3 reduces: i) capsaicin induced CGRP vasodilation in rat dura; ii) pain responses in rat hind-paw and eye following formalin and capsaicin exposure respectively; and iii) neuropathic pain associated with HIV reverse transcriptase therapy. As mentioned previously, the two variants (TAT-CBD3: A6K and R9–CBD3) of TAT-CBD3 displayed an increased ability to reduce neuropathic pain (Ju et al., 2013).

Following the initial in vivo studies, the neuroprotective properties of TAT-CBD3 peptide were assessed in traumatic brain injury and animal models of stroke. In a cortical impact mouse injury model, IP administration of TAT-CBD3 5 min after injury was shown to reduce hippocampal granular neuronal death (Brittain et al., 2011a). Similarly, in transient MCAO in the P10 rat, IP administration of TAT-CBD3 1-hour prior to occlusion significantly reduced infarct volume (Brittain et al., 2012). In a similar fashion, a 14 amino acid peptide derived from CRMP-2 (GVPRLYDGPCVEV_493–506; underlined sequence overlaps with CDB3 peptide) and fused to TAT (TAT-CRMP-2) when administered intra-cerebroventricularly 6 h prior to MCAO also reduced infarct volume (Bu et al., 2011).

2.2.3. Is the neuroprotective action of TAT-CBD3 mediated by TAT or the CBD3?

It is highly likely that the CBD3 peptide acts to potentiate the neuroprotective action of TAT. The CBD3 contains four arginine residues, resulting in a net charge for the TAT-CBD3 peptide of +11, as compared to +8 for TAT. In our opinion, difference in net charge should have the effect of increasing neuroprotective potency (Meloni et al., 2015). The importance of cationic charge and the presence of arginine residues in TAT-CBD3 peptide is further highlighted by evidence of increased efficacy with respect to inhibition of evoked calcium influx and pain suppression following substitution of an alanine by a lysine residue (TAT-CBD3-A6K: +12) and replacement of TAT with R9 (R9-CBD3: +12) (Feldman
& Khanna, 2013). The A6K modification results in increased binding affinity of CBD3: A6K Cav2.2, while replacement of TAT with R9 improves peptide cell transduction. Based on our findings for poly-arginine and arginine-rich peptide, both these modifications, especially the R9 substitution, would be expected to increase neuroprotective potency, most likely by enhancing the peptides’ endocytic properties. In this context, R9-CBD3 has been shown to be more potent than TAT-CBD3-A6K.

The control peptide used in most CBD3 studies has been a TAT-scrambled peptide (TAT-WEAKEMLYFEALVIE) that possesses no arginine residues and has a peptide charge of +5 due to the presence of several negatively charged glutamic acid residues. Due to its lower cationic charge and fewer number of arginine residues compared to TAT-CBD3, it is hardly surprising that this peptide was shown not to be neuroprotective or inhibitory to neuronal calcium influx. A more appropriate control would have been a scrambled CBD3 peptide fused to TAT, which we predict would possess neuroprotective properties due to its higher arginine content. However, in one study, a CBD3 peptide in reverse amino acid sequence fused to TAT peptide (TAT-LGRPVGERLEAKRSA) (which would alter CRMP2 peptide binding affinity) was used as a control in a DRG neuron depolarization-evoked calcium influx studies. While the more potent R9-CBD3 peptide at 10 μM reduced peak calcium influx by 50%, the reverse control peptide at the same concentration had no inhibitory effect. Based on the chemistry of the CBD3 reverse peptide, it is possible that higher concentrations may have had an inhibitory effect.

More remarkably, and in line with a non-specific endocytic-mediated down-regulation of cell surface structures, was the demonstration that TAT-CBD3 and TAT-CBD3: A6K peptides interfere with the function of N- (Cav2.2), T- (Cav3.1–3.3) and R-type (Cav2.3) calcium ion channels, the NMDA receptor and the NCX transporter. Moreover, there is evidence of TAT-CBD3 induced internalisation of the NMDA receptor subunit NR2B and NCX proteins (Brittain et al., 2011a; Brustovetsky et al., 2014). The finding of internalisation of NR2B by TAT-CBD3 was not confirmed in another study (Brustovetsky et al., 2014), possibly due to the fixation of cells (the initial study used live cells), a process which is likely to have altered the distribution of endosomal contents (Lundberg et al., 2003), including NR2B protein within the cell.

It should also be noted that siRNA down-regulation of CRMP2, which was used to confirm the specific action of the TAT-CBD3 peptide would be expected to suppress endocytosis (Nishimura et al., 2003). Consequently, in studies on CRMP2 down-regulation, the inability of TAT-CBD3 to induce NCX neuronal internalisation and attenuate NCX activity may well have been due to the suppression of CRMP2-associated endocytosis of the peptide rather than TAT-CBD3 not being able to interact with its intracellular target, CRMP2. Another anomalous finding was that TAT-CBD3 appeared to strengthen the CRMP2–NCX interaction rather than inhibit it (Brustovetsky et al., 2014), which is the opposite to what would be expected if the peptide was interfering with this protein-protein interaction.

2.4. mGluR1 and TAT-mGluR1 peptides

The 14 amino acid mGluR1 peptide (VIKPLTKSYQSGK928–942) is derived from the metabotropic glutamate receptor mGluR1. The peptide sequence is located at the intracellular carboxyl region of the mGluR1 protein and was initially selected based on its calpain cleavage site (Xu et al., 2007). Following glutamate-induced excitotoxicity, calpain cleavage of mGluR1 results in truncation of the protein at serine residue 936 (Xu et al., 2007). While truncation of mGluR1 does not inhibit the receptor’s ability to increase cytosolic calcium, it does inhibit receptor-mediated PI3K-Akt signalling, a process known to be associated with neuroprotection. In theory, blockage of calpain-mediated mGluR1 cleavage following excitotoxicity should enable neurons to better withstand excitotoxic insults due to neuroprotective signalling. Consequently, the TAT fused peptide TAT-mGluR1 was developed as a cell penetrable peptide to competitively inhibit calpain cleavage of the mGluR1 receptor.

2.4.1. In vitro and in vivo studies

In cultured cortical neurons exposed to NMDA excitotoxicity the TAT-mGluR1 peptide reduced cell death in a dose-dependent manner (Xu et al., 2007). The peptide was also neuroprotective in a hippocampal slice exposed to OGD. In other models (mouse kainic acid excitotoxicity and rat perinatal hypoxia–ischaemia; Table 5) pre-treatment with TAT-mGluR1 reduced mGluR1 truncation and neuronal degeneration.

Interestingly, the developers of TAT-mGluR1 were one of the first groups to recognise the intrinsic properties of the TAT peptide itself and concluded that TAT and mGluR1 are likely to act synergistically via independent mechanisms (Xu et al., 2008). These authors postulated that the TAT peptide exerted its neuroprotective effects by altering membrane proteins such as the NMDA receptor and affecting their function and/or structure.

2.4.2. Is the neuroprotective action of TAT-mGluR1 mediated by TAT or the mGluR1?

Xu et al. (2008), who first developed the TAT-mGluR1 peptide have suggested that the TAT and mGluR1 peptides are likely to act synergistically to produce a neuroprotective effect. We believe that this view is partly correct in that the mGluR1 peptide acts to potentiate the neuroprotective action of TAT, and that it is less likely the mGluR1 peptide is interacting with its cytoplasmic target. The two lysine residues in the mGluR1 peptide increase TAT/TAT-mGluR1 peptide net charge from +8 to +11, thereby increasing the peptide’s endocytic properties. The increase in peptide charge and the potentially increased stability due to increased length would more than likely result in greater neuroprotective potency, independently of any intracelular action of the mGluR1 peptide.

2.5. NR2Bct and TAT-NR2Bct peptides

NR2Bct is a 14 amino acid peptide (KKNRNKLRRQHSY1292–1306) derived from the NMDA receptor NR2B subunit. The peptide sequence is located at the intracellular carboxyl region of NR2B protein and was initially selected based on its binding affinity for the death–associated protein kinase 1 protein (DAPK1) (Tu et al., 2010). DAPK1 is a calcium–calmodulin regulated protein activated in neurons following NMDA receptor over-stimulation as occurs in ischaemia mediated excitotoxicity. The DAPK1 protein has been shown to interact with extra-synaptic NMDA receptors containing the NR2B subunit, a process that is thought to induce and/or exacerbate injurious calcium influx (Tu et al., 2010). Thus, activated DAPK1 is associated with other signalling pathways linked to ischaemia cell death (Pei et al., 2014; Wang et al., 2014). TAT-NR2Bct competitively inhibits activated DAPK1 binding to the NR2B subunit protein and thereby blocks subsequent downstream damaging cellular events caused by NMDA receptor over-activation.

2.5.1. In vitro and in vivo studies

In vitro TAT-NR2Bct is reported to block cortical neuron calcium influx following NMDA stimulation and following OGD exposure, whereas in vivo it reduces infarct volume following transient MCAO in the mouse (Tu et al., 2010; Table 5). By contrast, a TAT scrambled control peptide (TAT-NR2Bcts; TAT-NRRRNSLQLHKKY) does block NMDA or OGD induced calcium influx and is equally ineffective when administered before MCAO.

The TAT-NR2Bct peptide as well as a modified version containing an additional peptide motif (CTK: KFERQKILDQ) (NR2Bct-CTM) that directs the peptide for lysosomal degradation were also shown to reduce neuronal injury following transient MCAO in the rat with TAT-NR2Bct-CTM being more effective than TAT-NR2Bct (Fan et al., 2014; Table 5). The rationale for incorporating the CTK motif was to promote...
## Table 5

Studies using peptides fused to TAT (or TAT & R9 alone) in cerebral ischaemia/stroke and other CNS injury models.

<table>
<thead>
<tr>
<th>Peptide name &amp; sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Proposed target&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Injury model&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Route &amp; treatment schedule&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Dose</th>
<th>Neuroprotection&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Study</th>
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<tbody>
<tr>
<td>TAT-NR2R&lt;sub&gt;2&lt;/sub&gt;</td>
<td>DAPK1 Mouse: tMCAO; 60 min</td>
<td>IV: 1 h before or 1 h after MCAO</td>
<td>3060 nmol/kg</td>
<td>Yes, No scrambled control</td>
<td>Tu et al., 2010</td>
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<td></td>
<td>Control: NRRNSKLIQHHKY</td>
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<tr>
<td>TAT-NR2Bct-CTM</td>
<td>DAPK1 Rat (SD): tMCAO; 60 min</td>
<td>IV: 1 h after reperfusion</td>
<td>2010 nmol/kg</td>
<td>Yes</td>
<td>Fan et al., 2014</td>
<td></td>
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<tr>
<td></td>
<td>Control: NRRNSKLIQHHKY</td>
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<tr>
<td>TAT-p53DM</td>
<td>DAPK1 Mouse: tMCAO; 60 min</td>
<td>IV: 2 h after reperfusion</td>
<td>360 nmol/kg, 360, 720, 1440 or 1800 nmol/kg</td>
<td>No, Control Wang et al., 2014</td>
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<tr>
<td>RVCACPGRDRT Control: CCPGECVRTRRR</td>
<td>IV: 5 h after reperfusion</td>
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<tr>
<td>AIP (no CPP)</td>
<td>CalMK1 Mouse: tMCAO; 60 min</td>
<td>Intravitreal injection: at the NMDA exposure</td>
<td>3000 ng (2 nmol)</td>
<td>Yes Goebel, 2009</td>
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<td></td>
<td></td>
<td>IV: at time of reperfusion</td>
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<tr>
<td>TAT-CN21</td>
<td>CalMK1 Mouse: NM4A retinal injections</td>
<td>IV: 1 h after hypoxia</td>
<td>49220 nmol/kg</td>
<td>Yes Zhou et al., 2009</td>
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<td></td>
<td></td>
<td>IV: 2 h after hypoxia</td>
<td>16406 nmol/kg</td>
<td>Yes Xu et al., 2007</td>
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<tr>
<td>PYC6-TAT</td>
<td>AP-1 Mouse: tMCAO; 90 min</td>
<td>IV: 10 min after MCAO</td>
<td>7.6, 76 and 255 nmol/kg</td>
<td>No Gow et al., 2011</td>
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<td>TAT-PYC6-HP</td>
<td>AP-1 Rat (SD): pMCAO</td>
<td>IV: 1 h after MCAO</td>
<td>500, 1000 or 1500 nmol/kg</td>
<td>No Si et al., 2012</td>
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<td></td>
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<td>IV: 2 h after MCAO</td>
<td>250 nmol/kg</td>
<td>No Si et al., 2012</td>
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<tr>
<td>TAT-TN2</td>
<td>GluR5R CalMK1</td>
<td>IV: at time of reperfusion</td>
<td>250 nmol/kg</td>
<td>Yes Vest et al., 2010</td>
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<tr>
<td>TAT-CN21</td>
<td>N/A CalMK1</td>
<td>IV: 1 h after hypoxia</td>
<td>15.7 or 100 ng, 71.6, 716 nmol/kg</td>
<td>No Vaslin et al., 2009b</td>
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<td></td>
<td></td>
<td>IV: 2 h after MCAO</td>
<td>250 nmol/kg</td>
<td>No Vaslin et al., 2009b</td>
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<tr>
<td>D-TAT-GE5V</td>
<td>NOS1AP Mouse: tMCAO; 90 min</td>
<td>IV: 30 min or 4.5 h after reperfusion</td>
<td>3125 nmol/kg</td>
<td>No Control Zhang et al., 2013</td>
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<tr>
<td>TAT-NBD</td>
<td>SREBP-1 Rat (SD): pMCAO</td>
<td>IV: 45 min before MCAO or 30 min after reperfusion</td>
<td>2885 nmol/kg</td>
<td>Yes, No control Taghibiglou et al., 2009</td>
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<tr>
<td>TAT-sAβ-lan</td>
<td>CaMKII Mouse: tMCAO; 60 min</td>
<td>IV: at time of reperfusion</td>
<td>250 nmol/kg</td>
<td>Yes Vest et al., 2010</td>
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<tr>
<td>TAT-Indip</td>
<td>N/A Rat (SD): P12: pMCAO</td>
<td>IV: just after MCAO</td>
<td>15.7 or 100 ng, 71.6, 716 nmol/kg</td>
<td>No Vaslin et al., 2009b</td>
<td></td>
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<td></td>
<td></td>
<td>IV: 6 h after MCAO</td>
<td>100,000 ng</td>
<td>No Vaslin et al., 2009b</td>
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<tr>
<td></td>
<td></td>
<td>IV: 2 h after MCAO</td>
<td>250 nmol/kg</td>
<td>No Vaslin et al., 2009b</td>
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<tr>
<td>TAT-TN2</td>
<td>NOS1AP Mouse: tMCAO; 90 min</td>
<td>IV: at time of reperfusion</td>
<td>100 ng</td>
<td>No, Yes No control Li et al., 2013</td>
<td></td>
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<tr>
<td>TAT-NBD</td>
<td>NOS1AP Mouse: tMCAO; 90 min</td>
<td>IV: at time of reperfusion</td>
<td>100 ng</td>
<td>No, Yes No control Li et al., 2013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAT-CN21</td>
<td>NOS1AP Mouse: tMCAO; 90 min</td>
<td>IV: at time of reperfusion</td>
<td>100 ng</td>
<td>No, Yes No control Li et al., 2013</td>
<td></td>
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<tr>
<td></td>
<td>Control: GPEITSEVENCS</td>
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<tr>
<td>TAT-CN21</td>
<td>NOS1AP Mouse: tMCAO; 90 min</td>
<td>IV: at time of reperfusion</td>
<td>100 ng</td>
<td>No, Yes No control Li et al., 2013</td>
<td></td>
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<tr>
<td>TAT-sAβ-lan</td>
<td>NOS1AP Mouse: tMCAO; 90 min</td>
<td>IV: at time of reperfusion</td>
<td>100 ng</td>
<td>No, Yes No control Li et al., 2013</td>
<td></td>
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</tr>
<tr>
<td>TAT-NBD</td>
<td>NOS1AP Mouse: tMCAO; 90 min</td>
<td>IV: at time of reperfusion</td>
<td>100 ng</td>
<td>No, Yes No control Li et al., 2013</td>
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<tr>
<td>TAT-CN21</td>
<td>NOS1AP Mouse: tMCAO; 90 min</td>
<td>IV: at time of reperfusion</td>
<td>100 ng</td>
<td>No, Yes No control Li et al., 2013</td>
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<tr>
<td>TAT-CN21</td>
<td>NOS1AP Mouse: tMCAO; 90 min</td>
<td>IV: at time of reperfusion</td>
<td>100 ng</td>
<td>No, Yes No control Li et al., 2013</td>
<td></td>
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</tbody>
</table>

**Bold data indicate peptides used in studies.**

<sup>a</sup> Peptides synthesised using d-amino acids are represented in lowercase, TATD = rrrqrkkg, TAT = y4gkrkkqrr.  
<sup>b</sup> DAPK1: death-associated protein kinase 1 protein, CaMII: calcium/calmodulin-dependent protein kinase II, AP-1: activator protein 1, GluR6-9c: glutamate receptor 6, 9c: metabotropic glutamate receptor 1α, PTEN: Phosphatase and tensin homolog, SREBP-1: Sterol Regulatory Element-Binding Protein-1, NG1AP: nitric oxide synthase 1 adaptor protein, NP-kB: nuclear factor kappa-light-chain-enhancer of activated B cells, JNK: c-Jun N-terminal kinase, PKCs: Protein kinase C, iPKC: protein kinase C δ, PTPro: Protein tyrosine phosphatases α.  
<sup>d</sup> ICV: intracerebroventricularly; IP: intraperitoneally; IV: intravenously.  
<sup>e</sup> Neuroprotection: reduced infarct volume, tissue injury or neuronal cell death.
degradation of NR2Bct-CTM:DAPK1 complexes and thereby improve the peptide’s ability to block the action of DAPK1.

2.5.2. Is the neuroprotective action of TAT-NR2Bct and TAT-NR2Bct-CTM mediated by TAT or NR2Bct and NR2Bct-CTM?

TAT-NR2Bct has been shown to reduce neuronal calcium influx following excitotoxic insults (NMDA and OGD) suggesting that it is via this mechanism that it exerts its neuroprotective action. This raises the question of whether the reduction of calcium influx is due to the interaction of TAT-NR2Bct with the intracellular domain of the NMDA receptor subunit NR2B or is mediated via internalisation of calcium channels. In this context, OGD is known to activate neuronal calcium influx pathways other than via NMDA receptors (e.g. VGCC and reverse NOX activity) and it is difficult to reconcile this with the fact that TAT-NR2Bct is so effective at blocking net calcium influx in neurons exposed to OGD. Alternatively, if TAT-NR2Bct only acts by blocking the secondary injurious calcium influx following the interaction of activated DAPK1 with the NMDA receptor, the kinase should in theory still be available to stimulate other non-NMDA receptor associated cell death pathways resulting in neuronal degeneration.

Based on our hypothesis, the high arginine content and positive charge (+14.1) of the TAT-NR2Bct would predict that the peptide is neuroprotective and has neuronal calcium influx inhibiting properties. Consequently, it is surprising that it is reported (Tu et al., 2010) that the TAT fused scrambled NR2Bct peptide control (TAT-NR2Bcts) does not display any neuroprotective or calcium influx inhibitory properties despite having a similar arginine content and charge to the TAT-NR2Bct peptide. This anomaly led us to re-assess the effectiveness of the scrambled TAT-NR2Bcts peptide in cultured cortical neurons exposed to glutamate excitotoxicity, and as predicted our data showed that in our hands the peptide is neuroprotective (Fig. 4).

2.6. GluR6 and TAT-GluR6 peptides

The 12 amino acid GluR6 peptide (RLPGKETMA908) is derived from the carboxy terminal of kainic acid receptor GluR6 subunit, which binds to the PDZ1 domain of the PSD-95 protein (Pei et al., 2006; Table 5). As a result, the peptide has the capacity to inhibit GluR6 receptor-PSD-95 signalling, which following over-receptor stimulation can result in activation of JNK and other cell death associated pathways.

2.6.1. In vitro studies

Electrophysiological studies have shown that TAT-GluR6 (0.05 μM) has no inhibitory effects on kainic acid receptor function and is neuroprotective in vitro, significantly reducing hippocampal neuronal death following kainic acid exposure (Pei et al., 2006). By contrast, a control peptide in which the carboxy ETMA peptide motif is replaced by the amino acids AADD (RLPGKAADD; TAT-GluR6AA) is not neuroprotective.

2.6.2. In vivo studies

Treatment with TAT-GluR6 but not the control TAT-GluR6AA peptide reduced MLK3/PSD-95 assembly, signalling events associated with JNK activation (JNK and c-jun phosphorylation, Fas ligand expression) and CA1 hippocampal injury in rats in rats following intracerebroventricular administration of the peptides 40 min before the onset of global cerebral ischaemia (Pei et al., 2006; Table 5).

2.6.3. Is the neuroprotective action of TAT-GluR6 mediated by TAT or GluR6?

The TAT-GluR6 peptide has a charge of +9, while the TAT-GluR6AA peptide has a charge of +8, which is the same as the TAT peptide. Consequently, it is predicted that an additional charge should increase the neuroprotective potency of TAT-GluR6 peptide. However, the fact that the TAT-GluR6AA control peptide is not neuroprotective despite having the same charge as TAT is problematic, but may be explained by the presence of two introduced alanine residues, which may well have a negative impact on the peptide’s neuroprotective properties by impeding peptide endocytosis (Yang et al., 2014).

2.7. p53DM and TAT-p53DM peptides

p53DM is a 12 amino acid peptide (RVCACPGRDRRT271-282) derived from the p53 tumour suppressor protein (Wang et al., 2014). The peptide sequence is located within a p53 DNA-binding motif (DM) that also binds activated DAPK1, which can subsequently phosphorylate p53 at serine-23. Thus, the p53DM peptide acts to inhibit the DAPK1-p53 binding interaction and p53 phosphorylation, with phosphorylated p53 being known to induce the expression of pro-apoptotic proteins (e.g. Bax) and cell death.

2.7.1. In vitro and in vivo studies

Treatment of cortical neurons with a TAT-fused p53DM peptide (TAT-p53DM) has been shown to reduce the number of terminal deoxynucleotidyl transferase dUTP nick end labelled and propidium iodide positive neurons following OGD compared to a scrambled control peptide (TAT-s-p53DM; TAT-CCPGECVRTRRR) (Pei et al., 2014). However, in experiments involving OGD exposure, a vehicle control was not used. Treatment of mice with TAT-p53DM (but not the TAT-fused scrambled control peptide, TAT-s-p53DM) 3 or 6 h after the commencement of transient MCAO (60 min) similarly reduces brain injury and improves functional outcomes (Wang et al., 2014; Table 5).

2.7.2. Is the neuroprotective action of TAT-p53DM mediated by TAT or p53DM?

Both TAT-p53DM and TAT-s-p53DM are arginine-rich and have a net charge of +10.9 and therefore would be predicted to both have neuroprotective properties. Studies in our laboratory have confirmed that TAT-s-p53DM is neuroprotective in vitro in cortical neurons exposed...
to glutamic acid excitotoxicity (Fig. 4). TAT-s-p53DM peptide was however, slightly less effective than the TAT-p53DM peptide. One possible reason for the differential neuroprotective effects of the two peptides is that in s-p53DM, all arginine residues are located at the C-terminus of the peptide away from the TAT arginine (and lysine) residues. In contrast, p53DM has one of its arginine residues at the N-terminus adjacent to the TAT peptide and therefore may have a greater capacity to enhance the neuroprotective efficacy of TAT.

2.8. CN21, AIP, TAT-CN21 and TAT-AIP peptides

CN21 is a 21 amino acid peptide (KRPPKLGQCGRKRVVIEDRA43_63) derived from the calcium/calmodulin-dependent protein kinase II inhibitor (CaM-KII) protein. As its name suggests, CaM-KII is an inhibitor of kinase calcium/calmodulin-dependent protein kinase II (CaMKII). The CN21 is also a specific inhibitor of CaMKII (Vest et al., 2007). AIP (autocamtide-2-related inhibitory peptide) is a 13 amino acid peptide (RKKLRRQEAADAL) derived from but not homologous to the auto-regulatory domain of CaMKII, that can inhibit multiple members of the CaMKII-kinase family (Ishida et al., 1995; Smith et al., 1990).

The CaMKII protein is a mediator of many calcium associated signalling pathways. In the brain, CaMKII is involved in physiological glutamate receptor signalling but following receptor over-stimulation, the kinase is activated and autophosphorylated, processes believed to be involved in cell death pathways (Liu et al., 2012).

2.8.1. In vitro and in vivo studies

The TAT fused CN21 peptide (TAT-CN21) was shown to be neuroprotective in vitro following both glutamic acid and NMDA excitotoxicity in hippocampal and cortical neuronal cultures (Vest et al., 2010). By contrast, a TAT-fused CN21 reverse sequence (RRDEIVVRKSRGICLKPKPR) and a scrambled CN21 peptide (VKEPRIDKPKRLGQKSQDR) at 5 μM were shown to be equally ineffective as neuroprotective agents following glutamate excitotoxicity. In another study, both TAT-CN21 and TAT-AIP were established to be neuroprotective in cultured cortical neurons following glutamic acid excitotoxicity (Ashpole & Hudmon, 2011), with the TAT-AIP peptide having slightly greater potency than TATCN21. In the same study, TAT peptide (10 μM) was shown to be neuroprotective, while a control mutated TAT-CN21 peptide (TAT-CN21Aa; with seven alanine amino acid and one tryptophan substitutions: KAPAKAAWAAASKRVVIEDDR) had significantly lower neuroprotective properties. TAT-CN21 has also been shown to reduce infarct volume in a transient MCAO (60 min) mouse stroke model when administered after reperfusion (Vest et al., 2010; Table 5).

2.8.2. Is the neuroprotective action of TAT-CN21 and TAT-AIP mediated by TAT or CN21/AIP?

TAT-CN21 and TAT-AIP have net peptide charges of +12 and +11, respectively compared to +8 for the TAT peptide. Consequently, based on our hypothesis, it would be predicted that when fused to TAT, the additional cationic charge provided by CN21 and AIP should increase the neuroprotective potency of TAT. As discussed, it appears that TAT-AIP is slightly more neuroprotective than TAT-CN21. This may be due to the fact that all the positively charged arginine and lysine residues in AIP are located at its N-terminal region adjacent to the TAT peptide, whereas CN21 has a more even distribution of these residues across its structure. It is also hardly surprising that the TAT-CN21Aa control peptide displayed significantly lower neuroprotective properties compared to TAT-CN21 given its high alanine content and reduced charge (+9), factors of which we would be expected to lead to reduced peptidocytic efficiency (Yang et al., 2014).

2.9. Indip and TAT-Indip peptides

Indip is a 10 amino acid peptide (GEPHKFREW152_161) derived from insulin-induced gene 1 (Iinsig-1) protein (Taghibiglou et al., 2009). One of the roles of Insig-1 is to regulate the intracellular trafficking of sterol regulatory element binding protein-1 (SREBP-1). In non-stimulated neurons, SREBP-1 forms a complex with SREBP cleavage-activating protein (SCAP), which is retained in the endoplasmic reticulum (ER) due to the interaction of SCAP with the ER membrane protein Insig-1. Following NMDA receptor activation, Insig-1 lysine residues 156 and 158 are ubiquitinated and the protein degraded by the proteosome, thereby releasing SCAP from the ER membrane and allowing the protein to chaperone SREBP-1 to the Golgi apparatus. Within the Golgi apparatus, SREBP-1 is proteolytically cleaved to its transcriptional active N-terminal form (nt-SREBP-1), after which it translocates to the nucleus to stimulate the expression of genes containing sterol regulatory elements. The exact mechanisms associated with activated SREBP-1 and its role in neuronal injury are not known, however blocking Insig-1 degradation with the TAT-Indip peptide has been shown to be neuroprotective in NMDA induced excitotoxicity and OGD in cortical cultures (Taghibiglou et al., 2009). The Indip peptide was initially designed to competitively block ubiquitination of Insig-1 lysines residues 156 and 158, and thereby inhibit its degradation allowing maintenance of SREBP-1's anchorage to the ER membrane.

2.9.1. In vitro studies

The TAT fused Indip peptide (TAT-Indip) is reported to be neuroprotective (2 μM) in vitro in cultured neurons in both NMDA excitotoxicity and OGD (Taghibiglou et al., 2009). By contrast, a TAT fused mutated Indip peptide (2 μM; TAT-GEPRPREW; TAT-IndipK−R), in which lysine residues 156 and 158 are replaced with arginine residues, was shown to be ineffective.

2.9.2. In vivo studies

Treatment of rats with the TAT-Indip peptide 45 min before MCAO (90 min) or 30 min after reperfusion significantly reduces infarct volume (Taghibiglou et al., 2009; Table 5). The control peptide TAT-IndipK−R administered 30 min after reperfusion was shown to be ineffective resulting in infarct volumes equivalent to those in vehicle treated rats.

2.9.3. Is the neuroprotective action of TAT-Indip mediated by TAT or Indip?

Both the TAT-Indip and TAT-IndipK−R peptides have a net peptide charge +9.1 compared to +8 for the TAT peptide, which based on our hypothesis, leads us to predict that the TAT-IndipK−R due to its higher arginine content should be more neuroprotective than TAT-Indip. The fact that the opposite was observed is surprising. This led us to re-examine the potency of the two peptides in cortical neurons exposed to glutamate excitotoxicity. In our hands, both peptides are neuroprotective with the TAT-IndipK−R peptide appearing to be more potent than the TAT-Indip peptide as we predicted (Fig. 4).

2.10. Src, Src40–49-TAT and TAT-Src40–58 peptides

Src40–49 is a 10 amino acid peptide (KPASADGHRG39_48) equivalent to the mouse sequence peptide region 39–48) and Src40–58, a 19 amino acid peptide (PASADGHRGSPAAVFPPAA40_58), derivated from the protein kinase Src protein. It has been reported that the Src peptides can bind to the NADH dehydrogenase subunit 2 (ND2.1) protein (Liu et al., 2008). The Src protein can use ND2.1 as well as PSD-95 as adaptor proteins to indirectly bind to the NR2B NMDA receptor subunit. Intracellular signaling events following tissue injury and inflammation can trigger the Src protein to bind to and phosphorylate the NR2B subunit (at tyrosine residue 1472). This process can inhibit NMDA receptor endocytosis, and thus promote increased surface expression of the NMDA receptor (Zhang et al., 2008). Increased NMDA receptor expression/activity can be associated with chronic pain, pain hypersensitivity and emotional memory. The rationale for designing the Src40–49 peptide was to block the Src-ND2 protein interaction and thereby inhibit NMDA receptor phosphorylation and hyperactivity without disrupting normal memory. The rationale for designing the Src40–49 peptide was to block the Src-ND2 protein interaction and thereby inhibit NMDA receptor phosphorylation and hyperactivity without disrupting normal memory.
receptor function (Liu et al., 2008). The TAT-Src40–58 peptide was designed to block the interaction of Src with the PSD-95/NR2B protein complex and thereby reduce NMDA receptor surface expression.

2.10.1. In vitro studies
Studies using the longer TAT-Src40–58 peptide (TAT-PASADGHRG PSAAFPVPPAA) were performed in mouse amygdala neuronal cultures and brain slices (Sinai et al., 2010). Since it had been previously demonstrated that inhibiting phosphorylation of tyrosine (Y1472) on the NMDA receptor subunit NR2B causes receptor endocytosis (Zhang et al., 2008), it was predicted that adding TAT-Src40–58 to the neuronal cultures would decrease NR2B surface expression. As predicted, treatment of the neuronal cultures with peptide (20 μM) resulted in a >50% reduction in receptor protein surface expression in dendritic processes compared to controls (Sinai et al., 2010). Similarly, treating brain slices with the peptide reduced amygdalar long-term potentiation in the lateral nuclei of the basolateral complex. Controls consisting of TAT only or scrambled TAT-Srr40–58 peptides were not used in the experiments.

2.10.2. In vivo studies
IV administration of Src40–49-TAT (KPASADGHRG-TAT) 45 min before hind paw formalin injection, or intrathecal administration at the lumbar level 30 min before hindpaw injection reduced phase 2 NMDA receptor-dependent flinching (Li et al., 2008; Table 5). By contrast, a scrambled sSrc40–49 peptide (GAAKPSGDIH-TAT; sSrc40–49-TAT) and the TAT peptide alone did not have any effect. Moreover, IV and/or intrathecally administered Srrc40–49–TAT, but not vehicle or sSrc40–49–TAT, also reduced inflammatory pain and pain behaviour in a model of peripheral nerve injury (Liu et al., 2008). Coincidentally, Src40–49–TAT has also been shown to reduce Src binding to ND2.1 and NR2B phosphorylation in rat brain and spinal cord lysates, following IV or intrathecal peptide treatment in normal rats or rats subjected to inflammatory and neuropathic pain (Liu et al., 2008).

In another study, intravenous injection of the longer TAT-Src40–58 peptide (but not vehicle control or scrambled TAT-Src40–58) reduced NR2B tyrosine phosphorylation in the mouse amygdala compared to vehicle control and scrambled TAT-Src40–58 treated rats (Sinai et al., 2010; Table 5). IV administered TAT-Src40–58 also impaired amygdala-dependent cued fear conditioning and non-associative social recognition tasks in mice. It was concluded that both responses were linked to TAT-Src40–58 mediated reduced NMDA receptor surface expression.

2.10.3. Are the neurological effects induced by Src40–49TAT and TAT-Src40–58 mediated by TAT?
The Src40–49TAT and TAT-Src40–58 peptides are intriguing as their active components (Src40–49 and Src40–59 peptides) are predicted to cause NR2B/NMDA receptor endocytosis and reduced surface expression. Similarly, we have proposed that TAT-mediated peptide cellular uptake may result in NMDA receptor internalisation and reduced surface expression. There are several pieces of evidence suggesting that the latter mechanism is occurring. In both studies described above, the authors were unable to adequately reconcile why their peptides were so effective at reducing Src kinase NMDA receptor mediated responses when the closely related Src family member Fyn is another kinase capable of phosphorylating the NR2B subunit at tyrosine 1472, including when associated with neuropathic pain (Abe et al., 2005; Nakazawa et al., 2001). That is, Fyn kinase activity, which is not blocked by the Src40–49TAT and TAT-Src40–58 peptides, would be capable of phosphorylating NR2B and inhibit endocytosis of the NMDA receptor, and allow pain to occur. However, this was not the case and thus it is more likely that a TAT-mediated peptide mechanism was responsible for NMDA receptor endocytosis and reduced pain.

Moreover, it has been shown that Src, in addition to binding the ND2 protein can bind the PSD-95 protein to facilitate phosphorylation of tyrosine NR2B, a process that would not be blocked by the Src40–49-TAT and TAT-Src40–58 peptides. Given this, we argue that the reduced NMDA receptor activated processes reported above are the result of the TAT peptide’s endocytic properties. These endocytic effects may have been enhanced by the actions of the Src peptide to increase TAT’s stability or endocytic uptake independent of the cargo-peptide’s potential indirect cytoplasmic inducing endocytic effects.

3. Examination neuroprotective arginine- (and lysine-) rich peptides used in neuronal injury models
It is beyond the scope of this review to include every study that has used a neuroprotective TAT-fused peptide. For completeness, other in vivo studies using TAT-fused peptides are listed in Table 5. Evidently, several studies have identified arginine- and lysine-rich peptides not fused to TAT or to a CPP as being neuroprotective. We argue that it is possible that these peptides are intrinsically neuroprotective via mechanisms unrelated to their proposed action on a specific cell surface receptor or intracellular target. Like TAT, the neuroprotective effects of poly-arginine and arginine-rich peptides are likely to be related to their endocytosis-inducing properties. To pursue this idea further, here we review in detail the properties of two arginine- and lysine-rich peptides that meet these criteria, apolipoprotein E (APOE) derived peptides and an amyloid precursor protein (APP) derived peptide.

Animal studies that have used these peptides in CNS injury models are presented in Table 6.

3.1. APOE peptides — APOE141–149, COG133 and COG1410
The APOE141–1491 and COG133 peptides are derived form the APOE protein (LRKLRKRL141–149 and LRVRASHLRKRL133–149) and represent a region within the protein’s receptor binding domain. Interestingly, an early study using a dimer of the APOE141–1491 peptide showed that the peptide was capable of inducing significant increases in intracellular calcium influx in cultured neurons, especially at concentrations of ≥5 μM (Wang & Gruenstein, 1997). By contrast, the APOE141–1491 monomer peptide was less effective at inducing intracellular calcium influx even at high concentrations (100 μM). The mechanism associated with the COG1410 peptide induced calcium influx was proposed to be linked to peptide binding to the low-density lipoprotein receptor (Wang & Gruenstein, 1997).

A subsequent study showed that the COG133 peptide reduced cell death and calcium influx in mixed cortical–glial cultures following NMDA excitotoxicity (Aono et al., 2003). Neuroprotection was seen when the COG133 peptide was added to cortical cultures prior to or concurrently with NMDA but not a scrambled peptide of the identical size and amino acid composition. It is of interest that poly-arginine and arginine-rich peptides behave in a similar fashion to COG133 with respect to calcium influx and neuroprotection following glutamic acid excitotoxicity (Meloni et al., 2015).

In other studies it has been shown that intravascular administration of the COG133 peptide reduced the systemic and brain inflammatory responses induced by cytokines (e.g. TNF-α) and led to improved outcomes after closed head injury in the mouse (Lynch et al., 2003, 2005). Similarly, intraperitoneal administration of the COG133 peptide reduced disability and inflammatory infiltrates in to the spinal cord of a mouse model of multiple sclerosis (Li et al., 2006). A slightly shorter and modified COG133 peptide (COG1410: acetyl-AS-Alb-LRKL-AlbKRL-amide; Alb = 2-Aminoisobutyric acid) with superior anti-inflammatory properties has also been developed, and has shown to reduce vasospasm and/or lead to improved outcome in subarachnoid and intracerebral haemorrhage in the mouse (Gao et al., 2006; Laskowitz et al., 2012). In addition, COG1410 peptide improves functional and/or histological outcomes in different traumatic brain injury models (Hoane et al., 2007; Hoane et al., 2009; Jiang & Brody, 2012; Kaufman et al., 2010; Laskowitz et al., 2007), in transient MCAO (Tukhovskaya...
Table 6
Studies using arginine-rich peptides in CNS injury models.

<table>
<thead>
<tr>
<th>Peptide &amp; sequencea</th>
<th>Injury modelb</th>
<th>Route &amp; treatment schedulec</th>
<th>Dose</th>
<th>Neuroprotectiond</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>APOE receptor binding domain</td>
<td>Mouse: Inflammation; IV LPS</td>
<td>IV: At time of LPS</td>
<td>2765 nmol/kg</td>
<td>Reduced serum &amp; brain inflammatory markers (TNF, IL-6); COG133</td>
<td>Lynch et al., 2003</td>
</tr>
<tr>
<td>LRVRLASHLRKLRLL</td>
<td>Rat (W): P7; CCAO/2 h hypoxia</td>
<td>IP: Before hypoxia</td>
<td>100 to 5000 ng</td>
<td>Yes; COG133</td>
<td>McAdoo et al., 2005</td>
</tr>
<tr>
<td>COG112: Penetratin-COG133</td>
<td>Mouse: MS; EAE</td>
<td>IV: Every other day starting on day 2 &amp; ending 30 days post-immunisation</td>
<td>COG133: 469 nmol/kg</td>
<td>Yes, reduced symptoms; COG112 &gt; COG133</td>
<td>Lynch et al., 2005</td>
</tr>
<tr>
<td>COG112: Penetratin-COG133</td>
<td>Drosophila (APP transgenic); Alzheimer’s disease</td>
<td>Injected in abdomen; days 2, 5, 9, 13, 17, 21, 25 &amp; 29</td>
<td>0.2 μl: concentration not provided</td>
<td>Yes, COG133 &amp; COG112 reduced neurodegeneration</td>
<td>Sarantseva et al., 2009</td>
</tr>
<tr>
<td>COG1410</td>
<td>Mouse: TBI; contusion/diffuse</td>
<td>IV: 60 min post-TBI</td>
<td>461, 1843 or 3687 nmol/kg</td>
<td>No; COG133</td>
<td>Laskowitz et al., 2007</td>
</tr>
<tr>
<td>ASAibLRKLAibKRLL</td>
<td>Mouse: SAH</td>
<td>IV: Immediately after SAH, &amp; at 12 h intervals for 3 days</td>
<td>425 or 850 nmol/kg</td>
<td>Yes, reduced vasospasm, improved survival &amp; behaviour</td>
<td>Gao et al., 2006</td>
</tr>
<tr>
<td>COG1410</td>
<td>Mouse: TBI; contusion/diffuse</td>
<td>IV: 30 min post-TBI</td>
<td>285 or 570 nmol/kg</td>
<td>Yes, 570 nmol/kg. No 285 nmol/kg</td>
<td>Hoane et al., 2007</td>
</tr>
<tr>
<td>Mouse: TBI; contusion/diffuse</td>
<td>IV: 120 min post-TBI</td>
<td>213 or 426 nmol/kg</td>
<td>Yes, no control peptide (507 nmol/kg)</td>
<td>Laskowitz et al., 2007</td>
<td></td>
</tr>
<tr>
<td>COG1410</td>
<td>Mouse: TBI; contusion/diffuse</td>
<td>IV: 30 min &amp; 24 h post-TBI</td>
<td>570 nmol/kg</td>
<td>Yes, histologically &amp; functionally</td>
<td>Hoane et al., 2009</td>
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<tr>
<td>COG1410</td>
<td>Rat (SD): tMCAO; 90 min</td>
<td>30 min post-reperfusion</td>
<td>570 nmol/kg</td>
<td>Yes, histologically &amp; functionally</td>
<td>Tukhovskaya et al., 2009</td>
</tr>
<tr>
<td>Mouse: TBI; fluid percussion</td>
<td>IV: 2 &amp; 4 h post-TBI, &amp; then at 24, 48 &amp; 72 h</td>
<td>710 nmol/kg</td>
<td>Yes, histologically &amp; functionally</td>
<td>Kaufman et al., 2010</td>
<td></td>
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<tr>
<td>COG1410</td>
<td>Mouse: ICH</td>
<td>IV: 30 min</td>
<td>355, 710, 1420 or 2840 nmol/kg</td>
<td>Yes, functionally</td>
<td>Laskowitz et al., 2012</td>
</tr>
<tr>
<td>COG1410</td>
<td>Mouse: ICH</td>
<td>IV: 30 min, 1, 2 or 4 h after ICH, &amp; continued daily for 5 days</td>
<td>1420 nmol/kg</td>
<td>Yes, functionally. No reduction in haematoma volume</td>
<td>Laskowitz et al., 2012</td>
</tr>
<tr>
<td>COG1410</td>
<td>Mouse: TBI; cortical impact</td>
<td>IV: 30 min post-TBI, then every 24 h for 3 or 7 days</td>
<td>710 nmol/kg</td>
<td>Yes, reduced axonal APP immunoreactivity &amp; microglia activation. No reduction of peri-contusional white matter volume</td>
<td>Jiang &amp; Brody, 2012</td>
</tr>
<tr>
<td>Dynorphine A 1–13</td>
<td>Rat (SD): tMCAO; 90 min</td>
<td>IV: infusion started 1 h before MCAO &amp; continued for 4 h</td>
<td>30 μl: 1604, 16,040 or 160,400 ng (1, 10 or 100 nmol)</td>
<td>Yes, 16,040, 160,400 nmol. No 1604 ng</td>
<td>Kao et al., 2008</td>
</tr>
<tr>
<td>YGGFLRRIPKIK</td>
<td>Amyloid precursor protein</td>
<td></td>
<td></td>
<td></td>
<td>Corrigan et al., 2014</td>
</tr>
<tr>
<td>APP96–110</td>
<td>Mouse: TBI; cortical impact</td>
<td>IV: 30 min post-TBI</td>
<td>2 μl: 25 μM</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>

Bold data indicate peptides used in studies.

a Penetratin = RQIKIWFQNRRMKWKK, Aib = 2-Aminoisobutyric acid, APP: amyloid precursor protein.
c ICV: intracerebroventricularly; IP: intraperitoneally.
d Neuroprotection: reduced infarct volume, neural tissue injury, neuronal cell death or improved functional outcomes. Control peptide = SAibLRKLAibKR.
et al., 2009) and perinatal hypoxia–ischaemia models (McAdoo et al., 2005). Finally in a transgenic Drosophila model of Alzheimer’s disease, COG133 alone or when fused to the CPP penetratin (COG12) has been shown to reduce neurodegeneration and restore cognitive functions.

The mechanism whereby COG133 and its variant peptides exert their neuroprotective, calcium influx inhibitory and anti-inflammatory effects is not fully understood, but is assumed to be associated with the peptide’s binding to APOE receptors (e.g. low-density lipoprotein; LDL, lipoprotein receptor-related protein; LRP, apolipoprotein E receptor 2).

3.1.1. What is the neuroprotective mechanism of APOE141–I49, COG133 and COG1410 peptides?

Due mainly to their arginine and lysine residues the COG133 and COG1410 peptides have a net charge of +7.1 and +6.1 respectively, and therefore we would predict that these peptides should possess mild endocytopic properties and as has been reported to be neuroprotective and reduce calcium influx following excitotoxicity (Aono et al., 2003). Indeed our own assessment of the COG133 peptide in cultured cortical neurons exposed to glutamate excitotoxicity showed that the peptide has mild neuroprotective effects (Fig. 4).

3.2. APP peptide − APP96–110

The APP96–110 is a 15 amino acid peptide (NWCKRGRKQCKTHPH) derived from the heparin-binding site within the D1 domain of the APP (Corrigan et al., 2014). The APP96–110 peptide is thought to represent one of the neuroprotective regions (the other being the D6a domain) within the secreted amyloid-β precursor protein-α. The D1 domain is considered a growth factor-like domain and interestingly it has been hypothesised that its neuroprotective properties may be related to its ability to bind HSPGs (Corrigan et al., 2011). The APP96–110 peptide has been assessed for neuroprotective effects in traumatic brain injury models in APP−/− knock-out mice and normal rats (Corrigan et al., 2014). Intra-cerebroventricular peptide administration was shown to improve both histological and functional outcomes, and was effective whether administered pre- or post-insult. By contrast, a mutated form of the peptide (mAPP96–110: NWCNQGKQCKTHPH) specifically designed to reduce the ability of the peptide to bind heparin, by replacing positively charged arginine and lysine residues, was ineffective in traumatic brain injury in the rat.

3.2.1. What is the neuroprotective mechanism of APP96–100?

The APP96–110 peptide was synthesised with an acetylated N-terminal and an amidated C-terminal to minimise peptide proteolytic degradation and has a net charge of +5.1. The mutated form of the peptide has a net charge of +2.1. As mentioned above, it was hypothesised by the developers of APP96–110 that the peptide’s neuroprotective mechanism is related to its HSPG binding properties. The developers of APP96–110, have confirmed that this peptide had a strong binding affinity for the highly sulphated glycosaminoglycan molecule, heparin (Corrigan et al., 2014). By contrast, the mutated peptide mAPP96–110 has significantly reduced binding affinity for heparin. Despite the heparin binding properties of the APP96–110 peptide, its mechanism of action is not exactly known, although some form of interaction with cell surface receptors (e.g. FGFRs, APP, APLP1, APLP2) and the activation of signalling pathways has been proposed (Corrigan et al., 2014). However, it appears likely that APP96–100 behaves in a similar fashion to neuroprotective poly-arginine and arginine-rich peptides by binding cell surface HSPGs, and in doing so triggering peptide endosomal uptake. On this basis, we conclude that the endocytic properties of APP96–110 are integral to the peptide’s neuroprotective mechanism of action.

4. Discussion and concluding remarks

The main purpose of this review is to describe the neuroprotective properties of peptides fused to the arginine-rich CPP, TAT and in doing so provide evidence supportive of our hypothesis that neuroprotection is mediated not by the cargo molecule but largely by TAT itself. This hypothesis crystallised for us following the analysis of the neuroprotective and calcium influx inhibiting properties of a diverse set of peptides including: 1) arginine-rich CPPs (TAT, penetratin); 2) poly-arginine peptides (R3, R6, R7–15, R18); 3) several CPP-fused peptides (e.g. TAT-JNKI-1, TAT–PYC36, kFGF-JNKI-1); 4) several arginine-rich peptides (e.g. BEN0254, BEN1079, NCXB83, XIP); 5) poly-lysine–10 (K10) and a poly-arginine-9 fused poly-glutamic acid-9 peptide (E9/R9); and 6) a non-arginine, non-endocytic CPP (kFGF) (Meloni et al., 2015). What became increasingly clear from this analysis was that peptide neuroprotection is associated with peptide cationic charge. In particular, arginine and to a lesser extent lysine and tryptophan amino acid residues appear to be important determinants of neuroprotective potency.

Other studies have added weight to our hypothesis. For example, evidence that endocytosis is essential for TAT-JNKI-1 neuroprotection (Vaslin et al., 2011) and the observation that CPP-fused CD3 peptides can induce internalisation of NR2B, NCX and Cav2.2 membrane proteins. Another interesting and largely unexplained finding is that most Tat-fused and arginine-rich peptides are effective as neuroprotectants in neuronal excitotoxicity models in the μM range (e.g. 1–10μM) rather than the nM range (e.g. 10–100 nM or 0.01–0.1 μM). Our interpretation of this is that in order to be neuroprotective, a peptide must reach a critical concentration at the plasma membrane in order to induce endocytic internalisation of cell surface structures.

Recent findings suggests that cationic peptide charge conferred by arginine and lysine residues facilitates electrostatic interactions with cell surface HSPGs followed by arginine induced heparin sulphate clustering and endocytosis (Amand et al., 2012; Wallbrecher et al., 2014; Yang et al., 2014). Additionally, there is evidence that tryptophan residues within basic peptides can also promote proteoglycan binding and endocytosis (Rechard et al., 2013; Rydberg et al., 2012), while alanine residues have been shown to impede peptide–proteoglycan binding (Yang et al., 2014). Interestingly, the replacement of 1 to 3 arginine residues in poly-arginine-6 with the equivalent number of tryptophan residues increases the ability of the peptide to block NMDA receptor activity in amphibian oocytes (Ferrer-Montiel et al., 1988). Consistent with these observations, we have demonstrated that a 15mer consisting of 10 arginine and 5 tryptophan residues (WRRRWRRRRRWRRRW) is more neuroprotective than R15 in a cultured cortical neurons exposed to glutamic acid excitotoxicity, with an arginine/alanine peptide (ARRARRARRARRARRRA) being slightly less effective (Fig. 1).

There is emerging evidence that the presence of other amino acids or the amino acids sequence itself may decrease or increase peptide endocytosis and neuroprotective efficacy. In an earlier study, we reported that only 5 out of 19 TAT-fused peptides displayed significant neuroprotection in cortical neurons exposed to glutamic acid excitotoxicity (Meade et al., 2010b). Similarly, the addition of three amino acids to TAT (PKIRGKRRQRRG; AMBD-TAT) significantly increases peptide in vitro efficacy (Meade et al., 2010a). Therefore, it appears likely that in addition to arginine content and peptide charge, other amino acids most likely by influencing peptide secondary and tertiary structure can influence the endocytic and neuroprotective properties of arginine-rich and TAT-fused peptides. Ultimately, however, we recognise that additional in vitro and in vivo studies are required to unequivocally prove our hypothesis. Moreover, we cannot rule out the possibility that other peptide induced neuroprotective mechanisms are also operating, since it has been demonstrated that CPPs can exert intracellular biological effects by altering gene expression and the activity of kinases and proteolytic enzymes (Brugnano et al., 2010).

Finally, we speculate that by altering amino acid residue content and sequence within arginine-rich peptides it may be possible to develop
peptides with a greater capacity to target specific cell surface structures within the CNS and PNS, thereby providing a way to improve drug specificity for a range of neurological disorders such as epilepsy, pain, depression, Alzheimer’s disease and Parkinson’s disease. It may equally be possible to develop arginine-rich peptides that can target cell surface receptors associated with the pathogenesis of non-neurological disorders.

Conflict of interest

B.P. Meloni and N.W. Knuckey are named inventors of several patent applications regarding the use of arginine-rich peptides as neuroprotective agents. The other authors declare no conflict of interest.

Acknowledgments

This study was in support by the Department of Neurosurgery, Sir Charles Gairdner Hospital and by a Neurotrauma Research Program of Western Australia research grant.

References


Goebel, D. J. (2009). Selective blockade of CaMKIIα inhibits NMDA-induced caspase-3-dependent cell death but does not arrest PARP-1 activation or loss of plasma membrane selectivity in rat retinal neurons. Brain Res 1256, 190–204.
facilitative transport system for 2-deoxy-D-glucose, characterized by a novel membrane


effects of cell-penetrating peptides TAT, penetratin, Arg-9, and Pen-1 in glutamic acid, kainic acid,
and in vitro ischemia injury models using primary cortical neuronal cultures. Cell Mol Neuroprote

17(15–16), 830–860.

oligodeoxynucleotides protect against ischemia-induced neuronal death in the rat hippocampus.

oligodeoxynucleotides protect against ischemia-induced neuronal death in the rat hippocampus.


Chapter 3

General materials and methods
Materials and methods

In addition to information provided in publications (Chapters 4, 5, 6, 7 and 8), many of the materials and methods used in this project are also provided in this section of the thesis.

Peptides used in this thesis

Poly-arginine and arginine-containing peptides

The peptides used in animal and in *in vitro* studies are summarised in Tables 3.1 and 3.2. All peptides except protamine sulphate (referred as protamine; Ptm), which was purchased from Sanofi-Aventis (Perth, Australia), were purified using high performance liquid chromatography to at least 98% purity. With the exception of peptides used in the first study (R12, R15, R18) and protamine sulphate used for *in vivo* and *in vitro* studies, the peptides were subjected to hydrolysis and amino acid liquid chromatography analysis to obtain a precise measurement of peptide content (Mimotopes, Melbourne, Australia). For animal studies, all peptides were prepared in 0.9% sodium chloride for injection (Pfizer, Perth, Australia), aliquoted into a 650 µl volume within 3 ml syringes, and stored at -20°C until use. For additional details of protamine derived peptides see Table 3.2 and Chapter 7 of the thesis.

Table 3.1 Summary of the peptides used in the study.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence*</th>
<th>Arginine residues</th>
<th>Charge at pH 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>R12</td>
<td>H-RRRRRRRRRRRRRRR-OH</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>R15</td>
<td>H-RRRRRRRRRRRRRRRRRRR-OH</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>R18</td>
<td>H-RRRRRRRRRRRRRRRRRRRRRRRRRRRR-OH</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>R12W8a</td>
<td>H-WWRRRRRRRRRRRRRWWRRRRWW</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>TAT-NR2B9c (NA-1)</td>
<td>H-GRKKRRQRRR-KLSSIESDV-OH</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

* R = arginine, W = tryptophan, G = glycine, K = lysine, Q = glutamine, L = leucine, S = serine, I = isoleucine, E = glutamic acid, D = aspartic acid, V = valine.
Table 3.2  Protamine sulphate peptide used in the study.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence*</th>
<th>Arginine residues</th>
<th>Charge at pH7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ptm sulphate</td>
<td>Mixture of protamine peptides Ptm 1 – 4</td>
<td>≈21</td>
<td>21</td>
</tr>
<tr>
<td>Ptm 1</td>
<td>PRRRRRSSRPIRRRRRCPRASCCCRGRRRRRRRLRRRRRRGGRRRR</td>
<td>21</td>
<td>21</td>
</tr>
</tbody>
</table>
| Ptm 2     | PRRRRSSRRPVRRRRRPRVSSRRRGGRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRR
Middle cerebral artery occlusion (MCAO) stroke models

Most experimental stroke models rely on intravascular occlusion (Koizumi et al., 1986; Longa et al., 1989; Belayev et al., 1996) or extravascular ligation (Tamura et al., 1981a) to inhibit cerebral blood flow to a defined brain region. The most commonly used stroke models use an intraluminal filament to occlude the middle cerebral artery. An intraluminal filament procedure was utilised in this study and involved advancing a silicone coated filament into the internal carotid artery until it reached the middle cerebral artery bifurcation, and thereby blocking blood flow through the vessel. In this procedure, MCAO can be permanent, if the filament is tied in place, or transient if the filament is retracted, allowing for reperfusion.

Surgical procedure for the MCAO model

For this model, healthy Sprague Dawley (SD) rats weighing between 270 and 340 grams were used. Animals were fasted overnight, but allowed water ad libitum. Fasting was performed to provide a more consistent blood glucose range between rats, which improves model consistency. Anaesthesia was induced using a facemask with 4% isoflurane in a gas mixture of 30% O₂ and 70% of N₂O, reducing the isoflurane to 2.0–1.5% once the animal was anaesthetised, and during MCAO. If necessary animals were warmed with a fan heater during surgical procedures to maintain body temperature at 37.0-38.0°C. Eye ointment was applied during the procedure to avoid eye dryness. The tail artery was cannulated to allow blood pressure to be monitored and blood samples to be removed (between 50-200 µl) for blood gases (pO₂, pCO₂), pH and glucose measurements.

A midline incision was made in the scalp, and the top of the skull exposed. In the earlier studies a dental drill was used to thin the bone of the skull over the right cerebral hemisphere. In later studies, thinning of the skull bone was found to be unnecessary for laser Doppler recording and was not performed. A laser Doppler probe holder was attached to the skull using a fast-setting, non-toxic adhesive (loctite). Once attached, a laser probe was inserted into the probe holder. The laser Doppler measures cerebral blood flow by monitoring the velocity of moving red blood cells beneath the beam of the laser.
Through a longitudinal cervical skin incision, the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed. The ECA was ligated and a silicone coated nylon monofilament (Doccol, Redlands, CA, USA) advanced (19-21 mm) into the ICA to occlude the middle cerebral artery (MCA) (Belayev et al, 1996). In the permanent model, the thread was tied in place. However, in the transient model, following 90 - 180 minutes of MCA occlusion, reperfusion was achieved by retracting the filament. At the completion of the surgical procedure, the laser Doppler probe, probe holder, and adhesive were removed, the wounds sutured and the animal allowed to recover (Figure 3.1).

**Figure 3.1** Intraluminal filament MCAO model in the rat. (A) An intraluminal filament was advanced into the internal carotid artery until it blocked the MCA, which was confirmed by a laser Doppler. Blood pressure was monitored and temperature maintained at 37°C during surgical procedures. (B) & (C) Entire and coronal sections of rat brain stained with TTC (triphenyl tetrazolium chloride). The red area represented the viable tissue and the white area the brain infarct. CCA = common carotid artery, ECA = external carotid artery, ICA = internal carotid artery, MCA = middle cerebral artery, ACA = anterior cerebral artery.
**Post-surgical analgesia, animal body temperature monitoring and housing**

At the conclusion of surgery pethidine was administered intramuscularly (1 mg in 0.2 ml saline) and bupivacaine was administered subcutaneously (0.1 mg in 0.2 ml saline per site) to tail and head wounds. Animal body temperature was measured every 30–60 minutes using a lubricated rectal probe and closely monitored for at least 2 hours after surgery, and maintained between 37.0 and 37.8°C. To aid in the maintenance of animal body temperature during surgical recovery, animals were housed in an holding room maintained at 25-26°C, cages placed on heating pad and external heating via a fan heater applied if necessary.

**Functional testing**

Focal cerebral ischaemia or stroke causes sensorimotor deficits in rats. Therefore, to determine whether reduced brain infarction following treatment is also associated with improved sensorimotor outcomes, three neurological tests were performed. Functional assessment tests were performed before MCAO surgery (day before or on day of surgery) and at experiment end-point (24 hours after stroke onset).

**Neurological assessment test**

Scoring of neurological injury/impairment was performed using the modified Bederson’ scale (Bederson et al., 1986) and is summarised in Table 3.3. Because sensory disturbance and loss of motor function are common outcomes of stroke, the neurological assessment aims to assess the general animal well-being following stroke. Scores range from 0 for no deficits, 1 for flexed forepaw, 2 for inability to resist lateral push, 3 for circling, 4 for agitated circling and 5 for unresponsive to stimulation/stupor.
### Table 3.3 Neurological assessment test.

<table>
<thead>
<tr>
<th>Score</th>
<th>Neurological grading scale post-surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No deficits</td>
</tr>
<tr>
<td>1</td>
<td>Flexed forepaw</td>
</tr>
<tr>
<td>2</td>
<td>Inability to resist lateral push</td>
</tr>
<tr>
<td>3</td>
<td>Circling</td>
</tr>
<tr>
<td>4</td>
<td>Agitated circling</td>
</tr>
<tr>
<td>5</td>
<td>Stupor</td>
</tr>
</tbody>
</table>

**Adhesive tape paw removal test**

The bilateral asymmetry paw test assesses sensorimotor impairment. Each rat was tested three times for each forepaw (before and after the surgery), by placing a 10 x 10 mm piece of adhesive tape (Cryo-Babies; Diversified Biotech, Boston), on the palmar surface of the forepaw and the time taken for the first attempt to remove tape, the number of attempts to remove tape and the total time taken to remove tape recorded. A maximum of 120 seconds was allowed for animals to complete the task (normal rats usually take between 5 to 30 seconds to remove the tape). Animals that failed to remove the tape in the given time were scored 120 seconds.

**Rota-Rod test**

The Rota-Rod test was used to determine the balance and coordination of rats. This test involves assessing the rat’s ability to remain walking on a rotating rod as it speed of rotation is gradually increased from 4 to 40 revolutions per minute. The time in seconds at which each animal falls (15-20 cm) onto a foam/sponge mattress was recorded. Typically rats fall 15-100 seconds after placement on rod.
**Ex vivo assessment of brain injury**

*Tissue sectioning and triphenyl tetrazolium chloride (TTC) staining*

Twenty-four hours post-stroke animals were euthanised by lethal intraperitoneal injection of pentobarbitone (325 mg of pentobarbitone sodium in 1 ml solution). Infarct volume measurement was performed by brain slicing and TTC (triphenyl tetrazolium chloride) staining. The brain was carefully removed from the skull and placed in 0.9% saline and cooled for 7-10 minutes before being placed in a rodent brain matrix. Razor blades were inserted into the matrix to generate 2 mm coronal sections. The sections were incubated for 15–20 minutes in a 3% solution of TTC to stain viable non-infarcted tissue, and then fixed in 4% formalin for at least 12 hours.

*Measurement of ischaemic stroke damage in animal models*

Digital images of coronal sections from brain slices were acquired with a colour scanner and analysed using Image J Software (NIH) to calculate infarct volume. Unstained tissue was defined as ischaemic infarcted areas. The area of infarcted tissue and the area of both hemispheres were calculated for each brain slice. An oedema index (cerebral hemispheric swelling/oedema) was calculated by dividing the total volume of the stroke-affected hemisphere by the total volume of the contra-lateral hemisphere. The actual infarct volume adjusted for oedema by dividing the infarct volume for the oedema index. Once the infarct area for all coronal sections was defined the total volume of the infarct was calculated (Table 3.6).
Table 3.6  Description of steps used to for the measurement of infarct volume.

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1)</td>
<td>Open ImageJ.</td>
</tr>
<tr>
<td>2)</td>
<td>Click on File → open the image of interest. Check the spatial calibration and set the correct pixel size.</td>
</tr>
<tr>
<td>3)</td>
<td>Draw a line over the scale bar and select Analyze → Set Scale. In the set scale window enter the selected line into the ‘Known Distance’ box and set the ‘Unit of Measurement’. When the image is calibrated the number of pixels and the dimensions in the selected Unit of Length is displayed.</td>
</tr>
<tr>
<td>4)</td>
<td>Once measurement parameters are set, click on the Polygon Selections button. With the image open, trace the entire brain section by clicking and outlining the perimeter of the brain. When complete, click near the starting point and the contour selection of the brain will be shown. Select Analyze → Measure. A Results box will display the Area measurement and the parameters selected.</td>
</tr>
<tr>
<td></td>
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<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>5) Follow this same tracing method for the infarct area of the brain slice, and again select Analyze → Measure. The results block will show the area of the infarct selection.</td>
</tr>
<tr>
<td></td>
<td>6) You may do this for all your tissue sections. Determine the total volume of the infarct and brain, and then divide the infarct area by the brain area to obtain the infarct volume as a percentage of total brain volume.</td>
</tr>
</tbody>
</table>

**Perimeter of the infarct area**

**Example of infarct volume analysis using ImageJ**

**Statistical Analysis**

Data from physiological parameters, mean total infarct volume measurements (total and coronal slices), and cerebral oedema for each treatment group were evaluated by analysis of variance (ANOVA) followed by Fisher’s *post hoc* analysis. Data from adhesive tape and rota-rod tests were evaluated by analysis of variance (ANOVA) followed by Fisher’s *post hoc* analysis. Data from neurological assessment were analysed using Kruskal-Wallis test. A value of *p* < 0.05 was considered as significant for all data sets.
References


Chapter 4

Poly-arginine peptides reduce infarct volume in a permanent middle cerebral artery rat stroke model

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Poly-arginine peptides reduce infarct volume in a permanent middle cerebral artery rat stroke model

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Signed………………………………         Signed………………………………
Diego Milani                     Bruno Meloni (coordinating supervisor)
Poly-arginine peptides reduce infarct volume in a permanent middle cerebral artery rat stroke model

Diego Milani1,2,3, Vince W. Clark1,2,3, Jane L. Cross1,2,3, Ryan S. Anderton3,4, Neville W. Knuckey1,2,3 and Bruno P. Meloni1,2,3*

Abstract

Background: We recently reported that poly-arginine peptides have neuroprotective properties both in vitro and in vivo. In cultured cortical neurons exposed to glutamic acid excitotoxicity, we demonstrated that neuroprotective potency increases with polymer length plateauing at R15 to R18 (R = arginine residues). In an in vivo study in rats, we also demonstrated that R9D (R9 peptide synthesised with D-isoform amino acids) administered intravenously at a dose of 1000 nmol/kg 30 min after permanent middle cerebral artery occlusion (MCAO) reduces infarct volume. Based on these positive in vitro and in vivo findings, we decided to examine the neuroprotective efficacy of the L-isoform poly-arginine peptides, R12, R15 and R18 when administered at a dose of 1000 nmol/kg 30 min after permanent MCAO in the rat.

Results: At 24 h post-MCAO, there was reduced total infarct volume for R12 (12.8 % reduction) and R18 (20.5 % reduction), but this reduction only reached statistical significance for R18. Brain slice analysis revealed significantly reduced injury in coronal slices 4 and 5 for R18, and slice 5 for R12. The R15 peptide had no effect on infarct volume. Peptide treatment did not reveal any statistical significant improvement in functional outcomes.

Conclusion: While these findings confirm the in vivo neuroprotective properties of poly-arginine peptides, additional dose studies are required particularly in less severe transient MCAO models so as to further assess the potential of these agents as a stroke therapy.

Keywords: Poly-arginine peptides, Middle cerebral artery occlusion, Stroke, Neuroprotection

Background

Minimising brain injury following stroke is a critical clinical goal both to improve patient quality of life and to lessen the social and economic impacts of this devastating disorder. Currently, the most effective stroke therapy is to restore cerebral blood flow to a blocked artery using tPA and thrombectomy [1–3]. However, the current therapeutic window for coupled tPA ± thrombectomy therapy is so narrow (4.5 h) that the majority of stroke patients are unable to receive the treatment. Moreover, for those that do, up to 7 % develop intracranial haemorrhage as a complication. In addition, tPA ± thrombectomy is only available to patients having ready access to a hospital that has the facilities required for performing the procedures. Other treatments are only suitable for a small proportion of patients (e.g. hemicraniectomy to reduce intracranial pressure due to cerebral oedema) or provide only modest benefit (e.g. aspirin to reduce risk of clot propagation) [4]. As a consequence, while recent improvements in stroke therapy have been made, these have been limited and it is clear that there is urgent need for new, more widely applicable neuroprotective therapies that can be applied to stroke patients early by ambulance paramedics, in hospital emergency departments, and in remote locations away from tertiary hospitals. Additionally, any treatment that might improve the safety, therapeutic window...
and neuroprotective outcomes for tPA ± thrombectomy would be of great clinical significance.

Against the backdrop of the limited nature of current therapies, we have recently demonstrated that poly-arginine (and arginine-rich) peptides have potent neuroprotective properties in in vitro injury models that mimic the effects of stroke [5–7]. We have also established that poly-arginine peptides, as well as other arginine-rich peptides, including TAT and penetratin belonging to a class of peptide with cell penetrating properties also possess intrinsic neuroprotective properties [5–7]. Moreover, our in vitro data show that neuroprotective potency is enhanced with increasing arginine content (e.g. polymer length) [6]. As evidence of their clinical applicability, we have demonstrated that the poly-arginine R9D significantly reduces infarct volume in vivo following permanent middle cerebral artery occlusion (MCAO) in the rat [6]. A recent report [8] has also demonstrated that poly-arginine 7 (R7) containing peptides are neuroprotective in an in vivo retinal ganglion NMDA excitotoxicity model.

The neuroprotective properties of poly-arginine peptides in vitro and in vivo suggest that they may have potential as a neuroprotective therapy for stroke patients. To further investigate the efficacy of poly-arginine peptides in vivo and given the positive results obtained with the R9D peptide, in this study we assess the neuroprotective efficacy of the longer L-isoform poly-arginine peptides R12, R15 and R18 when administered 30 min after permanent MCAO. In addition, unlike in our earlier R9D trial, this study assesses functional outcomes using three behavioural tests as well as infarct volume to gain an understanding of the functional consequences of neuroprotection.

Results

Physiological and infarct volume measurements

Physiological measurements before or during surgery confirmed the absence of any significant differences between animal treatment groups (Table 1). Data on the mean total infarct volumes and representative TTC-stained coronal brain slices for each treatment group are presented in Fig. 1. These results show that the R18 peptide significantly reduced infarct volume (20.5 % reduction; \( P = 0.014 \)). The R12 peptide also reduced infarct volume (12.8 % reduction), but not to a statistical significant extent (\( P = 0.105 \)). By contrast, the R15 peptide had no effect on infarct volume. Rostral to caudal topographic analysis of infarcts in brain slices revealed that the R18 peptide significantly reduced brain injury in coronal slices 4 (\( P = 0.008 \)) and 5 (\( P = 0.01 \)) (Fig. 2). In addition, the R12 peptide significantly reduced brain injury in coronal slice 5 (\( P = 0.027 \)).

There were three post-treatment animal deaths that occurred the day following surgery, one in the vehicle and two in the R12-treated animals. While the animal deaths could be directly related to stroke severity and/or treatment, the exact cause of the deaths could not be precisely determined on autopsy.

Functional outcome assessment

Neurological scores using the modified Bederson’ scale for each treatment group are presented in Fig. 3. While neurological scores did not differ statistically between groups, the vehicle control group score was higher (1.9) than any of the scores for the peptide treatment groups (<1.4), indicative of a possible positive treatment effect. Results for the rota-rod assessment for each treatment group are presented in Fig. 4. Results were highly variable within groups and no significant differences were detected.

For the adhesive tape removal test pre- and post-MCAO measurements for time to detect tape, the number of attempts to remove tape and time taken to remove tape for each treatment group are presented in Fig. 5. As expected, the left paw was more adversely affected than the right paw, however there were no statistically significant differences between vehicle-treated versus peptide-treated groups. However, for the R12 peptide all parameters measured for the left paw, and two out of the three measurements obtained for the right paw showed a positive improvement, albeit not to a statistically significant extent.

### Table 1 Physiological parameters for experimental animals used in study

<table>
<thead>
<tr>
<th></th>
<th>Saline (N = 12)</th>
<th>R12 (N = 9)</th>
<th>R15 (N = 8)</th>
<th>R18 (N = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PaO₂ (mmHg)</td>
<td>115.10 ± 33.51</td>
<td>124.30 ± 18.40</td>
<td>112.80 ± 16.96</td>
<td>120.60 ± 20.34</td>
</tr>
<tr>
<td>PaCO₂ (mmHg)</td>
<td>42.92 ± 5.82</td>
<td>46.00 ± 4.21</td>
<td>39.38 ± 5.20</td>
<td>44.25 ± 7.74</td>
</tr>
<tr>
<td>pH</td>
<td>7.44 ± 0.09</td>
<td>7.33 ± 0.08</td>
<td>7.31 ± 0.09</td>
<td>7.42 ± 0.08</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>7.74 ± 1.27</td>
<td>7.42 ± 1.06</td>
<td>7.03 ± 1.11</td>
<td>7.13 ± 1.08</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td>89.00 ± 8.44</td>
<td>78.44 ± 6.98</td>
<td>88.00 ± 9.97</td>
<td>79.63 ± 12.65</td>
</tr>
<tr>
<td>Body temperature (°C)</td>
<td>37.48 ± 0.18</td>
<td>37.58 ± 0.13</td>
<td>37.46 ± 0.21</td>
<td>37.51 ± 0.06</td>
</tr>
</tbody>
</table>

PaO₂, PaCO₂, pH, blood pressure and glucose measured before MCAO. Body temperature data represent average over 2 h post-surgery monitoring period. Data are mean ± SD.
Weight loss measurement

At experiment end, all treatment groups recorded a loss in weight, with the greatest weight loss occurring in the R15 peptide treatment group ($P = 0.004$; Fig. 6).

Discussion

In a previous study, we demonstrated that the poly-arginine peptide R9D could reduce infarct volume by 20 % when administered intravenously 30 min post-MCAO [6], however no functional assessment was performed. The present study extends this previous study to include the poly-arginine peptides R12, R15 and R18 and explores their capacity to reduce infarct volume and improve functional outcomes when administered intravenously 30 min post-MCAO. Whereas R15 had no effect on infarct volume, R18 significantly reduced infarct volume (20.5 % reduction) and there was a trend towards reduced infarct volume with R12 (12.8 % reduction). Importantly, all peptide treatments displayed a trend towards improvement
in one or more of the neurological functional tests. Whilst the level of infarct volume reduction was modest (12.8–20.5 %), this most likely reflects the severity of the stroke model used in this particular study where up to 90 % of the affected brain hemisphere is infarcted by the stroke. It is also likely that the modest reductions in infarct volume, stroke severity and 24-h endpoint coupled with the small animal numbers used explain why the trend towards improvements in functional outcomes was not statistically significant. Despite the modest effects of the poly-arginine peptides following permanent MCAO, it is still possible that these peptides have potential clinical application, especially in less severe forms of stroke, stroke associated with cerebral reperfusion treatments (tPA ± thrombectomy) and haemorrhagic stroke. With respect to neuroprotective efficacy, further research is required to determine the optimal dose of the peptides to reduce infarct volume. It was particularly
as well as the findings of other studies, we have hypoth-
in cultured cortical neurons [6, 7]. Based on this finding,
reduce excitotoxic glutamic acid-induced calcium influx
shown that poly-arginine peptides have the capacity to
of action of peptides, but in previous studies we have
surrounding effective dosage for a range of poly-arginine
peptides in the in vivo stroke model. Studies are currently underway
in our laboratory to more definitively address questions
surrounding effective dosage for a range of poly-arginine
peptides in the in vivo stroke model.

The present study did not investigate the mechanism
of action of peptides, but in previous studies we have
shown that poly-arginine peptides have the capacity to
reduce excitotoxic glutamic acid-induced calcium influx
in cultured cortical neurons [6, 7]. Based on this finding,
as well as the findings of other studies, we have hypo-
thesised that these peptides have the capacity to inhibit cal-
cium influx by causing the internalisation of cell surface
structures such as ion channels and thereby reduce the
toxic neuronal calcium entry that occurs after excitotox-
icity and cerebral ischemia. We have speculated that due
to the cell penetrating properties of arginine-rich pep-
tides, including putative “neuroprotective peptides” fused
to the arginine-rich carrier peptide TAT, ion channel
receptor internalisation occurs during neuronal endocyt-
ic uptake of the peptides [6, 7]. Evidence that sup-
ports our hypothesis includes studies demonstrating that
arginine-rich peptides: (1) interfere with the function of
NMDA [9–14] and vanilloid receptors [15], voltage
gated calcium channels [16–18] and the sodium calcium
exchanger [13]; (2) cause internalisation or reduced sur-
face expression of neuronal ion channels [11, 13, 18]; and
(3) can induce the endocytic internalisation of epidermal
growth factor receptor and tumour necrosis factor recep-
tors in HeLa cells [19].

In support of the poly-arginine neuroprotective find-
ings in the present study, a recent report [8] has con-
firmed the neuroprotective properties of poly-arginine
7 (R7) containing peptides and other arginine-rich pep-
tides (TAT and TATNR2B9c) in an in vivo retinal gan-
glion NMDA excitotoxicity model. Moreover, the study
also provides evidence for an additional neuroprotective
mechanism associated with maintenance of mitochon-
drial function and integrity. Studies in our laboratory to con-
firm peptide-induced internalisation of cell surface receptors and other neu-
roprotective mechanisms are in progress. While we have
demonstrated that arginine-rich peptides have the capac-
ity to reduce excitotoxic calcium influx, it will be impor-
tant to obtain a more comprehensive understanding of
peptide neuroprotective mechanism of action. Never-
theless our findings indicate poly-arginine peptides have
both in vitro and in vivo neuroprotective properties and
warrant further evaluation in different stroke models and
other acute brain injury disorders.

Conclusion
The findings of this study further validates the neuropro-
tective properties of poly-arginine peptides [5–9], high-
lights their status a new class of neuroprotective agent
and provides justification for their evaluation in different
stroke models and other acute brain injury disorders. The
findings also further question the mechanism of action of
the many reported “neuroprotective peptides” fused to
arginine-rich carrier peptides, which are thought to act
through interaction with specific intracellular proteins,
but which our data suggest may act through a common
mechanism of action relating to peptide arginine content
and positive charge.

Methods
Peptides
The R12 (H-RRRRRRRRRRRR-OH), R15 (H-RRRRRR
RRRRRRRRRR-OH) and R18 H-RRRRRRRRRRRRRR
RRRR-OH) peptides used in the study were synthesised
by China Peptides (Shanghai, China). The peptides were
HPLC purified to >94 % purity. All peptides were pre-
pared in 0.9 % sodium chloride for injection (Pfizer,
Perth, Australia) aliquoted into 650 µl volumes in 3 ml
syringes and stored at −20 °C until use.

Rat permanent middle cerebral artery occlusion procedure
This study was approved by the Animal Ethics Commit-
tee of the University of Western Australia and follows
guidelines outlined by the Australian Code for the Care
and use of Animals for Scientific Purposes. The experi-
mental procedure for performing the permanent mid-
dle cerebral artery occlusion (MCAO) stroke model

![Graph](image_url)
has been described previously [20, 21]. Briefly, male Sprague-Dawley rats weighing 270–320 g were kept under controlled housing conditions with a 12 h light–dark cycle and with free access to food and water. Experimental animals were fasted overnight and subjected to filament permanent MCAO. In order to monitor blood pressure and withdraw blood samples, a cannula was inserted in the tail artery. Between 50 and 200 µL of blood was used for glucose (glucometer; Medisense Products, Abbott Laboratories, Bedford, MA, USA) and other measurements (PaO2, PaCO2, pH; ABL5, Radiometer, Copenhagen, Denmark). The MCAO procedure was considered successful based on a >25% decrease from baseline in cerebral blood flow (CBF) after insertion of filament, as measured by laser Doppler flowmetry. During surgery temperature was closely monitored using a rectal probe (Physitemp Instruments, Clifton, USA) and maintained at 37.5 ± 0.5 °C, with fan heating or cooling.

Thirty minutes post-MCAO, rats were intravenously treated with the peptide (1000 nmol/kg in 600 µL over 6 min) or vehicle (0.9 % sodium chloride for injection; 600 µL over 6 min). Treatments were administered via the right internal jugular vein and infusion pump. Treatments were randomised and all procedures were performed blinded to treatment.

Twenty-four hours post-MCAO, infarct area assessment was performed by preparing 2 mm thick cerebral coronal brain slices, and incubating in 3% 2,3,5 triphenyltetrazolium chloride (TTC; Sigma-Aldrich, St. Louis, USA) at 37 °C for 20 min, followed by fixation in 4% formalin at room temperature overnight. Digital images of coronal sections were acquired using a colour scanner and analysed by an operator blind to treatment status, using ImageJ software (3rd edition, NIH, Bethesda, USA). The total infarct volume was determined by measuring the areas of infarcted tissue on both sides of the 2 mm sections. These measured areas were corrected for cerebral oedema by multiplying the infarct volume for the oedema index (calculated by dividing the total volume of the stroke-affected hemisphere by the total volume of the contralateral hemisphere) [22].

A total of 42 animals were used in the trial. Five animals were excluded from the study; two animals were euthanased due to subarachnoid haemorrhage, one animal was excluded due to insufficient decrease in CBF, one animal was excluded due to pyrexia, and one died during surgical recovery for an unknown reason.

Post-surgical monitoring

Following surgery animals were placed in a clean cage with free access to food and water. The body temperature of animals was measured every 30–60 min using a rectal probe for at least 2 h post-surgery, and maintained between 37.0 and 37.8 °C. To avoid hypothermia, rat cages were placed on a heating mat during the post-surgical monitoring and housed in a holding room maintained at 26–28 °C. If necessary, additional heating or cooling was performed by applying fan heating or cold water spray.

Behavioural testing

To determine if peptide treatment was associated with improved sensorimotor outcomes, three neurological tests were performed 24 h post-stroke.

Neurological assessment test

The scoring system was performed using the modified Bederson’ scale. Scores range from 0 for no deficits, 1 for flexed forepaw, 2 for inability to resist lateral push, 3 for circling, 4 for agitated circling and 5 for unresponsive to stimulation/stupor [23].

Adhesive tape removal test

This is a bilateral asymmetry paw-test, which assesses sensorimotor impairment [24]. Adhesive tape (Diversified Biotech, Dedham, USA) 10 mm × 10 mm in size was placed on the palmar surface of the forepaw and the time taken for the first attempt to remove tape, the number of attempts to remove tape and the total time taken to remove tape recorded. Each forelimb was assessed sequentially starting with the unaffected side (right side) with animals having a maximum of 120 s to complete the task (normal rats usually take between 5 and 30 s to remove the tape). Animals were tested a total of six times, three times on the day before surgery and three times 24 h post-MCAO. Mean values were calculated for each forepaw for the pre- and post-surgery trials.

Rota-rod test

This test assesses balance and coordination by assessing a rat’s ability to remain walking on a rotating rod when its speed of rotation gradually increases from 4 to 40 revolutions per minute. The time at which the animal falls is recorded. Typically rats fall 27–137 s after placement on the rod.

Statistical analysis

Mean infarct volume measurements (total and coronal slices) for each treatment group was compared to the vehicle control group by analysis of variance (ANOVA) followed by the Fisher’s post hoc analysis. Data from neurological assessment were analysed using Kruskal–Wallis test [25]. Data from adhesive tape and rota-rod tests were analysed using ANOVA followed by post hoc analysis using Scheffe’s multiple comparison procedure. A value
of $P < 0.05$ was considered significant for all data sets. Data in figures are presented as mean ± standard deviation (SD).

**Authors’ contributions**

DM, VC and JC contributed to animal procedures, post-surgical monitoring, functional assessment, infarct volume analysis or statistical analysis. BM, DM, NK and RA contributed to experimental design and manuscript preparation. All authors read and approved the final manuscript.

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**Competing interests**

B. P. Meloni and N. W. Knuckey are the holders of several patents regarding the use of arginine-rich peptides as neuroprotective treatments. The other authors declare no competing interests.

**Compliance with ethics requirements**

This study was approved by the Animal Ethics Committee of the University of Western Australia and follows guidelines outlined by the Australian Code for the Care and use of Animals for Scientific Purposes and National Health and Medical Research Council of Australia.

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Chapter 5

The R18 poly-arginine peptide is more effective than the TAT-NR2B9c (NA-1) peptide when administered 60 minutes after permanent middle cerebral artery occlusion in the rat.

Published: Stroke Research and Treatment
The R18 poly-arginine peptide is more effective than the TAT-NR2B9c (NA-1) peptide when administered 60 minutes after permanent middle cerebral artery occlusion in the rat

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Neville Knuckey: 4%
Ryan Anderton: 3%
Jane Cross: 3%

Signed……………………………………………
Signed……………………………………………

Diego Milani
Bruno Meloni (coordinating supervisor)
Research Article

The R18 Polyarginine Peptide Is More Effective Than the TAT-NR2B9c (NA-1) Peptide When Administered 60 Minutes after Permanent Middle Cerebral Artery Occlusion in the Rat

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We examined the dose responsiveness of polyarginine R18 (100, 300, and 1000 nmol/kg) when administered 60 minutes after permanent middle cerebral artery occlusion (MCAO). The TAT-NR2B9c peptide, which is known to be neuroprotective in rodent and nonhuman primate stroke models, served as a positive control. At 24 hours after MCAO, there was reduced total infarct volume in R18 treated animals at all doses, but this reduction only reached statistical significance at doses of 100 and 1000 nmol/kg. The TAT-NR2B9c peptide reduced infarct volume at doses of 300 and 1000 nmol/kg, but not to a statistically significant extent, while the 100 nmol/kg dose was ineffective. The reduction in infarct volume with R18 and TAT-NR2B9c peptide treatments was mirrored by improvements in one or more functional outcomes (namely, neurological score, adhesive tape removal, and rota-rod), but not to a statistically significant extent. These findings further confirm the neuroprotective properties of polyarginine peptides and for R18 extend its therapeutic time window and dose range, as well as demonstrating its greater efficacy compared to TAT-NR2B9c in a severe stroke model. The superior neuroprotective efficacy of R18 over TAT-NR2B9c highlights the potential of this polyarginine peptide as a lead candidate for studies in human stroke.

1. Introduction

While the incidence of stroke is falling in developed countries, it remains a leading cause of death and disability worldwide, with an increasing global disease burden due to an aging population, as well as the ongoing epidemics of diabetes, hypertension, and obesity [1]. In terms of acute therapies, for ischaemic stroke, reperfusion therapy using tPA (tissue plasminogen activator) alone or more recently in combination with thrombectomy is by far the most effective treatment intervention currently available [2–6]. However, despite the success of tPA/thrombectomy therapy, the number of stroke patients that receive this treatment is relatively small. This is due to a combination of factors including the narrow therapeutic time window for tPA/thrombectomy (3–4.5 h after stroke), delays in patients obtaining medical care, the requirement for a brain scan to exclude haemorragic stroke, and the need for highly trained personnel and specialised equipment to perform the intervention. Given these limitations, the search continues for a neuroprotective agent that can be safely administered early after stroke onset to limit the extent of brain injury after stroke and that can be used when reperfusion interventions cannot be implemented. Additionally, any neuroprotective treatment that improves the efficacy, safety, and therapeutic window for tPA/thrombectomy would be of great clinical significance.

In terms of neuroprotective agents, our laboratory has recently demonstrated that polyarginine and arginine-rich peptides have potent neuroprotective properties in in vitro injury models that mimic the effects of stroke [7–9]. Moreover, we have extended these in vitro findings by demonstrating that the polyarginine peptides R9, R12, and R18
significantly reduce infarct volume in a permanent middle cerebral artery occlusion (MCAO) stroke model [8, 10]. Based on these in vitro and in vivo findings, we have recently proposed [8, 9] that arginine-rich peptides including “neuroprotective peptides” fused to arginine-rich cell penetrating peptides (e.g., TAT-NR2B9c [11] and TAT-JNKI-1 [12]) represent a new class of neuroprotective agents for which arginine residues are critical for neuroprotection.

In the present study, we further evaluate the efficacy of the R18 polyarginine peptide by examining its dose responsiveness and by extending the treatment administration time from 30 minutes to 60 minutes after permanent MCAO. In parallel, the study compares the efficacy of R18 with that of TAT fused NR2B9c peptide (TAT-NR2B9c), which has previously been demonstrated to be neuroprotective in various rodent and nonhuman primate stroke models and to reduce ischaemic brain lesions in humans following endovascular repair of ruptured aneurysms [11, 13–15].

2. Materials and Methods

2.1. Peptides Used in the Study. The R18 (H-RRRRRRRRRRRRRRRRRR-OH) and TAT-NR2B9c (H-YKRRQRRRRRKLSSIESDV-OH, also known as NA-1) peptides used in the study were synthesised by Mimotopes (Melbourne, Australia). The peptides were HPLC-purified to 98% purity and were subject to peptide hydrolysis and amino acid liquid chromatography analysis to obtain a precise measurement of peptide content (Mimotopes). The peptides were prepared in 0.9% sodium chloride for injection (Pfizer, Perth, Australia), aliquoted into a 650 µL volume in a 3 mL syringe and stored at −20°C until use.

2.2. Surgical Procedure for Permanent Middle Cerebral Artery Occlusion. The surgical procedures for permanent middle cerebral artery occlusion (MCAO) as well as behavioural and histologic assessment were performed in accordance with the Animal Ethics Committee of the University of Western Australia and following the guidelines outlined by the Australian Code for the Care and Use of Animals for Scientific Purposes.

The filament permanent MCAO stroke model as performed in our laboratory has been described previously [10, 16]. Briefly, male Sprague-Dawley rats weighing 275–340 g that had been fasted overnight underwent facemask anaesthesia with 4% of isoflurane (mix 30% oxygen/70% nitrous oxide) and maintenance with 2% isoflurane. The tail artery was cannulated to allow blood pressure monitoring and for measurement of arterial blood gases (pO₂, pCO₂), pH, and glucose. The MCAO procedure was considered successful if there was a >25% decrease from baseline in cerebral blood flow after insertion of the filament, as measured by laser Doppler flowmetry. During surgery, body temperature was closely monitored using a rectal probe (Physit mnemonic Instruments, Clifton, USA) and maintained at 37–37.8°C, with fan heating or cooling, as required.

At sixty minutes after MCAO rats were treated intravenously through the right internal jugular vein using an infusion pump with the vehicle (0.9% sodium chloride, 600 µL over 6 min) or with three different doses of the peptide (R18 or TAT-NR2B9c: 100, 300, or 1000 nmol/kg, 600 µL over 6 min). Treatments were randomised and all procedures were performed while being blinded to treatments.

Fifty male Sprague-Dawley rats underwent surgery for permanent MCAO. Eight animals were excluded from the study: five animals were excluded due to an insufficient decrease in cerebral blood flow following MCAO, one was excluded due to death during anaesthetic induction, and two animals were excluded because no obvious infarct lesion was detected 24 hours after MCAO (one saline and one R18 100 nmol/kg treated animal). A further six animals died several hours before the 24-hour post-MCAO study end-point but were still included in the final infarct volume analysis. These animals comprised two R18 treated (one 100 nmol/kg and one 1000 nmol/kg) and four TAT-NR2B9c treated (two 100 nmol/kg, one 300 nmol/kg, and one 1000 nmol/kg) animals. While the exact cause of the deaths could not be determined, it is possible that it reflects the severity of the stroke in this model, which is known to result in up to 90% of the affected hemisphere being infarcted by the stroke. For infarct volume analysis each treatment group consisted of six animals. Due to animal deaths before the 24-hour study end-point, four to six animals per group were available for behavioural testing.

2.3. Postsurgical Monitoring. The body temperature of animals was measured every 30–60 minutes using a rectal probe for at least 2 hours after surgery and maintained between 37.0 and 37.8°C. To avoid hypothermia, rat cages were placed on a heating mat during the postsurgical monitoring and housed in a holding room maintained at 26–28°C. If necessary, additional heating or cooling was performed by applying fan heating or a cold water spray.

2.4. Infarct Volume Assessment. Infarct volume was assessed 24 hours after MCAO as previously described [17]. Briefly 2 mm cerebral coronal brain slices were stained in 3% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich, St. Louis, USA). Digital images of coronal brain slices were acquired and analysed using ImageJ (3rd edition, NIH, Bethesda, USA) by an operator blind to treatment status. The total infarct volume was determined by measuring the areas of infarcted tissue on both sides of the 2 mm sections and corrected for cerebral oedema [17].

2.5. Behavioural Testing. In order to assess if any treatment improved sensorimotor outcomes, three behavioural tests were performed 24 hours after MCAO. A neurological assessment was performed using a five-point scale (0–5) developed by Bederson et al. [18]. Scores range between 0 for no deficits, 1 for flexed forepaw, 2 for inability to resist lateral push, 3 for circling, 4 for agitated circling, and 5 for unresponsiveness to stimulation/stupor. The adhesive tape removal test is a bilateral asymmetry paw test to assess sensorimotor impairment [19]. Adhesive tape (Diversified Biotech, Dedham, USA) 10 mm × 10 mm in size was placed on the palmar surface of the forepaw and the time taken for the first attempt to remove tape (time to detect tape), the
time taken to remove the tape, and the number of attempts to remove tape were recorded. Each forelimb was assessed sequentially starting with the unaffected side (right side) with animals having a maximum of 120 seconds to complete the task (normal rats usually take between 5 and 30 sec to remove the tape). Each rat was assessed three times on the day prior to the surgery and once 24 hours after MCAO. The rota-rod test assesses balance and coordination by assessing a rat’s ability to keep walking on a rotating rod, with the speed of rotation being progressively increased from 4 to 40 revolutions per minute. The time the animal falls was recorded.

2.6. Statistical Analysis. Total infarct volume and physiological parameters were evaluated by analysis of variance (ANOVA) followed by Fisher’s post hoc analysis. The neurological assessment measurements were analysed using Kruskal-Wallis test. Data from adhesive tape removal and rota-rod tests were analysed using ANOVA followed by Scheffé’s multiple comparison post hoc analysis. $P < 0.05$ was considered as significant. Data are presented as mean ± standard deviation (SD).

3. Results

3.1. Physiological Data, Infarct Volume Measurements, and Animal Deaths. Physiological parameters measured during surgery and before MCAO were within the normal range and did not differ significantly between animal treatment groups (Table 1).

Data on the mean total infarct volumes for each treatment group are presented in Figure 1. These results show that the R18 peptide significantly reduced infarct volume at doses of 100 nmol/kg and 1000 nmol/kg by 19.7% ($P = 0.043$) and 24% ($P = 0.013$), respectively, while, at the 300 nmol/kg, infarct volume was reduced by 12% ($P = 0.19$), albeit not to a statistically significant extent. By contrast, while the TAT-NR2B9c peptide at doses of 300 nmol/kg and 1000 nmol/kg reduced infarct volume by 6.8% ($P = 0.56$) and 7% ($P = 0.55$), respectively, these effects were not statistically significant. At 100 nmol/kg, TAT-NR2B9c was ineffective in reducing infarct volume. In comparative terms, at 100 nmol/kg R18 was significantly more effective in reducing infarct volume than TAT-NR2B9c (19.7% versus 1.1%, $P = 0.045$).

3.2. Functional Outcome Assessment. Although not statistically significant, there was a trend towards improvement in the performance in some of the behavioural parameters measured for R18 and TAT-NR2B9c treatment groups (Figures 2–4). Neurological scores for 100, 300, and 1000 nmol/kg R18 treatment animals showed improved outcomes compared with the vehicle-treated controls (Figure 2). By contrast, for TAT-NR2B9c, only the 1000 nmol/kg treatment group was associated with an improvement in neurological score compared to vehicle. Measurements for the adhesive tape test after MCAO were variable; however, treatment with the 300 nmol/kg R18 or 1000 nmol/kg TAT-NR2B9c appeared to improve the time required to detect tape from the right paw of the nonaffected forelimb, while the 100 nmol/kg TAT-NR2B9c treatment appeared to improve the time required to detect tape from the left paw (Figure 3(a)). Similarly, the number of attempts required to remove the tape from the right and left paw was increased in animals treated with 1000 nmol/kg and 100 nmol/kg TAT-NR2B9c, respectively (Figure 3(b)). Additionally, treatment with 1000 nmol/kg R18 was associated with the shortest time to remove tape from the right paw (Figure 3(c)). For the rota-rod test, the group receiving 100 nmol/kg R18 was the only treatment group

### Table 1: Physiological parameters (mean ± SD).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle (saline)</th>
<th>R18 (nmol/kg)</th>
<th>TAT-NR2B9c (nmol/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PaO$_2$, before MCAO</td>
<td>11.79 ± 2.44</td>
<td>11.66 ± 1.34</td>
<td>11.16 ± 1.37</td>
</tr>
<tr>
<td>PaCO$_2$, before MCAO</td>
<td>38.33 ± 2.94</td>
<td>39 ± 3.57</td>
<td>39.5 ± 3.50</td>
</tr>
<tr>
<td>pH, before MCAO</td>
<td>7.36 ± 0.05</td>
<td>7.37 ± 0.04</td>
<td>7.44 ± 0.06</td>
</tr>
<tr>
<td>Glucose (mmol/L), before MCAO</td>
<td>8.0 ± 1.37</td>
<td>8.15 ± 0.68</td>
<td>8.11 ± 1.00</td>
</tr>
<tr>
<td>BP (mmHg), average during surgery</td>
<td>87.33 ± 4.17</td>
<td>88.33 ± 7.06</td>
<td>91.6 ± 5.44</td>
</tr>
<tr>
<td>Temperature (°C), average 2h after surgery</td>
<td>37.51 ± 0.16</td>
<td>37.37 ± 0.25</td>
<td>37.46 ± 0.29</td>
</tr>
</tbody>
</table>
that displayed an increased time to remain on the rotating cylinder when compared to vehicle (118 sec versus 77 sec, Figure 4).

3.3. Weight Loss Measurement. All groups recorded a loss in body weight 24 hours after MCAO ranging from 28.5 grams for the TAT-NR2B9c 100 nmol/kg treatment group to =34.5 g for the 300 nmol/kg R18 and TAT-NR2B9c treatment groups (Figure 5).

4. Discussion

The results of the present study add to our previous findings, which showed that 1000 nmol/kg R18 when administered 30 minutes after permanent MCAO significantly reduces infarct volume in the rat [10]. Importantly, we now show that R18 is effective over an even wider therapeutic window (60 min) and broader dose range (100–1000 nmol/kg) and that, on balance, R18 is more effective than the extensively characterised neuroprotective peptide, TAT-NR2B9c. Treatment with R18, as well as to a lesser extent TAT-NR2B9c, resulted in some functional recovery as assessed by behavioural tests, but not to statistically significant levels, which most likely reflects the severity of the stroke model used coupled with the relatively small numbers of animals in the study. Notwithstanding these limitations, our findings highlight the potential clinical applicability of R18 as a therapeutic intervention in stroke, especially in light of evidence that it is superior as a neuroprotective agent to TAT-NR2B9c, which is planned to enter a phase 3 clinical trial in stroke patients [20]. The superior neuroprotective efficacy of R18 compared to TAT-NR2B9c is consistent with our in vitro findings in a glutamic acid induced neuronal excitotoxicity model of cell death [8].

The TAT-NR2B9c peptide has been shown to be neuroprotective in rodent [11, 21–25] and nonhuman primate stroke models [13, 14] and has been found to be safe and cause a nonsignificant reduction in ischaemic brain lesions in patients undergoing aneurysm surgery [15]. The NR2B9c peptide (KLSSIESDV) is derived from the intracellular terminal carboxyl region of the N-methyl-D-aspartate (NMDA) receptor NR2B subunit protein [11] and is fused to the arginine-rich TAT peptide (YRKKKRRKQRRR) to allow entry into the brain and neuronal cells. The NR2B9c peptide was designed to act as a competitive inhibitor of the PSD-95 adaptor protein (postsynaptic density-95) binding to the NR2B subunit protein and, in doing so, to block downstream cell signaling associated with overstimulation of the NMDA receptor, leading to nitric oxide synthase activation and subsequent production of nitric oxide; however, we [9] and others [25, 26] have proposed other mechanisms for neuroprotection.

As an alternative mechanism, we have proposed that the neuroprotective properties of TAT-NR2B9c are largely mediated by the TAT peptide itself [9], which we [7, 27] and others [28, 29] have previously reported to display modest neuroprotective properties. Furthermore, due to the TAT peptide’s arginine content and positive charge, it is likely to possess a similar mode of action as polyarginine and arginine-rich peptides [8, 9]. For arginine-rich peptides, we have previously hypothesised that at least in part neuroprotection is related to the ability of these peptides to transverse cell membranes and, in doing so, decrease the levels of cell surface ion channels and receptors, thereby reducing the toxic influx of calcium that occurs in neurons following cerebral ischaemia [8, 9]. This mechanism of action is in line with the confirmed ability of arginine-rich peptides to reduce glutamate excitotoxic calcium influx in cortical neurons [8, 9, 30–32] and evoke receptor currents in NR1-NR2 NMDA receptor-expressing oocytes [33], as well as the observation that peptide neuroprotective efficacy correlates with peptide endocytic or cell membrane transversing properties [34]. In addition, several studies have demonstrated that TAT fused peptides and arginine-rich cell penetrating peptides can reduce the expression of cell surface ion channels and receptors in neurons [26, 31, 35–38] and other cells [39].

There is evidence to indicate that arginine residues are critical elements for peptide and protein mitochondrial uptake [40–43] and that arginine-rich peptides exert beneficial effects on mitochondria. For example, in isolated rat liver mitochondria, cationic tetra- and polycationic peptides and especially those containing arginine were highly effective in blocking calcium induced mitochondrial swelling and in maintaining membrane potential [44]. Similarly, cationic compounds including tetrapeptides containing an arginine residue (e.g., SS-20, SS-31) or biguanidines (e.g., metformin) have been shown to target mitochondria and exert positive effects on the organelle by limiting complex I activity and reactive oxygen species production [41, 45], inhibiting the opening of the mitochondrial permeability transition pore [46], protecting cristae architecture [47], accelerating ATP recovery [47], and preventing cytochrome c release [48]. While the exact mechanisms for these beneficial effects on mitochondria are not fully known, the ability of cationic guanidino groups to interact with anionic phosphate groups of mitochondrial membrane phospholipids especially the inner membrane phospholipid cardiolipin (−2 net charge) may be a contributing factor.

Recently, Marshall et al. [49] confirmed the neuroprotective properties of polyarginine peptides in an in vivo NMDA-induced retinal ganglion cell excitotoxicity model and provided evidence that the peptides reduce neuronal mitochondrial oxidative stress. Furthermore, it was demonstrated in HEK293 cells that polyarginine peptides localise to

**Figure 2:** Neurological grading scores 24 hours after permanent MCAO (0 = no deficit, 5 = major deficit) for saline (vehicle) and peptide (R18 and TAT-NR2B9c at 100, 300, or 1000 nmol/kg) treatment groups. Lines on graph indicate range and median for neurological scores.
Figure 3: Functional assessment measurements using adhesive tape removal test before and 24 hours after MCAO for saline (vehicle) and peptide (R18 and TAT-NR2B9c at 100, 300, or 1000 nmol/kg) treatment groups. (a) Time to detect tape. (b) Number of attempts to remove tape. (c) Time to remove tape. Values are mean ± SD. Maximum time allowed for adhesive tape removal was 120 seconds.
Taken together, the results from the current study support other findings from our laboratory [8–10] and suggest that polyarginine and arginine-rich peptides may represent a new class of neuroprotective agents with enormous clinical potential for the treatment of acute and chronic neurological injuries. Importantly, it is possible that the reported beneficial effects of arginine-rich cell penetrating peptides fused to a “neuroprotective peptide” in animal studies of acute brain injury are largely attributable to the effects of the arginine residues contained within the peptide [9]. This adds to the growing weight of evidence suggesting that arginine-rich peptides (including R18) may be beneficial in a range of acute clinical neurological disorders. There is also evidence that arginine-rich peptides may improve functional recovery from central nervous system injury as evidenced by experimental studies on the effects of TAT-NR2B9c in stroke [60] and TAT-ISP in spinal cord injury [61]. Consequently, there is a growing body of evidence that supports the need for clinical studies on the effects of arginine-rich peptides to establish whether these peptides are equally beneficial in patients with stroke or other acute and chronic neurological disorders.

**Competing Interests**

B. P. Meloni and N. W. Knuckey are the holders of several patents regarding the use of arginine-rich peptides as neuroprotective treatments. The other authors declare no conflict of interests.

**Authors’ Contributions**

B. P. Meloni developed the theory and experimental design of study. D. Milani performed the stroke animal experiments, supervised the animal care, and analysed and interpreted the results. N. W. Knuckey and R. S. Anderton contributed to the development of the experimental design and analysis of results. J. L. Cross contributed to animal care and animal processing. D. Milani and B. P. Meloni wrote the first draft and final version of the paper.

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**References**


Chapter 6

Neuroprotective efficacy of poly-arginine R18 and NA-1 (TAT-NR2B9c) peptides following transient middle cerebral artery occlusion in the rat

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Neuroprotective efficacy of poly-arginine R18 and NA-1 (TAT-NR2B9c) peptides following transient middle cerebral artery occlusion in the rat

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Neuroprotective efficacy of poly-arginine R18 and NA-1 (TAT-NR2B9c) peptides following transient middle cerebral artery occlusion in the rat

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\textbf{A B S T R A C T}

We examined the efficacy of R18 in a transient MCAO model and compared its effectiveness to the well-characterized neuroprotective NA-1 peptide. R18 and NA-1 peptides were administered intravenously (30, 100, 300, 1000 nmol/kg), 60 min after the onset of 90 min of MCAO. Infarct volume, cerebral swelling and functional outcomes (neurological score, adhesive tape and rota-rod) were measured 24 h after MCAO. R18 reduced total infarct volume by 35.1% ($p = 0.008$), 24.8% ($p = 0.059$), 12.2% and 9.6% for the respective 1000 to 30 nmol/kg doses, while the corresponding doses of NA-1 reduced lesion volume by 26.1% ($p = 0.047$), 16.6%, 16.5% and 7%, respectively. R18 also reduced hemisphere swelling by between 46.1% (1000 and 300 nmol/kg; $p = 0.009$) and 24.4% (100 nmol/kg; $p = 0.066$), while NA-1 reduced swelling by 25.7% (1000 nmol/kg; $p = 0.054$). In addition, several R18 and NA-1 treatment groups displayed a significant improvement in at least one parameter of the adhesive tape test. These results confirm the neuroprotective properties of R18, and suggest that the peptide is a more effective neuroprotective agent than NA-1. This provides strong justification for the continuing development of R18 as a neuroprotective treatment for stroke.

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\textbf{1. Introduction}

Although endovascular recanalization therapy using thrombolysis (tissue plasminogen activator) alone, or more recently, in combination with thrombectomy provide a proven means of minimizing brain injury and improving outcomes after ischemic stroke (Rabinstein, 2016), it is estimated that only 7–15% of patients will be eligible for these interventions (Henninger and Fisher, 2016). Therefore, the need for an effective neuroprotective agent that can minimize ischemic brain injury when used alone and/or in combination with thrombolysis/thrombectomy cannot be overstated. For this reason, it is now important that any potential neuroprotective agent be evaluated in experimental stroke models that incorporate cerebral reperfusion since this mimics recanalization therapy (Sutherland et al., 2016).

A well-characterized and promising neuroprotective agent is the NR2B9c peptide fused to the arginine-rich carrier peptide TAT, which was originally known as TAT-NR2B9c and has been renamed NA-1. The NA-1 peptide is neuroprotective in a number of rodent (Aarts et al., 2002; Soriano et al., 2008; Sun et al., 2008; Bratane et al., 2011; Bell et al., 2013; Srejic et al., 2013; Teves et al., 2016; Xu et al., 2015) and non-human primate (Cook et al., 2012a,b) stroke models, and can reduce ischemic brain lesions in humans following endovascular aneurysm repair (Hill et al., 2012). We recently demonstrated that the R18 poly-arginine peptide was neuroprotective in a permanent middle cerebral artery occlusion (MCAO) stroke model in the rat (Milani et al., 2016a,b), and that the efficacy was superior to that of NA-1 (Milani et al., 2016b).

Mechanistically, we have shown that neuroprotective poly-arginine and arginine-rich peptides including the TAT-fused
neuroprotective peptides NA-1 and JNKI-1-TAT have the capacity to reduce excitotoxic neuronal calcium influx (Meloni et al., 2015a,b). With respect to the mechanism of action, we have proposed that poly-arginine and arginine-rich peptides including putative “neuroprotective peptides” fused TAT act through a common mechanism(s) largely mediated by arginine residues and the peptide’s positive charge (Meloni et al., 2015a,b). Beyond this, based on the known endocytic features of TAT, poly-arginine and arginine-rich cell penetrating peptides (Fotin-Mleczek et al., 2005; Kauffman et al., 2015) we have hypothesized (Meloni et al., 2015b) that one mechanism responsible for the ability of these peptides to reduce calcium influx is by reducing the levels of neuronal cell surface calcium ion channels. Consistent with this hypothesis, we have recently demonstrated that the poly-arginine peptide R12, as well the NA-1 peptide can reduce the neuronal cell surface expression of the glutamate receptor subunit NR2B (unpublished observation).

Other neuroprotective actions of arginine-rich peptides may be related to their ability to target mitochondria (Horton et al., 2008) through effects on limiting complex I activity and reactive oxygen species production, increasing tolerance to calcium influx and the opening of the mitochondrial permeability transition pore, protecting cristae architecture, accelerating ATP recovery and preventing cytochrome c release (Rigobello et al., 1995; Zhao et al., 2004; Szeto et al., 2011; Marshall et al., 2015; Birk et al., 2015). There is also the possibility that arginine-rich peptides may exert neuroprotective effects following stroke due to their ability to inhibit proteolytic enzymes such furin (Cameron et al., 2000) cathepsin C (Horn et al., 2000) and the proteasome (Wojcik and Di Napoli, 2004; Kloss et al., 2009; Doepner et al., 2015), and by binding to the apelin receptor (Zhou et al., 2003) to stimulate pro-survival cell signaling (Gu et al., 2013; Yang et al., 2016).

The results of the present study with R18 in primary MCAO studies (Milani et al., 2016a,b), the potential multiple neuroprotective modes of action of arginine-rich peptides and the recent positive endovascular recanalization trials, the present study examined the neuroprotective efficacy and dose responsiveness of R18 in a transient MCAO/reperfusion stroke model in the rat. The NA-1 peptide, which has undergone rigorous testing in previous preclinical stroke experiments and is also being explored clinically (Internet Stroke Centre, 2016), was included as a benchmark.

2. Materials and methods

2.1. Peptides used in the study

The R18 (H-RRRRRRRRRRRRRRRR-OH) and NA-1 (TAT-NR2B9c: H-YGRKKRRQRRR-KLSSIESDV-OH) peptides used in the study were synthesised by Mimotopes (Melbourne, Australia). Peptides were purified using high performance liquid chromatography to at least 98% purity, and were subject to peptide hydrolysis and amino acid liquid chromatography analysis to obtain a precise measure of peptide content (Mimotopes). Peptides were prepared in 0.9% sodium chloride for injection (Pfizer, Perth, Australia), aliquoted into a 650 μl volume in 3 ml syringes, and stored at −20 °C prior to use.

2.2. Animals ethics and study design

This study was approved by the Animal Ethics Committee of the University of Western Australia and follows the guidelines outlined in the “Australian Code for the Care and Use of Animals for Scientific Purposes”. In the design of these studies, every effort was made to follow both the STAIR (Fisher, 2011) and ARRIVE (Kilkenny et al., 2010) guidelines as appropriate.

2.3. Transient middle cerebral artery occlusion

Rats were subjected to 90 min of transient MCAO induced using an intraluminal filament as described previously (Campbell et al., 2013). Briefly, rats that had been fasted overnight underwent face-mask anesthesia with 4% isoflurane (mix 30% oxygen/70% nitrous oxide) and maintenance with 1.5–2% isoflurane. The tail artery was cannulated to allow for blood pressure monitoring and withdrawal of blood samples for measurement of arterial blood gases (PaO2, PaCO2), pH (ABL5, Radiometer, Copenhagen, Denmark) and plasma glucose levels (MediSense Optium, Abbott Laboratories, USA). The MCAO procedure was considered successful if there was a >25% decrease from baseline in cerebral blood flow after insertion of the filament to occlude the right middle cerebral artery, as measured by laser Doppler flowmetry. During surgery, body temperature was closely monitored using a rectal probe (Physitemp Instruments, Clifton, USA) and maintained between 37 and 37.8 °C, with fan heating or cooling, as required.

Sixty minutes after MCAO, treatments were administered (600 μl over 6 min) intravenously through the right internal jugular vein using an infusion pump. Treatment groups consisted of the vehicle (0.9% sodium chloride), and R18 or NA-1 peptides at four different doses (30, 100, 300 or 1000 nmol/kg). Treatments were randomized and all procedures were performed while being blinded to treatments.

2.4. Post-surgical analgesia and monitoring

At the conclusion of surgery pethidine was administered intramuscularly (1 mg in 0.2 ml saline) and bupivacaine was administered subcutaneously (0.1 mg in 0.2 ml saline per site) to tail and head wounds. The body temperature of animals was measured every 30–60 min using a rectal probe for at least 2 h after surgery, and maintained between 37 and 37.8 °C. To avoid hypothermia, rats were placed on a heating mat during the post-surgical monitoring and housed in a holding room maintained at 26–28 °C. If necessary, additional heating or cooling was performed by applying fan heating or a cold water spray.

2.5. Animals used and sample size

Sixty healthy male Sprague-Dawley rats weighing 275–340 g housed under controlled conditions on a 12-h light-dark cycle and with free access to food and water ad libitum underwent surgery for transient MCAO. Five animals were excluded from the study, one due to an insufficient decrease in cerebral blood flow following MCAO, two due to animal death during the surgical procedure, and two because no obvious infarct lesion was detected 24 h post-MCAO (treated with R18 at 100 or 300 nmol/kg). Another animal (treated with NA-1 at 300 nmol/kg) died several hours before the 24 h post-MCAO study end-point for an unknown reason, but was still included in the final infarct volume analysis. For infarct volume analysis, the vehicle treatment group consisted of seven animals while each peptide treatment group consisted of six animals.

In order to use the minimum number of animals to achieve our aims, we used an adaptive sample size trial design (Mehta and Patel, 2006). After interim analysis of data (N’s = 6–7) we uncovered statistically significant treatment effects and therefore achieving the main aims of the study without the need for additional animals.

2.6. Infarct volume measurement, cerebral edema and functional assessment

Infarct volume was assessed 24 h after MCAO as previously described (Campbell et al., 2013). Briefly, 2 mm thick cerebral coronal brain slices were stained with 2,3,5 triphenyltetrazolium
chloride (TTC; Sigma–Aldrich, St. Louis, USA) and digital images analysed using ImageJ software (3rd edition, NIH, Bethesda, USA) by an operator blind to treatment status. The total infarct volume was determined by measuring the areas of infarcted tissue on both sides of the 2 mm slices. The ratio of contralateral to stroke affected hemisphere areas was used to correct for cerebral edema. The cerebral edema ratio was also used to determine percentage cerebral swelling of the stroke affected hemisphere. In order to determine if peptide treatment improved sensorimotor outcomes, three functional tests were undertaken, namely neurological assessment, the adhesive tape removal test and the rota-rod test. These were performed by an operator blind to treatment status 24 h after MCAO, as previously described (Milani et al., 2016a).

2.7. Statistical analysis

Physiological parameters, mean total infarct volume measurement and data from adhesive tape removal and rota-rod tests for each treatment group were evaluated by analysis of variance (ANOVA) followed by Fisher’s post-hoc analysis. Data from the neurological assessment measurements were analysed using Kruskall–Wallis test. A value of p < 0.05 and/or p < 0.1 were considered as significant.

3. Results

3.1. Physiological data, infarct volume measurements and cerebral edema

Animal physiological parameters measured before, during and after MCAO did not show any significant differences between treatment groups (Supplementary Table 1).

Data on mean total infarct volume, percentage infarct volume reduction, and percentage cerebral hemisphere swelling as well as representative images of brain slices for each treatment group are presented in Fig. 1a–d. These results show that the R18 peptide significantly reduced infarct volume at doses of 1000 nmol/kg (by 35.1%; p = 0.008) and 300 nmol/kg (24.8%; p = 0.058). The R18 doses of 100 and 30 nmol/kg reduced infarct volume, albeit not statistically significantly, by 13.2% and 9.6% respectively. In comparison, the NA-1 peptide significantly reduced infarct volume at the 1000 nmol/kg by 26.1% (p = 0.047). The NA-1 doses of 300, 100 and 30 nmol/kg also reduced infarct volume by 16.7%, 16.6% and 7.2% respectively, but these effects were not statistically significant.

R18 reduced cerebral hemisphere swelling at doses of 1000, 300 and 100 nmol/kg doses by 46.1% (p < 0.009), 46.1% (p = 0.009) and 24.4% (p = 0.066), respectively, while NA-1 only reduced swelling at the highest 1000 nmol/kg dose by 25.7% (p = 0.054).

3.2. Functional outcomes and weight loss

R18 at doses of 30, 300 and 1000 nmol/kg and NA-1 at doses of 30, 100 and 1000 nmol/kg treatment led to significant improvement in one or more of the parameters assessed as part of the adhesive tape test (Fig. 2a–h). Although not statistically significant, several R18 and NA-1 treatment groups also displayed a trend towards improvements in the other functional test used (Fig. 2d and e). All treatment groups recorded a loss in body weight ranging from 18.2 g (R18; 300 nmol/kg) to 32.4 g (NA-1; 300 nmol/kg) (Supplementary Fig. 1).

4. Discussion

In previous permanent MCAO stroke studies, we demonstrated the neuroprotective effectiveness of poly-arginine peptides (R9, R12 and R18) (Meloni et al., 2015a; Milani et al., 2016a,b) and the superior efficacy of R18 compared to the well-characterized NA-1 peptide (Milani et al., 2016b). In the present study, using a transient MCAO with reperfusion stroke model we confirm the dose-dependent neuroprotective effect of R18 showing that the peptide reduces infarct volume (e.g., 35% vs 26% reduction at the 1000 nmol/kg dose) and cerebral edema (e.g., 46% vs 5% reduction at the 300 nmol/kg dose) to a greater extent than NA-1.

While several of the R18 and NA-1 treatment groups showed a significant improvement in at least one functional parameter of the adhesive tape test, in some cases this was for a p value of <0.1 or for a low dose treatment group (30 nmol/kg) that did not significantly reduce infarct volume. Similarly, there was a trend towards improvement in several of the R18 and NA-1 treatment groups in the other functional tests used (i.e., neurological assessment and rota-rod) but these differences were not statistically significant. The modest behavioral improvements obtained in the study most likely reflect the number of animals used, the high variability associated with behavioral outcomes after stroke and the short 24-h study end-point. In addition, the behavioral improvement for the low peptide dose is suggestive that at low concentration the peptides may have a positive effect on synaptic connectivity and/or plasticity that improves function, as has been recently demonstrated for the closely related NA-1 peptide TAT-HA-NR2B9c (Zhou et al., 2015). Although additional studies are required (e.g., long-term histological and function end-points, assessment in different stroke models, in different animal species, in aged animals and in female animals) to further evaluate the potential of R18 as a neuroprotective therapy in stroke, our present and past (Milani et al., 2016b) findings strongly indicate that R18 may be even more potent than NA-1, the efficacy of which is currently being evaluated in a Phase 3 clinical stroke trial (Internet Stroke Centre, 2016).

The apparent superior neuroprotective efficacy of the R18 peptide compared to the NA-1 peptide is consistent with findings in vitro excitotoxicity models. For example, NA-1 is neuroprotective following mild excitotoxicity (1 h exposure of cortical neurons to 100 μM NMDA in the presence CNQX and nimodipine to block secondary activation of AMPARs and VGCCs, respectively) (Aarts et al., 2002), but is ineffective against severe excitotoxicity (5 min exposure of cortical neurons to 100 μM glutamic acid), whereas R18, is highly neuroprotective (Meloni et al., 2015a).

As mentioned (see Introduction) arginine-rich peptides have to capacity to reduce excitotoxic neuronal calcium influx (Meloni et al., 2015a,b), exert beneficial effects on mitochondria (Rigobello et al., 1995; Zhao et al., 2004; Szeto et al., 2011; Marshall et al., 2015; Birk et al., 2015), inhibit proteolytic enzymes (Horn et al., 2000; Kloss et al., 2009) and induce cell survival signaling (Gu et al., 2013; Yang et al., 2016). Additionally, the capacity of R18 to reduce brain edema may be related to the ability of the peptide to indirectly inhibit activation of matrix metalloproteinases (MMP) and thereby maintain the blood brain barrier (BBB). Poly-arginine peptides and arginine-rich cell penetrating peptides are potent inhibitors of proprotein convertases, including furin (Cameron et al., 2000; Kacprzak et al., 2004; Fugere et al., 2007; Ramos-Molina et al., 2015). Furin is a ubiquitously expressed convertase that is up-regulated in the ischemic brain (Yokota et al., 2001; Yang et al., 2007), and can activate MMP2, MMP3 and MMP14 (also known as MT1-MMP) (Tian et al., 2011). Furthermore, MMP3 can activate MMP1, MMP7 and MMP9, and MMP14 can activate MMP2 and MMP2 can activate MMP9 (Yang and Rosenberg, 2015; Turner and Sharp, 2016). Significantly, following stroke MMP2, MMP3, MMP9 and MMP14 are associated with BBB disruption (Hosomi et al., 2005; Yang et al., 2007; Yang and Rosenberg, 2015; Turner and Sharp, 2016; Hafez et al., 2016), while MMP inhibition reduces BBB degradation, cerebral edema and tissue injury (Yang et al., 2007;
Fig. 1. Infarct volume analysis, cerebral edema, and representative images of coronal brain slices for the different treatment groups as determined 24 h after transient MCAO (90 min). Treatments were administered intravenously (saline vehicle or R18 and NA-1 peptide, doses in nmol/kg) 60 min after MCAO. (A) Infarct volume measurements. Values are mean ± SD. *p < 0.05 and #p < 0.1 when compared to the vehicle control group. †Denotes animal that died several hours before the 24-h post-MCAO study end-point, but was still included in the final infarct volume analysis. (B) Percentage infarct volume reduction for the different R18 and NA-1 doses when compared to the saline vehicle group. Values are means ± SD. (C) Percentage cerebral hemisphere swelling for vehicle and the different R18 and NA-1 doses. Values are means ± SD. *p < 0.05 and #p < 0.1 when compared to the vehicle control group. †p < 0.05 when compared to NA-1 300 nmol/kg dose. (D) Representative images of TTC stained coronal brain slices from vehicle, R18 and NA-1 treatment groups.

In summary, we have confirmed that the R18 peptide has the capacity to reduce infarct volume, and cerebral edema and improve at least some functional outcomes in a stroke model incorporating reperfusion, and have shown that R18 has even greater neuroprotective efficacy than the highly characterized NA-1 peptide. This proof of effectiveness of R18 in a stroke model associated with cerebral reperfusion is further evidence that the peptide has the potential to be of clinical benefit in stroke patients directly and by extending the therapeutic window of endovascular recanalization therapy. The positive results for R18 in this and previous studies supports the conclusion that poly-arginine peptides constitute a new class of neuroprotective agent with enormous potential as a treatment for stroke and other acute and chronic neurological disorders.

Yang and Rosenberg, 2015; Turner and Sharp, 2016; Hafez et al., 2016.)
Fig. 2. Functional measurements using the adhesive tape removal test, neurological assessment and rota-rod 24 h after MCAO for saline (veh = vehicle) and peptide (R18 and NA-1, doses in nmol/kg) treatment groups. (A) Time (sec = seconds) to detect tape. (B) Number of attempts to remove tape. (C) Time (sec = seconds) to remove tape. Values are means ± SD. Maximum time allowed for adhesive tape removal was 120 seconds. *P < 0.05 and #P < 0.1 when compared to the vehicle control group. (D) Neurological grading scores (0 = no deficit, 5 = major deficit) for saline (vehicle) and peptide (R18 and NA-1, doses in nmol/kg) treatment groups. Vertical lines on graph indicate the range and median for neurological scores. (E) Rota-rod performance (time remaining on rod; sec = seconds) for saline (vehicle) and peptide (R18 and NA-1, doses in nmol/kg) treatment groups. Values are means ± SD.
Authors contribution

B. P. Meloni developed the theory of the study. D. Milani and B. P. Meloni developed the experimental design and wrote the first draft and final version of the paper. D. Milani performed the animal experiments, supervised the animal care and analysed and interpreted the results. N. W. Knuckey, R. S. Anderton and D. J. Blacker contributed to the development of the experimental design and analysis of results. J. L. Cross contributed to animal care and animal processing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neures.2016.09.002.

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Chapter 7

Assessment of the neuroprotective effects of arginine-rich protamine peptides, poly-arginine peptides (R12-cylic, R22) and arginine-tryptophan containing peptides following \textit{in vitro} excitotoxicity and/or permanent middle cerebral artery occlusion in rats

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Assessment of the neuroprotective effects of arginine-rich protamine peptides, poly-arginine peptides (R12-cyclic, R22) and arginine-tryptophan containing peptides following in vitro excitotoxicity and/or permanent middle cerebral artery occlusion in rats

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Neville Knuckey: 2%
Jane Cross: 2%
Vince Clark: 8%
Adam Edwards: 4%
Ryan Anderton: 2%
David Blacker: 2%

Signed ………………………………… Signed …………………………………
Diego Milani                      Bruno Meloni (coordinating supervisor)
Assessment of the Neuroprotective Effects of Arginine-Rich Protamine Peptides, Poly-Arginine Peptides (R12-Cyclic, R22) and Arginine–Tryptophan-Containing Peptides Following In Vitro Excitotoxicity and/or Permanent Middle Cerebral Artery Occlusion in Rats

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Abstract We have demonstrated that arginine-rich and poly-arginine peptides possess potent neuroprotective properties with arginine content and peptide positive charge being particularly critical for neuroprotective efficacy. In addition, the presence of other amino acids within arginine-rich peptides, as well as chemical modifications, peptide length and cell-penetrating properties also influence the level of neuroprotection. Against this background, we have examined the neuroprotective efficacy of arginine-rich protamine peptides, a cyclic (R12-c) poly-arginine peptide and a R22 poly-arginine peptide, as well as arginine peptides containing tryptophan or other amino acids (phenylalanine, tyrosine, glycine or leucine) in in vitro glutamic acid excitotoxicity and in vivo rat permanent middle cerebral artery occlusion models of stroke. In vitro studies demonstrated that protamine and poly-arginine peptides (R12-c, R22) were neuroprotective. Arginine–tryptophan-containing peptides were highly neuroprotective, with R12W8a being the most potent arginine-rich peptide identified in our laboratory. Peptides containing phenylalanine or tyrosine substituted in place of tryptophan in R12W8a were also highly neuroprotective, whereas leucine, and in particular glycine substitutions, decreased peptide efficacy. In vivo studies with protamine administered intravenously at 1000 nmol/kg 30 min after MCAO significantly reduced infarct volume and cerebral oedema by 22.5 and 38.6%, respectively. The R12W8a peptide was highly toxic when administered intravenously at 300 or 100 nmol/kg and ineffective at reducing infarct volume when administered at 30 nmol/kg 30 min after MCAO, unlike R18 (30 nmol/kg), which significantly reduced infarct volume by 20.4%. However, both R12W8a and R18 significantly reduced cerebral oedema by 19.8 and 42.2%, respectively. Protamine, R12W8a and R18 also reduced neuronal glutamic acid-induced calcium influx. These findings further highlight the neuroprotective properties of arginine-rich peptides and support the view that they represent a new class of neuroprotective agent.

Keywords Poly-arginine · Protamine · Tryptophan · Excitotoxicity · Middle cerebral artery occlusion (MCAO) · Neuroprotection · Stroke

Introduction

Despite the fact that no neuroprotectants are currently clinically available for stroke and other acute brain injuries, there is widespread recognition that neuroprotection remains a legitimate and achievable therapeutic target (Tymianski 2014; Henninger and Fisher 2016; Hoque et al. 2016). Given this, it is imperative that the search for novel
neuroprotective agents continues with the goal of developing future neuroprotective therapeutics. To this end, we have recently established that cationic arginine-rich and poly-arginine peptides possess potent neuroprotective properties and have proposed that they represent a new class of neuroprotective agent (Meloni et al. 2014, 2015a, b; Milani et al. 2016a, b, 2017). Arginine-rich and poly-arginine peptides also possess cell-penetrating properties, a feature which appears to correlate with neuroprotective efficacy (Meloni et al. 2015a, b).

Our research has demonstrated in cortical neuronal cultures exposed to glutamic acid that arginine-rich peptides are highly neuroprotective with efficacy increasing with increasing arginine content and peptide charge and have identified poly-arginine R18 as one of the most potent poly-arginine peptides (Meloni et al. 2015a, b). Moreover, we have established that poly-arginine peptides exert a preconditioning neuroprotective effect lasting up to 4 h post-treatment and have the capacity to reduce excitotoxic calcium influx (Meloni et al. 2015a). In support of a mechanism whereby poly-arginine peptides act by reducing calcium influx, we have observed that R12, as well as the TAT-fused neuroprotective peptide TAT-NR2B9c (also known as NA-1), reduces neuronal cell surface expression of the glutamate receptor subunit protein, NR2B (MacDougall et al. 2017). Consistent with the findings of in vitro studies, we have also demonstrated that peptides R9, R12 and R18 reduce infarct volume in permanent and/or transient middle cerebral artery occlusion (MCAO) stroke models and that our lead peptide, R18, is consistently more effective than the extensively characterised NA-1 neuroprotective peptide (Meloni et al. 2015a; Milani et al. 2016b, 2017).

Given the evidence we have amassed of the neuroprotective effects of different poly-arginine and arginine-rich peptides both in vitro and in vivo, the primary aim of the present study was to identify additional and potentially more efficacious arginine-rich peptides. As the presence of other amino acids in arginine-containing peptides is known to influence the degree of neuroprotection (Meloni et al. 2015b), and given that neuroprotective efficacy appears to correlate with peptide cell-penetrating properties (Meloni et al. 2015a), we were particularly interested in exploring the influence of tryptophan residues on the degree of neuroprotection, as this amino acid is known to increase peptide endocytosis and cellular uptake (Bechara et al. 2013, 2015; Jobin et al. 2015; Rydberg et al. 2012; Walrant et al. 2012). In addition, protamine, which is an arginine-rich peptide consisting of approximately 30–32 amino acids (20–21 arginine) and has been in clinical use for over 50 years, was also examined. Protamine is administered intravenously to reverse the anti-coagulant effects of heparin, as well as added to an insulin preparation known as neutral protamine Hagedorn to slow the absorption of insulin. Furthermore, we also examined a low molecular weight protamine (LMWP) peptide derived from protamine, which has been described as a potential alternative heparin reversal agent, as well as a cell-penetrating carrier peptide (He et al. 2014).

Therefore, this study examines the neuroprotective efficacy of arginine–tryptophan-containing peptides or arginine-rich peptides containing other amino acids (i.e. phenylalanine, tyrosine, glycine or leucine), as well as arginine-rich protamine peptides, a cyclic poly-arginine peptide (R12-c) and the poly-arginine R22 peptide, in vitro following glutamic acid excitotoxicity. Following the neuroprotective assessment of peptides using the in vitro excitotoxicity model, protamine and the most neuroprotective arginine–tryptophan-containing peptide were assessed for neuroprotective efficacy following permanent MCAO in the rat. In addition, selected peptides were examined for their capacity to reduce glutamic acid-induced neuronal calcium influx. Previously characterised poly-arginine peptides, R11, R12, R15 or R18 were used as benchmarks for efficacy in the different studies.

**Materials and Methods**

**Neuronal Cultures**

Establishment of rat primary cortical cultures in Neurobasal/B27 supplement (Life Technologies, Australia) using cortical tissue obtained directly from E18 day embryos was as previously described (Meloni et al. 2014); however, some cultures were established from cortical tissue stored in Hibernate-E (Life Technologies)/2% B27 supplement for 2–7 days at 5 °C. Neurons were seeded into 96-well plastic plates (Nunc, Australia) or 96-well glass wells (7 mm diameter, ProTech, Australia) and maintained in a CO₂ incubator (5% CO₂, 95% air balance, 98% humidity) at 37 °C until use on days in vitro 10 to 14. Under these conditions, cultures routinely consist of >95% neurons and 1–5% astrocytes.

**Peptides**

Details of the peptides used in this study are provided in Tables 1 and 2. The poly-arginine, low molecular weight protamine and arginine-containing peptides also containing tryptophan, phenylalanine, tyrosine, glycine or leucine residues were synthesised by Mimotopes (Australia). Protamine sulphate for injection (referred to as protamine) was purchased from Sanofi-Aventis, and other protamine peptides (protamine 1–5; Ptm 1–5) were synthesised by Pepmic (China). All synthesised peptides were purified by
Table 1  Protamine peptides used in study

<table>
<thead>
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<th>Peptidea</th>
<th>Sequenceb</th>
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<tr>
<td>Protamine sulphate; Protamine</td>
<td>Mixture of protamine peptides Ptm 1–4</td>
</tr>
<tr>
<td>Protamine 1; Ptm 1</td>
<td>PRRRRRSSSRPSSSSSSRPRASRRRRRGGRRRR</td>
</tr>
<tr>
<td>Protamine 2; Ptm 2</td>
<td>PRRRRSSSRPVRRRRRPRVSRRRRRGGRRRR</td>
</tr>
<tr>
<td>Protamine 3; Ptm 3</td>
<td>PRRRRSSSRPVRRRRRPRVSRRRRRGGRRRR</td>
</tr>
<tr>
<td>Protamine 4; Ptm 4</td>
<td>PRRRASRRRRRPRVSRRRRRGGRRRR</td>
</tr>
<tr>
<td>Protamine 5; Ptm 5</td>
<td>PRRRRSSSRPVRRRRRPRVSRRRRRGGRRRR</td>
</tr>
<tr>
<td>Low molecular weight protamine; LMWP</td>
<td>VSRRRRRGGRRRR</td>
</tr>
</tbody>
</table>

a Ptm 1–4 were identified following high-performance liquid chromatography and peptide sequence analysis of commercial preparations of protamine sulphate produced from salmon milt (Hoffmann et al. 1990). Protamine sulphate is likely to consist of Ptm 1–4 as the sulphate salt. Ptm 5 is the protamine peptide sequence provided in the SwissProt database. The LMWP sequence is derived from Ptm 5 (sequence underlined). Ptm 1–5 peptides have H and OH amino and carboxyl terminals.
b Arginine residues are indicated in bold text, while other amino acids are indicated in regular text.
R arginine, S serine, A alanine, V valine, G glycine, P proline, I isoleucine.

Table 2  Arginine peptides used in study

<table>
<thead>
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<th>Peptide</th>
<th>Sequencea</th>
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<tbody>
<tr>
<td>R11</td>
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<td>R12</td>
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<td>R15</td>
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<td>R18</td>
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<td>R12G8</td>
<td>GRRRRRRRRRRRRGG</td>
</tr>
</tbody>
</table>

a Unless indicate all peptide have H and OH amino and carboxyl terminals. Arginine residues are indicated in bold text, while other amino acids are indicated in regular text. R arginine, W tryptophan, F phenylalanine, Y tyrosine, L leucine, G glycine. R12-c is a cyclic peptide. D-isoform amino acids indicated by lower case.
high-performance liquid chromatography to 93–99% purity, with the exception of the cyclic R12 peptide (R12-c), which was 84% pure. An arginine–tryptophan peptide (R10W4D) was also synthesised containing D-isof orm acids. All peptides were prepared as 100× stocks (500 μM) in water for irrigation (Baxter, Australia).

Glutamic Acid Excitotoxicity Model and Peptide Treatments

Peptides were added to culture wells 10 min prior to glutamic acid (L-glutamic acid; Sigma-Aldrich) exposure by removing media and adding 50 μL of MEM/B27 (Minimal Essential Medium plus 2% B27 supplement; Life Technologies) containing the specific peptide. To induce excitotoxicity, 50 μL of MEM/B27 containing glutamic acid (200 μM; final concentration 100 μM) was added to the culture wells and incubated at 37 °C in the CO₂ incubator for 5 min. After the 5-min exposure, media were replaced with 100 μL of Neurobasal/B27 and cultures incubated for a further 24 h at 37 °C in the CO₂ incubator. Untreated controls with or without glutamic acid treatment underwent the same incubation steps and media additions. Note that in this model neurons are exposed to peptide for 10 min prior to, and during (half-peptide concentration) the 5-min glutamic acid insult.

Intracellular Calcium Kinetics

Intracellular calcium influx was monitored in neuronal culture wells (glass wells) using Fura-2 AM in real-time using a fluorescence plate reader. The aim of these experiments was to determine the relative change in intracellular calcium before and after glutamic acid exposure. Cells were loaded with the fluorescent calcium ion indicator Fura-2-AM (5 μM) in 50 μL MEM/B27, 0.1% Pluronic F-127, for 20 min at 37°C in the CO₂ incubator. Fura-2-AM solution was removed from wells, replaced with 50 μL MEM/B27 containing peptide (0.02–5 μM) or glutamate receptor blockers (5 μM MK801/5 μM CNQX) and incubated for 10 min at 37°C in the CO₂ incubator. Control cultures received 50 μL of MEM/B27 only. Following the 10-min incubation period, media in wells were replaced with 50 μL of balanced salt solution (116 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄ and 1 mM NaH₂PO₄, pH 7) and wells were transferred to a spectrophotometer (BMG Labtec, CLARIOstar) while maintaining the temperature at 37 °C. Fifty microlitres of MEM/B27 containing glutamic acid (200 μM; 100 μM final concentration) was added to wells, and every 5 s starting 30 s before and for 2 min after glutamic acid addition, spectrophotometer measurements (excitation: 355 nm/emission 495 nm) were recorded. Experiments were performed in triplicate and fluorescent intensity data were converted to percentage change in fluorescence. The average non-glutamic acid control well readings were taken as baseline (0%), and the average glutamic acid-treated control well readings after glutamic acid addition were taken as 100% calcium influx.

Neuronal Viability Assessment

At different times after treatments (e.g. 0.5–4 h and 18–24 h), cultures were examined by light microscopy to qualitatively assess neuronal cell viability. Neuronal viability was quantitatively measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulphophenyl)-2H-tetrazolium salt (MTS) assay (Promega, Australia). MTS absorbance data were converted to reflect proportional cell viability relative to both the untreated (no insult) and treated (glutamic acid) controls, with the untreated control taken as 100% viability. Based on light microscopy, neuronal cell survival in glutamic acid-treated control wells typically ranges from 2 to 15%. All data are presented as mean ± standard error (SE). As previously reported, in some studies different concentrations of peptide increased the ability of neurons to reduce the MTS substrate above no insult control levels following glutamic acid excitotoxicity (Meloni et al. 2014; Meloni et al. 2015a) and likely represent a direct or indirect effect of the peptide to increase the capacity of neurons to reduce MTS and/or to reduce background cell death in neuronal cultures.

Permanent Middle Cerebral Artery Occlusion Rat Stroke Model

This study was performed in accordance with the Animal Ethics Committee of the University of Western Australia and the guidelines outlined by the Australian Code for the Care and Use of Animals for Scientific Purposes. Healthy male Sprague–Dawley rats weighing 275–340 g were housed under controlled conditions with 12 h light/dark cycle and had ad libitum access to food and water. Experimental animals were fasted overnight and subjected to permanent MCAO induced using an intraluminal filament as described previously (Milani et al. 2016a). Briefly, rats that had been fasted overnight underwent facemask anaesthesia with 4% of isoflurane (mix 30% oxygen/70% nitrous oxide) and maintenance with 1.5–2% isoflurane. The tail artery was cannulated to allow for blood pressure monitoring and withdrawal of blood samples for measurement of arterial blood gases (PaO₂, PaCO₂), pH (ABL5, Radiometer, Copenhagen, Denmark) and plasma glucose levels (MediSense Optium, Abbott Laboratories, USA). The MCAO procedure was considered successful if
there was a >25% decrease from baseline in cerebral blood flow after insertion of the filament to occlude the right middle cerebral artery, as measured by laser doppler flowmetry. During surgery, body temperature was closely monitored using a rectal probe (Physitemp Instruments, Clifton, USA) and maintained between 37 and 37.8°C, with fan heating or cooling, as required.

Thirty minutes after MCAO, treatments were administered (600 µl over 6 min) intravenously through the right internal jugular vein using an infusion pump. In one study, treatment groups consisted of the vehicle (0.9% sodium chloride) and protamine 1000 nmol/kg (Protamine study). In a second study, treatment groups consisted of the vehicle (0.9% sodium chloride), and R12W8a or R18 peptides at 30 nmol/kg (R12W8a study). In addition, two animals were treated with R12W8a at the 300 nmol/kg dose and one animal at 100 nmol/kg dose. Treatments were randomised and all procedures were performed while being blinded to treatments.

Post-surgical Analgesia and Monitoring

At the conclusion of surgery, pethidine was administered intramuscularly (1 mg in 0.2 ml saline) and bupivacaine was administered subcutaneously (0.1 mg in 0.2 ml saline per site) to tail and head surgical wounds. The body temperature of animals was measured every 30–60 min using a rectal probe for at least 2 h after surgery and maintained between 37 and 37.8°C. To avoid hypothermia, rat cages were placed on a heating mat during the post-surgical monitoring and housed in a holding room maintained at 26–28°C. If necessary, additional heating or cooling was performed by applying fan heating or a cold water spray.

Animals Used and Sample Size

Protamine study: Twenty-five rats underwent surgery for permanent MCAO. Five animals were excluded from the study: two animals died shortly after surgery due to sub-arachnoid haemorrhage, one animal was excluded due to an insufficient decrease in cerebral blood flow following insertion of the monofilament, one animal was excluded due to pyrexia, and one died during surgical recovery for an unknown reason. The vehicle treatment group consisted of twelve animals, and the protamine treatment group consisted of eight animals.

R12W8a study: Twenty-three rats underwent surgery for permanent MCAO. Two animals were excluded from the study due to an insufficient decrease in cerebral blood flow following insertion of the monofilament. The vehicle treatment group consisted of seven animals, the R18 treatment group consisted of six animals, and the R12W8a treatment group consisted of eight animals.

In order to use the minimum number of animals to achieve our aims in the protamine and R12W8a studies, we used an adaptive sample size trial design (Mehta and Patel 2006). After processing 6–7 animals per treatment group, interim analysis of infarct volume data uncovered statistically significant treatment effects, therefore achieving the main aims of the study without the need for the inclusion of additional animals.

Infarct Volume Measurement and Functional Assessment

Infarct volume was assessed 24 h after MCAO as previously described (Campbell et al. 2008). Briefly, 2-mm-thick cerebral coronal brain slices were stained with 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich, St. Louis, USA) and digital images analysed using ImageJ software (3rd edition, NIH, Bethesda, USA) by an operator blind to treatment status. The total infarct volume was determined by measuring the areas of infarcted tissue on both sides of the 2-mm slices. The ratio of contralateral to stroke affected hemisphere areas was used to determine and correct for cerebral oedema. In order to determine if peptide treatment improved sensorimotor outcomes, three functional tests were undertaken, as previously described (Milani et al. 2016a), namely neurological assessment, the adhesive tape removal test and the rota-rod test. An operator blind to treatment status performed the functional tests 24 h after MCAO.

Statistical Analysis

Neuronal viability data were analysed by ANOVA, followed by post hoc Fisher’s PLSD test, with \( P < 0.05 \) values considered statistically significant. At least four wells were used in all assays and usually repeated a minimum of two times independently. Physiological parameters, mean total infarct volume measurement and data from adhesive tape removal and rota-rod tests for each treatment group were evaluated by analysis of ANOVA followed by Fisher’s post hoc analysis. Data from the neurological assessment measurements were analysed using Kruskal–Wallis test. A value of \( P < 0.05 \) was considered significant.

Results

Effects of Different Arginine-Rich Protamine Peptides on Cultured Neurons Exposed to Glutamic Acid Excitotoxicity

Protamine consists of four highly homologous arginine-rich peptides (Ptm 1–4; Table 1) and is used
intravenously to reverse the anti-coagulant effects of heparin. In addition, a closely related protamine peptide (Ptm 5) has been described, as well as a low molecular weight protamine (LMWP) peptide derived from the last 14 carboxy-terminal amino acids of Ptm 5. We observed that protamine, Ptm 1–5, and to a lesser extent LMWP were neuroprotective following glutamic acid excitotoxicity (Fig. 1a–c). In addition, protamine was neuroprotective when neuronal cultures were pre-treated with peptide for 10 min 1 or 2 h before glutamic acid exposure (Fig. 1d). In contrast, pre-treatment with LMWP 1 or 2 h before the glutamic acid insult was not neuroprotective (Fig. 1d).

**Effects of Cyclic R12 (R12-c), Poly-Arginine R22 and Arginine–Tryptophan-Containing Peptides (R11W2, R10W4D) on Cultured Neurons Exposed to Glutamic Acid Excitotoxicity**

We first assessed whether peptide cyclisation, increasing poly-arginine peptide length beyond R18 (current lead peptide) to R22, or the addition of tryptophan residues (R11W2), would significantly enhance peptide neuroprotective efficacy. We observed that the R12-c, R22 and R11W2 peptides displayed a high degree of neuroprotection, but that efficacy did not appear to be significantly greater than the R12 and R18 peptides (Fig. 2).

**Fig. 1** Glutamic acid excitotoxicity model: protamine peptide dose response (a–c) and pre-treatment experiments (d). a–c Peptides present in neuronal cultures for 10 min before and during 5-min glutamic acid exposure. R15 peptide used as a positive control. d Peptides present in neuronal cultures for 10 min only at 0, 1 or 2 h before glutamic acid exposure. Neuronal viability measured 20–24 h following glutamic acid exposure. Concentration of peptide in μM. Glut. cont. glutamic acid control. MTS data were expressed as percentage neuronal viability with no insult control taken as 100% viability. Values are mean ± SE; n = 4; *P < 0.05
the high degree of neuroprotection observed with R11W2, which contains only 11 arginine residues, appears to suggest that tryptophan residues are beneficial in terms of improving peptide efficacy (e.g., R11W2 versus R18 at 0.5 μM; Fig. 2). In addition, the D-isoform peptide, R10W4D, displayed a level of neuroprotective similar to that with R11W2 (Fig. 4a).

Effects of Increasing Tryptophan Content in Arginine-Containing Peptides on Neuroprotection in Cultured Neurons Exposed to Glutamic Acid Excitotoxicity

Given the level of neuroprotection obtained with R11W2 and R10W4D, we examined the neuroprotective efficacy of peptides containing increasing numbers of tryptophan and/or arginine residues. Peptides containing 2–3 arginine residues and 6–8 tryptophan residues did not display any neuroprotection following glutamic acid excitotoxicity (Fig. 3a). Peptides containing 10–13 arginine residues and 2–5 tryptophan residues displayed a neuroprotective effect similar to R18 (Fig. 3b, c). In contrast, peptides containing ≥10 arginine residues and ≥6 tryptophan residues displayed a high degree of neuroprotection, in most cases achieving 80–100% neuroprotection at 0.2 μM (Fig. 3d, e). In comparison, R18 showed little to no neuroprotection at 0.2 μM following glutamic acid excitotoxicity (data not shown). To assess if the sequence arrangement of arginine and tryptophan residues influences peptide neuroprotective efficacy, the efficacy of peptides R12W8a, R12W8b and R12W8c was compared following glutamic acid excitotoxicity. While all three arginine–tryptophan-containing peptides displayed a high degree of neuroprotection, R12W8a showed the greatest efficacy, with the peptide achieving 100% neuroprotection at 0.01 and 0.02 μM concentrations, while R12W8b and R12W8c peptides only achieved around 20% neuroprotection at 0.01 μM and 50–60% neuroprotection at 0.02 μM (Fig. 3e).

Effect of Substituting Tryptophan Residues in R12W8a with Phenylalanine, Tyrosine, Leucine or Glycine on Neuroprotection in Cultured Neurons Exposed to Glutamic Acid Excitotoxicity

Given the positive results obtained with arginine and tryptophan-containing peptides, we examined the effect of substituting tryptophan residues in R12W8a with phenylalanine (R12F8), tyrosine (R12Y8), leucine (R12L8) or glycine (R12G8) residues on neuroprotective efficacy following glutamic acid excitotoxicity. The results revealed that R12F8, and to lesser extent R12Y8, displayed high neuroprotective efficacy, but not to the same level as displayed by R12W8a. In contrast, R12L8 and in particular R12G8 displayed significantly reduced neuroprotective efficacy compared to R12W8a (Fig. 4a, b).

Calcium Influx Studies

To investigate the mechanism of action of neuroprotective peptides, we compared the effects relative to controls (no peptide) of protamine, R18, and R12W8a on intracellular calcium levels follow glutamic acid exposure. In line with our previous findings (Meloni et al. 2015a), all peptides to varying degrees were shown to reduce intracellular neuronal calcium levels when administered for 10 min before glutamic acid exposure (Fig. 5a–c).
Fig. 3 Glutamic acid excitotoxicity model; poly-arginine and arginine–tryptophan peptide dose response experiments (a–e). Peptides present in neuronal cultures for 10 min before and during 5-min glutamic acid exposure. R11, R15 and R18 peptides used as a positive controls. Neuronal viability measured 20–24 h following glutamic acid exposure. Concentration of peptide in μM. Glut. cont. glutamic acid control. MTS data were expressed as percentage neuronal viability with no insult control taken as 100% viability. Values are mean ± SE; n = 4; *P < 0.05

Fig. 4 Glutamic acid excitotoxicity model; dose response experiments of peptides containing arginine–tryptophan, arginine–phenylalanine, arginine–tyrosine, arginine–glycine or arginine–leucine (a and b). Peptides present in neuronal cultures for 10 min before and during 5-min glutamic acid exposure. Neuronal viability measured 20–24 h following glutamic acid exposure. Concentration of peptide in μM. Glut. cont. glutamic acid control. MTS data were expressed as percentage neuronal viability with no insult control taken as 100% viability. Values are mean ± SE; n = 4; *P < 0.05
Animal Neuroprotection Studies

Based on the positive results obtained for protamine and tryptophan–arginine-containing peptides following glutamic acid excitotoxicity, we undertook studies to assess the neuroprotective efficacy of protamine (protamine study) and R12W8a (R12W8a study) following permanent MCAO in the rat. In the R12W8a study, the R18 peptide was included as a positive benchmark. Note that the R18 peptide had already been assessed at the 1000 nmol/kg dose when administered 30 min after permanent MCAO (Milani et al. 2016a) and therefore...
Physiological Data, Infarct Volume, Cerebral Oedema and Adverse Events

Protamine study: data from 20 animals were available for the study. Animal physiological parameters measured before and during MCAO did not show significant differences between treatment groups (Table 3). When compared with the vehicle treatment group, infarct volume was reduced by 22.5% in the protamine treatment group \( (P = 0.018) \) (Fig. 6a, b), while cerebral oedema was reduced by 38.6% \( (P = 0.001) \) (Fig. 6c).

R12W8a study: Data from 21 animals were available for the study. Animal physiological parameters measured before and during MCAO did not show significant differences between treatment groups (Table 4). When compared with the vehicle treatment group, infarct volume was reduced in the R18 treatment group by 20.4% \( (P = 0.034) \), while R12W8a was ineffective in reducing infarct volume (Fig. 7a, b). In contrast, cerebral oedema was significantly reduced in both the R18 and R12W8a treatment groups by 42.2% \( (P < 0.001) \) and 19.8% \( (P = 0.027) \), respectively, compared with the vehicle treatment group (Fig. 7c).

In addition to the animals treated with R12W8a at a dose of 30 nmol/kg, two animals were treated at a dose of 300 nmol/kg and one animal at a dose of 100 nmol/kg. These animals displayed a reduction in blood pressure commencing 1 min into the 6-min intravenous peptide infusion, a reduction that continued to gradually decrease to 30–50 mmHg by several minutes after the completion of the infusion, after which time animals became cyanotic. Ten to 15 min after the completion of the peptide infusion, blood pressure levels and cyanotic features in the animals did not improve and therefore the animals were euthanased. The blood pressure and cyanotic adverse effects were not observed for R12W8a at a dose of 30 nmol/kg.

Table 3 Protamine study: physiological parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle (saline)</th>
<th>Protamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>PaO2, before MCAO</td>
<td>115.1 ± 33.51</td>
<td>122.0 ± 16.66</td>
</tr>
<tr>
<td>PaCO2, before MCAO</td>
<td>44.92 ± 5.82</td>
<td>40.63 ± 4.53</td>
</tr>
<tr>
<td>pH, before MCAO</td>
<td>7.44 ± 0.09</td>
<td>7.36 ± 0.05</td>
</tr>
<tr>
<td>Glucose, before MCAO</td>
<td>7.74 ± 1.27</td>
<td>6.56 ± 1.2</td>
</tr>
<tr>
<td>BP, average during surgery</td>
<td>89.0 ± 8.44</td>
<td>76.06 ± 28.96</td>
</tr>
<tr>
<td>Temp, average 2 h after surgery</td>
<td>37.48 ± 0.18</td>
<td>37.5 ± 0.2</td>
</tr>
</tbody>
</table>

PaO2, PaO2, and BP in mmHg, glucose in mmol/L and temperature in °C. BP mean arterial blood pressure, Temp temperature. Data are mean ± SD

Behavioural Outcomes and Weight Loss Measurements

Protamine study: compared to the vehicle treatment group, no significant differences were observed with protamine in the neurological, rota-rod and adhesive tape tests (Fig. 6d–h) or in weight loss (data not shown).

R12W8a study: only a neurological assessment was undertaken in the study and compared to the vehicle treatment group, no significant differences were detected for R12W8a and R18 (Fig. 7d). Moreover, there were no significant differences in weight loss between the any of the treatment groups (data not shown).

Discussion

The present study extends our previous findings regarding the neuroprotective effects of poly-arginine and arginine-rich peptides (Meloni et al. 2014, 2015a, b; Milani et al. 2016a, b, 2017). In doing so, the results for the first time demonstrate the neuroprotective action of arginine-rich protamine peptides following in vitro neuronal excitotoxicity or in vivo following permanent MCAO. These findings are hardly surprising given the arginine content and positive charge of arginine-rich protamine peptides, and given our previous studies highlighting the critical importance of arginine residues and peptide positive charge for neuroprotection. Interestingly, and consistent with the features associated with arginine-rich peptides, the LMWP peptide has been previously used as a cell-penetrating peptide (He et al. 2014). In addition, due to the side effects associated with protamine, including anaphylactic reactions and systemic and pulmonary hypotension, LMWP along with the poly-arginine R15 peptide has been evaluated as safer heparin reversal agents (Byun et al. 1999; Li et al. 2015).

Also of interest is a study demonstrating that mice fed a nucleoprotamine diet for 1 week prior to transient forebrain ischaemia displayed significantly reduced CA1 hippocampal neuronal injury, compared to mice fed similar diets not containing protamine (Matsunaga et al. 2003). However, the neuroprotective mechanism associated with the nucleoprotamine diet was attributed to increased cerebral blood flow during forebrain ischaemia. It was postulated that the nucleoprotamine diet increased levels of circulating arginine available for vascular endothelial cells to generate nitric oxide, through endothelial nitric oxide synthase. Whether this neuroprotective mechanism applies to intravenously administered protamine and poly-arginine peptides is presently unknown. Nevertheless, as previously demonstrated for arginine-rich peptides, we were able to confirm that protamine reduces excitotoxic neuronal intracellular calcium levels. Interestingly, protamine can...
inhibit endoplasmic reticulum ryanodine receptor activity (Koulen and Ehrlich 2000; Diaz-Sylvester and Copello 2009), and the release of calcium from this organelle through ryanodine channels is known to contribute to neuronal excitotoxicity (Ruiz et al. 2009). As the positive charge of protamine was attributable to its inhibitory action on the ryanodine receptor, it is likely that other cationic arginine-rich peptides will have a similar effect on the receptor.

The current study also demonstrated that the cyclic polyarginine peptide, R12-c, is neuroprotective, a finding that suggests that structural conformations associated with linear peptides (e.g. α-helix, β-sheets, β-turns) are not critical for neuroprotection. These findings also indicate that a more generic mechanism may be involved in eliciting neuroprotection, particularly one that is dependent on electrostatic interactions of an arginine-rich peptide with target molecules, such as negatively charged plasma.

Fig. 6 Infarct volume measurements, representative images of coronal brain slices, cerebral edema and functional assessment data 24 h after permanent MCAO for protamine study (A–H). Treatments were administered intravenously (saline vehicle or protamine peptide at 1000 nmol/kg; 600 μl volume over 6 min) 30 min after MCAO. Unless indicated, values are mean ± SD. *P < 0.05 when compared to the vehicle control group. a Mean total infarct volume. b Representative images of TTC-stained coronal brain slices. c Percentage increase in cerebral edema as compared to contralateral hemisphere. d Neurological grading scores (0 = no deficit, 5 = major deficit). Lines on graph indicate range and median for neurological scores. e Rota-rod performance. f–h Adhesive tape removal test. Protamine = Ptm; (F) time to detect tape, (G) time to remove tape, and (H) number of attempts to remove tape. Maximum time allowed for adhesive tape removal was 120 s.
Table 4 R12W8a study: physiological parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle (saline)</th>
<th>R18</th>
<th>R12W8a</th>
</tr>
</thead>
<tbody>
<tr>
<td>PaO2, before MCAO</td>
<td>117.33 ± 9.79</td>
<td>122.50 ± 10.67</td>
<td>121.33 ± 19.82</td>
</tr>
<tr>
<td>PaCO2, before MCAO</td>
<td>41.85 ± 1.46</td>
<td>41.83 ± 1.94</td>
<td>42.42 ± 0.78</td>
</tr>
<tr>
<td>pH, before MCAO</td>
<td>7.33 ± 0.02</td>
<td>7.33 ± 0.06</td>
<td>7.34 ± 0.06</td>
</tr>
<tr>
<td>Glucose, before MCAO</td>
<td>5.67 ± 1.7</td>
<td>6.21 ± 2.05</td>
<td>5.42 ± 1.0</td>
</tr>
<tr>
<td>BP, average during surgery</td>
<td>82.6 ± 10.28</td>
<td>84.20 ± 5.44</td>
<td>86.25 ± 6.67</td>
</tr>
<tr>
<td>Temp, average 2 h after surgery</td>
<td>37.24 ± 0.28</td>
<td>37.38 ± 0.3</td>
<td>37.21 ± 0.33</td>
</tr>
</tbody>
</table>

PaO2, PaO2, and BP in mmHg, glucose in mmo/L and temperature in °C. BP mean arterial blood pressure, Temp temperature. Data are mean ± SD.

Fig. 7 Infarct volume measurements, representative images of coronal brain slices, cerebral oedema and neurological grading scores 24 h after permanent MCAO for R12W8a study (a–d). Treatments were administered intravenously (saline vehicle or R18 and R12W8a peptide at 30 nmol/kg; 600 μl volume over 6 min) 30 min after MCAO. Unless indicated, values are mean ± SD. *P < 0.05 when compared to the vehicle control group.

b Representative images of TTC-stained coronal brain slices. c Percentage increase cerebral oedema as compared to contralateral hemisphere.

d Neurological grading scores (0 = no deficit, 5 = major deficit). Lines on graph indicate range and median for neurological scores.
membrane structures such a heparan sulphate proteoglycans, phospholipid head groups or receptors and anionic domains of proteins. With respect to interactions with the plasma membrane, we have proposed that the ability of arginine-rich peptides to reduce excitotoxic neuronal intracellular calcium levels involves a process whereby the peptides decrease the levels of cell surface ion channels and receptors, possibly by endocytosis (Meloni et al. 2015b). For example, we have shown that R12 and TAT-NR2B9c can reduce neuronal cell surface expression of the NR2B NMDA receptor subunit protein (McDougall et al. 2016). This mechanism of action is in line with the confirmed ability of arginine-rich peptides to reduce the activity of other calcium ion channels and receptors (Tu et al. 2010; Brittain et al. 2011; Brustovetsky et al. 2014; Meloni et al. 2015a, b; Moutal et al. 2014; Ferrer-Montiel et al. 1998), and the fact that neuroprotective efficacy correlates with peptide endocytic or cell membrane transversing properties (Mitchell et al. 2000; Bechara et al. 2013, 2015; Jobin et al. 2015; Rydberg et al. 2012; Walrant et al. 2012). In addition, the arginine-rich cell-penetrating peptides TAT, penetratin and R9 induce the internalisation of EGFR and TNFR in HeLa cells (Fotin-Mleczek et al. 2005). Since a critical step involved in peptide endocytosis is an electrostatic interaction with negatively charged plasma membrane moieties, this could explain why cyclic poly-arginine peptides retain neuroprotective properties. Cyclic arginine-rich peptides are also known to retain cell-penetrating properties (Oh et al. 2014; Traboulsi et al. 2015; Qian et al. 2016).

In an attempt to identify what are the most effective arginine-rich peptides compared to R18, we demonstrated that the R22 peptide was less effective, while tryptophan–arginine-containing peptides were more effective. The improvement in neuroprotective efficacy with arginine-rich peptides containing the aromatic hydrophobic amino acid, tryptophan, is not surprising. Tryptophan residues increase the endocytic uptake of arginine-rich cell-penetrating peptides (Bechara et al. 2013, 2015; Jobin et al. 2015; Rydberg et al. 2012; Walrant et al. 2012), and we have previously demonstrated that peptide neuroprotective efficacy correlates with peptide endocytic properties (Meloni et al. 2015a, b). In addition, tryptophan residues are reported to improve the neuroprotective actions of arginine-containing 6-mer peptides (Ferrer-Montiel et al. 1998). Our results also indicate that the two other aromatic hydrophobic amino acids, phenylalanine and tyrosine, are able to enhance neuroprotective efficacy. Hydrophobic amino acids such as tryptophan are believed to improve cell-penetrating peptide endocytosis/uptake by enhancing penetration into the plasma membrane lipid bilayer following electrostatic interaction of the peptide with the cell surface. Based on these present results as well as data from previous studies, it appears that along with positively charged lysine, the presence of tryptophan, phenylalanine, tyrosine and cysteine residues can improve the neuroprotective efficacy of arginine-containing peptides (Marshall et al. 2015; Meloni et al. 2014, 2015a, b). Interestingly, these findings may also explain why the peptide, penetratin (RQIKIWFQNRRMKWKK), which only contains 3 arginine residues, but has 4 lysine, 2 tryptophan and 1 phenylalanine, displays such a high level of neuroprotection following glutamic acid excitotoxicity (Meloni et al. 2014).

While the R12W8a peptide was highly neuroprotective in vitro, it caused severe hypotension and cyanotic side effects when administered intravenously at relatively low doses of 100 and 300 nmol/kg. The hypotensive and cyanotic effects of arginine-rich peptides, particularly protamine, are well documented (Starbuck et al. 1967; Horrow 1985; DeLucia et al. 1993), but are usually associated with high dosages and rapid bolus infusion. The hypotensive effects of protamine are not fully understood, but are believed to be associated with vasodilation, possibly due to the stimulation of endovascular nitric oxide production (Raikar et al. 1996; Pevni et al. 2000). In the case of R12W8a, it appears its side effects are linked with its increased neuroprotective potency. Based on the potential of the R12W8a and protamine to antagonise plasma membrane and endoplasmic reticulum ion channel receptor levels/function, it is possible that the vasodilatory effects induced by these peptides are the result of altered calcium levels in vascular smooth muscle cells and as a consequence, failure of the cells to contract. In addition, it is possible that the cyanosis is caused by peptide electrostatic interactions with the lung surfactant phospholipid molecule, phosphatidylglycerol (+1 net charge), leading to increase surface tension of fluids within alveoli, collapse of alveoli and the reduced transfer of gases between the blood and alveolar air.

Importantly, while both protamine, R18 and R12W8a were shown to reduce infarct volume and/or cerebral oedema following permanent MCAO stroke, no significant improvements in functional parameters were observed, a finding which most likely reflects the high variability associated with behavioural outcomes after stroke, the short 24-hour study end-point, and stroke severity. As a consequence, additional studies are required to examine long-term histological and functional end points, as well as the assessment of arginine-rich and other peptides in less severe models of stroke and in stroke models using aged animals, female animals and larger mammalian species.
Conclusions

The results from this and previous studies (Meloni et al. 2014; Marshall et al. 2015; Meloni et al. 2015a, b; Milani et al. 2016a, b, 2017) further validate the neuroprotective properties of poly-arginine and arginine-rich peptides and support their recognition as a new class of neuroprotective agent with enormous potential as a treatment for stroke and other acute and chronic neurological disorders. Furthermore, we have demonstrated that while peptide neuroprotective potency could be increased considerably by the addition of tryptophan and other aromatic hydrophobic amino acid residues (i.e. phenylalanine and tyrosine), increased peptide potency was associated with toxicity when administered intravenously. As a consequence, if more potent arginine-rich peptides are to be developed, a CNS route of delivery (e.g. nasal or intracerebral artery) that avoids potential systemic side effects may need to be developed.

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Compliance with Ethical Standards

Conflict of interest Bruno P. Meloni and Neville W. Knuckey are the holders of several patents regarding the use of arginine-rich peptides as neuroprotective treatments. The other authors declare no conflict of interest.

Animal Rights All animal studies were approved by the University of Western Australia Animal Ethics Committee, and experiments were carried out according to guidelines outlined by the Australian Code for the Care and Use of Animals for Scientific Purposes.

References


peptides is linked to the attenuation of stress-induced hyperpolarization of the inner mitochondrial membrane potential. *Journal of Biological Chemistry*, 290(36), 22030–22048.


Chapter 8

Delayed 2-hour post-stroke administration of R18 and NA-1 (TAT-NR2B9c) peptides after permanent and/or transient middle cerebral artery occlusion in the rat

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Delayed 2-hour post-stroke administration of R18 and NA-1 (TAT-NR2B9c) peptides after permanent and/or transient middle cerebral artery occlusion in the rat

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Jane Cross: 4%
Ryan Anderton: 2%
David Blacker: 2%
Neville Knuckey: 2%

Signed…......................... Signed…………………………
Diego Milani Bruno Meloni (coordinating supervisor)
Research report

Delayed 2-h post-stroke administration of R18 and NA-1 (TAT-NR2B9c) peptides after permanent and/or transient middle cerebral artery occlusion in the rat

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\section*{A R T I C L E   I N F O}

\textbf{Keywords:}
Stroke
Middle cerebral artery occlusion
R18
NA-1
TAT-NR2B9c

\section*{A B S T R A C T}
Following positive results with the poly-arginine peptide R18 when administered intravenously 30 or 60 min after permanent and/or transient middle cerebral artery occlusion (MCAO; 90 min) in the rat, we examined the effectiveness of the peptide when administered 2 h after MCAO. R18 was administered intravenously (1000 nmol/kg via jugular vein) after permanent MCAO or a transient 3-h MCAO or when administered intra-arterially (100 nmol/kg via internal carotid artery) immediately after reperfusion following a transient 2-h MCAO. In the transient MCAO studies, the neuroprotective NA-1 peptide was used as a positive control. Infarct volume, cerebral edema and functional outcomes were measured 24 h after MCAO. Following permanent or transient MCAO, neither R18 nor NA-1 significantly reduced infarct volume. However, following permanent MCAO, R18 appeared to reduce cerebral edema ($p = 0.006$), whereas following a transient 3-h MCAO, R18 improved the time to remove adhesive tape ($p = 0.04$) without significantly affecting cerebral edema. There was also a trend ($p = 0.07$) towards improved rota-rod performance with R18 in both permanent and transient 3-h MCAO. Following a transient 2-h MCAO, R18 had no significant effects on cerebral edema or neurological score but did lessen the extent of weight loss. Overall, while R18 had no effect on infarct volume, the peptide reduced cerebral edema after permanent MCAO, and improved some functional outcomes after transient MCAO.

\section*{1. Introduction}

Recently, we have demonstrated that poly-arginine (e.g. R9, R12 and R18) and arginine-rich peptides (e.g. TAT, penetratin and protamine) are neuroprotective in \textit{in vitro} injury models that mimic the effect of ischemic stroke (Meloni et al., 2014, 2015a,b, 2017). In line with these \textit{in vitro} studies, we have also demonstrated that poly-arginine peptides R9, R12 and R18 reduce infarct volume following permanent and/or transient middle cerebral artery occlusion (MCAO) in the rat (Milani et al., 2016a,b, 2017) and that our lead peptide, R18 is consistently more effective than the extensively characterised TAT-NR2B9c neuroprotective peptide, now known as NA-1 (Meloni et al., 2015a, 2017; Milani et al., 2016b, 2017).

While these findings are very encouraging, they are based on studies that have only assessed the neuroprotective efficacy of the R18 and/or NA-1 peptides when administered at 30 or 60 min post-MCAO. For example, R18 has been shown to reduce infarct volume when administered 30 or 60 min after permanent MCAO (Meloni et al., 2017; Milani et al., 2016a,b, 2017), while NA-1 was largely ineffective at 60 min post-occlusion (Milani et al., 2016b). Similarly, following a 90-min duration of transient MCAO, both R18 and NA-1 reduced infarct volume when administered 60 min after occlusion (Milani et al., 2017). Therefore these positive results obtained with our lead peptide R18 in the stroke models, led us to explore the neuroprotective efficacy of the peptide when administered 2-h after the onset of permanent MCAO or a 3-h duration of MCAO. A R18 peptide dose of 1000 nmol/kg was chosen as this dose has previously been demonstrated to be effective when administered 60 min after permanent and transient MCAO.

In addition, the effectiveness of thrombectomy using endovascular clot retrieving catheters in ischemic stroke (Rabinstein, 2016) may
provide the opportunity to deliver neuroprotective agents intra-arterially prior to or shortly after clot removal and thereby further improve outcomes post-stroke (Lyden et al., 2016). This is a potentially important clinical application of neuroprotective agents, which led to a secondary aim of this paper to assess the effectiveness of the R18 and NA-1 peptides when administered intra-arterially immediately following a 2-h occlusion of the middle cerebral artery. Due to direct delivery of the peptide to the brain region affected by the MCAO and in order to avoid any potential neurotoxicity, the peptide dose was reduced to 100 nmol/kg. As this was primarily an exploratory study, measurement of infarct volume, cerebral edema and functional outcomes were performed 24 h post-MCAO.

2. Materials and methods

2.1. Animal ethics and study design

This study was approved by the Animal Ethics Committee of the University of Western Australia and follows the guidelines outlined in the "Australian Code for the Care and Use of Animals for Scientific Purposes". In the design of these studies, every effort was made to follow both the STAIR (Fisher, 2011) and ARRIVE (Kilkenny et al., 2010) guidelines, as appropriate.

2.2. Peptides used in the study

The R18 (H-RRRRRRRRRRRRRRRRRRH- OH) and NA-1 (H-YRKKRRQRRR-KLSSIESDVR-OH; also known as TAT-NR2B9c) peptides used in the study were synthesised by Mimotopes (Melbourne, Australia). The peptides were purified by high performance liquid chromatography to 98% purity and subject to peptide hydrolysis and amino acid liquid chromatography analysis to obtain a precise measurement of peptide content (Mimotopes). The peptides were prepared in 0.9% sodium chloride for injection (Pfizer, Perth, Australia), aliquoted into 650 μl volumes in 3 ml syringes and stored at –20 °C until use.

2.3. Induction of permanent and transient middle cerebral artery occlusion and treatment administration

Healthy male Sprague Dawley rats were subjected to permanent or transient MCAO using an intraluminal filament as described previously (Campbell et al., 2008a; Milani et al., 2016a,b, 2017). Briefly, rats underwent facemask anesthesia with isoflurane (mix 30% oxygen/70% nitrous oxide). The tail artery was cannulated to allow for blood pressure monitoring and withdrawal of blood samples for measurement of arterial blood gases (PaO2, PaCO2), pH (ABL5, Radiometer, Copenhagen, Denmark) and plasma glucose levels (MediSense Optium, Abbott Laboratories, USA). The MCAO procedure was considered successful if there was a > 25% decrease from baseline in cerebral blood flow after insertion of the filament to occlude the right middle cerebral artery, as measured by laser Doppler flowmetry. During surgery, body temperature was closely monitored using a rectal probe (Physitemp Instruments, Clifton, USA) and maintained between 37 and 37.8 °C, with fan heating or cooling, as required. Depending on study (see below) treatments were administered intravenously via the right internal jugular vein or intra-arterially via the right internal carotid artery using an infusion pump (600 μl over 6 min). Treatments were randomized and all procedures (i.e. treatment administration, infarct volume analysis and functional assessment) were performed while being blinded to treatments.

2.4. Study 1: permanent MCAO and intravenous peptide administration

Treatment groups consisted of vehicle (0.9% sodium chloride) and R18 peptide (1000 nmol/kg) administered intravenously, 2-h after MCAO. Twenty-one animals underwent surgery for MCAO. Animals generating an infarct volume ≤75 mm3 were excluded from the study as an infarct of this volume is consider small following permanent MCAO, and indicative of an animal with excellent collateral circulation and/or only partial MCAO. Additionally, animals were excluded if they experienced a significant drop in blood pressure (i.e. < 60 mmHg) prior to treatment administration.

Five animals were excluded from the study, one due to an insufficient decrease in cerebral blood flow following MCAO, one due to a lack of infarct lesion at 24-h post-MCAO (treated with vehicle), two due to a drop in blood pressure prior to treatment administration, and one animal died several hours before the 24-h post-MCAO study end-point for an unknown reason (treated with R18).

2.5. Study 2: transient 3-h MCAO and intravenous peptide administration

Treatment groups consisted of vehicle and R18 (1000 nmol/kg) or NA-1 (1000 nmol/kg) peptides administered intravenously 2-h after a MCAO in rats subjected to a 3-h MCAO. Twenty-four animals underwent surgery for MCAO. Animals generating an infarct volume ≤40 mm3 were excluded from the study as an infarct of this volume is considered small following a 3-h MCAO, and indicative of an animal with excellent collateral circulation and/or only partial MCAO. In addition, generating an infarct volume ≥400 mm3 were excluded from the study, as an infarct of this volume is more consistent with a permanent MCAO, possible due to a thrombus occluding the middle cerebral artery.

Five animals were excluded from the study, one due to an insufficient decrease in cerebral blood flow following MCAO, four due to a lack of an infarct lesion at 24-h post-MCAO (two treated with NA-1, one with R18 and one with vehicle). One animal died shortly before the 24 h end-point for an unknown reason, but was still included in the final infarct volume analysis (treated with NA-1).

2.6. Study 3: transient 2-h MCAO and intra-arterial peptide administration

Treatment groups consisted of vehicle and R18 (100 nmol/kg) or NA-1 (100 nmol/kg) peptides administered intra-arterially immediately following filament retraction after a 2-h duration of MCAO. Forty-eight animals underwent surgery for MCAO. Animals generating an infarct volume ≤40 mm3 or ≥200 mm3 were excluded from the study for reasons described above.

Twenty animals were excluded from the study, two due to an insufficient decrease in cerebral blood flow following MCAO, eight due to a lack of infarct lesion at 24-h post-MCAO (three treated with R18, two with NA-1 and three with vehicle), five due to an infarct volume ≤40 mm3 (two treated with R18, one with NA-1 and two with vehicle), four due to an infarct volume ≥200 mm3 (three treated with R18, and one treated with NA-1), and one animal died 1 h after the completion of surgery for an unknown reason (treated with R18). One animal died shortly before the 24 h end-point for an unknown reason, but was still included in the final infarct volume analysis (treated with vehicle).

2.7. Post-surgical analgesia and animal monitoring

At the conclusion of surgery, pethidine was administered intramuscularly (1 mg in 0.2 ml 0.9% sodium chloride) and bupivacaine was administered subcutaneously (0.1 mg in 0.2 ml 0.9% sodium chloride per site) to tail and head surgical wounds. Rats were subsequently placed in a clean cage with free access to food and water with the body temperature measured every 30–60 min using a rectal probe for at least 2 h post-surgery, and maintained between 37.0–37.8 °C. To avoid hypothermia, rat cages were placed on a heating mat during the post-surgical monitoring and housed in a holding room maintained at 26–28 °C. If necessary, additional heating or cooling was performed by applying fan heating or a cold water spray.
2.8. Functional testing and infarct volume assessment and cerebral edema calculation

In order to determine if peptide treatment improved sensorimotor outcomes, three behavioural tests (neurological score, adhesive tape and rota-rod) were performed 24 h after MCAO as described previously (Milani et al., 2016a,b, 2017). Infarct volume was determined using 2 mm thick cerebral coronal brain slices stained with 3% 2,3,5 triphenytltetrazolium chloride 24 h after MCAO as previously described (Campbell et al., 2008b). The ratio of contralateral hemisphere volume to stroke-affected hemisphere volume was used to correct for cerebral edema when calculating infarct volume (i.e. unaffected hemisphere volume/affected hemisphere volume x infarct volume = corrected infarct volume). In addition, the cerebral hemisphere swelling measurements were used to determine percentage cerebral edema of the stroke-affected hemisphere (i.e. affected hemisphere volume – unaffected hemisphere volume x 100/unaffect ed hemisphere volume). 

2.9. Statistics

Physiological parameters, mean total infarct volume, percentage cerebral edema and data from adhesive tape removal and rota-rod tests for each treatment group were evaluated by analysis of variance (ANOVA) followed by Fisher’s post-hoc analysis. Data from the neurological assessment measurements were analysed using Kruskall-Wallis test. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Study 1: permanent MCAO and intravenous peptide administration

Physiological parameters did not show significant differences between treatment groups (Table 1). Mean total infarct volume was not significantly different in the R18 treated rats compared to the vehicle controls (Fig. 1A), however cerebral edema was significantly lower in the R18 treated group (Fig. 1C), neurological outcomes (i.e. neurological score, adhesive tape removal test and rota-rod) did not differ significantly between R18 treated rats and vehicle treated controls (Fig. 1C-E). A positive trend was observed for time on rota-rod and to a lesser extent the neurological score for the R18 treated group compared to controls but the differences were not statistically significant. Weight loss was also not significantly different in R18 versus vehicle treated controls (Fig. 1F).

3.2. Study 2: transient 3-h MCAO and intravenous peptide administration

Physiological parameters did not differ significantly between treatment groups (Table 2). Moreover, mean total infarct volume and cerebral edema for both the R18 and NA-1 peptides were not significantly different from vehicle treated controls (Fig. 2A, B). There was a trend towards improved performance in tape removal tasks in R18 treated rats compared to controls, which reached statistical significance for time to remove tape ($p = 0.04$) (Fig. 2D). A positive trend, albeit not statistically significant, was also observed for time on rota-rod and to a lesser extent the neurological score in R18 treated rats (Fig. 2C, E). By contrast, functional outcomes did not differ significantly in NA-1 and vehicle treated animals (Fig. 2C-E). Weight loss was not significantly different in R18, NA-1 and vehicle treated animals (Fig. 2F).

3.3. Study 3: transient 2-h MCAO and intra-arterial peptide administration

Physiological parameters did not differ significantly between treatment groups (Table 3). Similarly, mean total infarct volume and cerebral edema for animals treated with the R18 or NA-1 peptides were not significantly different from values in vehicle treated animals (Fig. 3A, B). Neurological score did not differ significantly between R18, NA-1 and vehicle treated groups (Fig. 3C). Similarly, weight loss was not significantly different in R18, NA-1 and the vehicle treated groups (Fig. 3D), however there was a trend towards reduced weight loss in the R18 treated group.

4. Discussion

In previous studies we have demonstrated that the R18 and NA-1 peptides reduce infarct volume when administered 60 min after the onset of a 90-min MCAO in the rat (Milani et al., 2017), while R18, but not NA-1, significantly reduced infarct volume when administered 60 min after the onset of permanent MCAO (Milani et al., 2016b). In this exploratory study, administration of R18 and/or NA-1 2-h after the onset of permanent or a 2- or 3-h duration of transient MCAO did not have a significant effect on infarct volume. These findings indicate that at least in the stroke models used in the current study, the therapeutic window for R18 and NA-1 to reduce ischemic brain injury would appear to be < 2-h. However, consistent with previous studies, R18 reduced severity of cerebral edema and led to the improvement of some functional parameters (Milani et al., 2017). Reduced cerebral edema may be the result of R18 acting to maintain blood brain barrier integrity by reducing matrix metalloproteinase activity (Cameron et al., 2000; Kacprzak et al., 2004; Fuggere et al., 2007), while positive effects on functional outcomes may be the result of the peptide improving synaptic connectivity and/or plasticity, as has been recently demonstrated for the TAT-HA-NR2B9c peptide (Zhou et al., 2015). In addition, while intra-arterial administration of R18 and NA-1 immediately after a 2-h duration of MCAO did appear to improve histological and functional outcomes, no adverse effects were observed, suggesting that further studies examining different intra-arterial doses of the peptides after shorter durations of MCAO are warranted, especially in light of the increased use intra-arterial thrombectomy for acute ischemic stroke (Lyden et al., 2016).

Previous studies on NA-1 in permanent MCAO, have demonstrated efficacy when administered at doses of 300 or 3000 nmol/kg, 60 min after occlusion in the rat (Sun et al., 2008; Bratane et al., 2011), while a 3000 nmol/kg dose administered 30 or 60 min after occlusion in mice led to early (6 h), but not sustained (24 h) neuroprotection (Bach et al., 2012; Kleinschnitz et al., 2016). There are no reports examining the efficacy of NA-1 when administered beyond 60-min after permanent MCAO in rodents. In transient of MCAO of 90 min duration of occlusion in the rat, NA-1 has displayed efficacy at doses ranging from 30 to 3000 nmol/kg when administered before occlusion (45 min) or before (30 min) or after (60 min, 90 min) reperfusion (Aarts et al., 2002; Sun et al., 2008; Bratane et al., 2011), however a recent study has reported a lack of efficacy when administered at the time of reperfusion (Kleinschnitz et al., 2016). Interestingly, in mice following a 30- or 60-min duration of MCAO, and peptide administration at the time of reperfusion at a 10000 nmol/kg dose, but not 3000 nmol/kg was neuroprotective (Teves et al., 2016), while a different study reported a lack of efficacy for both doses (Kleinschnitz et al., 2016). More promising results have been obtained for NA-1 in non-human primates (macaque) with the peptide shown to reduce infarct volume and improve

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle (saline)</th>
<th>R18</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{PaO}_2$, before MCAO</td>
<td>117.5 ± 22.31</td>
<td>105.5 ± 10.37</td>
</tr>
<tr>
<td>$\text{PaCO}_2$, before MCAO</td>
<td>43.2 ± 2.21</td>
<td>41.3 ± 2.62</td>
</tr>
<tr>
<td>pH, before MCAO</td>
<td>7.37 ± 0.04</td>
<td>7.38 ± 0.05</td>
</tr>
<tr>
<td>Glucose, before MCAO</td>
<td>7.4 ± 0.46</td>
<td>7.67 ± 0.77</td>
</tr>
<tr>
<td>BP, average during surgery</td>
<td>85.0 ± 8.75</td>
<td>90.2 ± 9.62</td>
</tr>
<tr>
<td>Temperature, average 2 h after surgery</td>
<td>37.35 ± 0.12</td>
<td>37.40 ± 0.40</td>
</tr>
</tbody>
</table>

Notes: PaO2, PaCO2, and BP in mmHg, glucose in mmol/L and temperature in °C. BP = mean arterial blood pressure. Data are means ± SD.
functional outcomes when administered at a dose of 1083 nmol/kg, 60 min after the onset of a 90-min or 4.5-h “severe” MCAO (no collateral blood flow to the distal MCA) (Cook et al., 2012). Similarly, NA-1 has been shown to be also effective when administered 3 h after the onset of 3.5-h “mild” MCAO (collateral blood flow to the distal MCA) (Cook et al., 2012). In addition, administration of NA-1 after endovascular aneurysm surgery in humans is reported to reduce the number, but not volume of ischemic lesions (Hill et al., 2012). As a result of these positive findings two clinical phase III studies of NA-1 in acute ischemic stroke are underway (FRONTIER and ESCAPE-NA1; Internet Stroke Centre, 2017).

The above findings indicate that, while NA-1 holds promise as a potential neuroprotective treatment for stroke, the pre-clinical assessment of the peptide performed in the rat, mouse, and macaque produced a range of differential neuroprotective outcomes. This is not totally unexpected, as neuroprotective efficacy for any given agent in different stroke models is influenced by several factors such as the rate of tissue infarction, stroke severity, collateral circulation, amount of potentially salvageable brain tissue at the time of treatment administration, drug neuroprotective mechanism of action and variable drug pharmacokinetics in different animal species. Consequently, the lack of a significant and consistent neuroprotective effect with respect to infarct volume reduction with NA1 and R18 as demonstrated in the present study does not necessarily invalidate the potential clinical use of these peptides as a stroke therapeutic. For example, considering the previous positive results obtained in rodent and non-human primate (macaque) stroke models with NA-1 and/or R18, a lack of efficacy in reducing ischemic brain injury could be due to the rapid development of infarction, and hence the presence of no or minimal salvageable tissue at the time of peptide administration. Indeed, MRI data obtained in a previous study examining the ability of the NA-1 peptide to “freeze the penumbra” in rats following MCAO indicated that the amount of potentially salvageable penumbral tissue after 2 h of occlusion is limited (Bratane et al., 2011). Therefore, as recently used in thrombectomy trials in ischemic stroke, it would be important to confirm the presence or absence of any potential salvageable penumbral tissue prior to peptide treatment (Fisher, 1997; Fisher and Brott, 2003; Campbell et al., 2015; Wetterling et al., 2016). However, even without direct

Table 2
Physiological parameters: Transient MCAO (study 2).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle (saline)</th>
<th>R18</th>
<th>NA-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>PaO2, before MCAO</td>
<td>103.5 ± 28.6</td>
<td>128.5 ± 15.4</td>
<td>134.8 ± 14.2</td>
</tr>
<tr>
<td>PaCO2, before MCAO</td>
<td>47.3 ± 3.50</td>
<td>49.3 ± 3.00</td>
<td>43.3 ± 4.04</td>
</tr>
<tr>
<td>pH, before MCAO</td>
<td>7.33 ± 0.09</td>
<td>7.32 ± 0.01</td>
<td>7.31 ± 0.05</td>
</tr>
<tr>
<td>Glucose, before MCAO</td>
<td>7.39 ± 0.68</td>
<td>7.06 ± 0.60</td>
<td>7.43 ± 1.41</td>
</tr>
<tr>
<td>PaO2, during MCAO</td>
<td>111.7 ± 27.7</td>
<td>122.3 ± 19.4</td>
<td>101.2 ± 9.8</td>
</tr>
<tr>
<td>PaCO2, during MCAO</td>
<td>47.7 ± 8.5</td>
<td>42.5 ± 3.4</td>
<td>40.9 ± 5.5</td>
</tr>
<tr>
<td>pH, during MCAO</td>
<td>7.38 ± 0.05</td>
<td>7.34 ± 0.02</td>
<td>7.33 ± 0.04</td>
</tr>
<tr>
<td>PaO2, after MCAO</td>
<td>107.6 ± 26.0</td>
<td>116.1 ± 9.4</td>
<td>112.8 ± 5.5</td>
</tr>
<tr>
<td>PaCO2, after MCAO</td>
<td>43.2 ± 5.4</td>
<td>41.8 ± 7.3</td>
<td>39.2 ± 3.5</td>
</tr>
<tr>
<td>pH, after MCAO</td>
<td>7.35 ± 0.03</td>
<td>7.33 ± 0.04</td>
<td>7.38 ± 0.01</td>
</tr>
<tr>
<td>BP, average during surgery</td>
<td>97.8 ± 4.3</td>
<td>95.4 ± 8.7</td>
<td>102.7 ± 6.6</td>
</tr>
<tr>
<td>Temperature, average 2 h after surgery</td>
<td>37.56 ± 0.35</td>
<td>37.65 ± 0.28</td>
<td>37.41 ± 0.23</td>
</tr>
</tbody>
</table>

Notes: PaO2, PaCO2, and BP in mmHg, glucose in mmo/L and temperature in °C. BP = mean arterial blood pressure. Data are means ± SD.
comparative brain imaging techniques to detect penumbral tissue, previous NA-1 animal studies indicate that the evolution of ischemic brain injury can differ in rodent and macaque stroke models (Bratane et al., 2011; Cook et al., 2012; Kleinschnitz et al., 2016). Against this background, data generated in experimental stroke models using different animal species need to be interpreted appropriately, rather than being used as simple measures to validate or invalidate the results obtained in one stroke model compared to another, as has recently been suggested (Kleinschnitz et al., 2016). Furthermore, as used in the thrombectomy trials (Campbell et al., 2015), it is imperative that any future stroke trial assessing a neuroprotective agent only includes patients with potentially salvageable penumbral tissue.

Based on previous studies in our laboratory assessing the neuroprotective efficacy of arginine rich-peptides (e.g. protamine, LMWP, XIP, TAT), including poly-arginine peptides (e.g. R9, R12 and R18) and putative "neuroprotective peptides" fused to arginine-rich carrier peptides (e.g. TAT-NR2B9c, JNKI-1-TATD, TAT-p53DM/TAT-s-p53DM, TAT-NR2Bct/TAT-NR2Bcts, Indip/IndipK-R), we believe that the neuroprotective mechanism of action of many of the peptides is predominately related to their arginine content, positive charge and cell membrane traversing properties (Meloni et al., 2014, 2015a,b, 2017). To this end, we have demonstrated that arginine-rich, poly-arginine peptides and the TAT fused neuroprotective peptides TAT-NR2B9c and JNKI-1-TATD have the capacity to reduce neuronal excitotoxic calcium influx (Edwards et al., 2016; Meloni et al., 2015a,b, 2017). In addition, poly-arginine R12 and TAT-NR2B9c reduce neuronal cell surface expression of the NR2 B NMDA receptor subunit protein (MacDougall et al., 2016). It is for these reasons, as well as supportive data from other studies (Ferrer-Montiel et al., 1988; Marshall et al., 2015) that we have proposed that arginine-rich peptides be recognised as a new class of neuroprotective agent with potential application as a therapeutic for stroke and other forms of acute brain injury, and possibly even for chronic neurological disorders.

Despite the lack of significant neuroprotective effects as assessed histologically for R18 and NA-1 when administered 2-h after stroke onset in the rat, previous studies by others with NA-1 in macaque stroke models as well as previous studies from our laboratory demonstrating the superior efficacy of R18 versus NA-1 in rat stroke models provides evidence that these arginine-rich peptides have significant potential as stroke therapeutics. While NA-1 has progressed to clinical trial, further pre-clinical assessment of R18 (e.g. assess long-term histological and function end-points, assessment in a large animal, in female animals and in animals with co-morbidities) is required before human safety and clinical stroke studies can be undertaken.

Disclosures

Bruno P. Meloni and Neville W. Knuckey are the holders of several patents regarding the use of arginine-rich peptides as neuroprotective treatments. The other authors declare no conflict of interest.
Acknowledgements

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Fig. 3. Transient 2-h MCAO study results obtained 24-h after occlusion. Treatments were administered intraarterially (vehicle or R18 and NA-1 peptides at 100 nmol/kg) immediately after reperfusion (filla-ment retraction). Values are mean ± SD, except for neurological score where lines on graph indicate range and medium. (A) Infarct volume. Denotes animals that died several hours before the 24-h post-MCAO study end-point. (B) Cerebral edema. (C) Neurological score. Lines on graph indicate range and median for neurological scores. (D) Weight loss.

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Milani, D., Knuckey, N.W., Anderton, R.S., Cross, J.L., Meloni, B.P., 2016b. The R18 polyarginine peptide is more effective than the TAT-NR2B9c (NA-1) peptide when administered 60 minutes after permanent middle cerebral artery occlusion in the rat. Stroke Res. Treat. 2016, 2372710.


Assessment of dual laser Doppler probe cerebral blood flow recording following MCAO

In this Chapter, an additional aim in Study 3 was to assess the usefulness of dual laser Doppler probe recording to identify animals with patent collateral circulation between cerebral hemispheres, and which may result in smaller or variable infarct volumes. Consequently, if these animals are able to be identified following MCAO and before the administration of treatment they could be excluded in future studies, and in doing so reduce experimental variability in terms of stroke outcomes.

The usefulness of this approach in a transient MCAO stroke model in rats was recently demonstrated by Cuccione et al. (2016)¹. The procedure involves using one laser Doppler probe (Probe 1) to detect the cerebral perfusion in the territory of the middle cerebral artery (MCA) (as per normal), and a second probe (Probe 2) to detect the level of co-lateral blood flow at the border zone between the anterior cerebral artery (ACA) and MCA territories. A failure to register a significant perfusion deficit (at least 15% change from baseline) by Probe 2 after MCAO, is indicative of the presence of collateral communicating anastomosis between the ACA and the MCA. As the study in this Chapter was primarily aimed at assessing the usefulness of the information provided by using a second laser Doppler probe no animals were excluded from the study, even if Probe 2 registered a drop in cerebral blood flow of at least 15%.

The measurements for the reduction in cerebral blood flow (from baseline) obtained for the two laser Doppler probes following MCAO are summarised in the linear regression graphs provided in the Appendix. Measurements were obtained from 42 rats. Linear regression analysis revealed a poor correlation between a reduction in cerebral blood flow and infarct volume for both probes (Probe 1: $R^2 = 0.11$ and Probe 2: $R^2 = 0.01$).

Chapter 9

General discussion
Introduction

Ischaemic stroke occurs when there is a reduced blood supply to a region of the brain, resulting in neuronal loss and brain injury. Currently, the most effective stroke therapy is to restore blood flow to the territory of the blocked cerebral artery using tPA thrombolysis (tissue plasminogen activator) and/or thrombectomy (mechanical removal of clot). However, to be effective, reperfusion therapies need to be administered within 4.5 hours after stroke onset, and thus it is estimated that only 5-15% of eligible stroke patients receive this therapy (Donnan et al., 2008; Henninger and Fisher, 2016). For the majority of patients with ischaemic stroke there is still no proven effective therapy to reduce the severity of the stroke and extent of brain injury.

Currently, there are no clinically available neuroprotective therapeutic agents that can be administered to stroke patients to reduce the severity and extent of brain injury. The availability of a neuroprotective agent for stroke would provide the potential to reduce brain injury in patients who do not receive thrombolysis/thrombectomy and further improve outcomes in those patients who do undergo endovascular recanalisation therapy. In addition, the early administration of a neuroprotective agent could prolong neuronal viability within the ischaemic penumbra and thereby also extend the therapeutic window of thrombolysis/thrombectomy recanalisation therapy. Therefore, there is an urgent need for the development of new more widely applicable neuroprotective therapies that can be applied to stroke and other forms of cerebral ischaemia. Additionally, any treatment that can be applied in the field and/or improve the safety of thrombolysis/thrombectomy would also be highly advantageous.

Against this backdrop, recent studies in A/Prof Meloni’s laboratory have demonstrated that poly-arginine and arginine-rich peptides are highly neuroprotective agents for \textit{in vitro} injury models that mimic the effect of stroke (i.e. excitotoxicity and oxygen glucose deprivation), and in the case of poly-arginine-9 (R9), following permanent MCAO in the rat (Meloni et al., 2014; Meloni et al., 2015ab). These earlier studies have provided the rationale for this thesis to further investigate poly-arginine peptides as a neuroprotective therapy for stroke.
Key findings arising from this thesis

Assessment of poly-arginine peptides and protamine in permanent MCAO stroke model

The initial aim of this study was to identify a potential lead neuroprotective poly-arginine or arginine-rich peptide as a focus for further investigation. Four peptides were selected for assessment: poly-arginine peptides R12, R15, R18 and the arginine-rich peptide protamine. Selection of the appropriate poly-arginine peptides was based on previous in vitro studies, demonstrating that peptide neuroprotective efficacy increased with increasing arginine polymer length, plateauing at around R15 to R18 (Meloni et al., 2015a). Protamine was selected based on its high neuroprotective efficacy in vitro, high arginine content (21 arginine residues), its clinical use as a heparin reversal agent (Van Ryn-McKenna et al., 1990), and its use to slow the absorption of insulin (Horvath et al., 2007; Mathew et al., 2015). Initial assessment of the peptides was performed using a permanent MCAO stroke model, since most ischaemic stroke patients do not receive reperfusion therapy, and as recommended by the Stroke Treatment Academic Industry Roundtable guidelines (STAIR, 1999).

The permanent MCAO stroke study involved a single peptide dose (1000nmol/kg) administered 30 minutes after MCAO (Chapters 4 and 7) and revealed neuroprotective effects for R12, R18 and protamine, whereas R15 did not show any obvious neuroprotection. Considering that the R15 peptide had previously been shown to have comparable neuroprotective efficacy to R18 in an in vitro neuronal glutamate excitotoxicity model (Meloni et al., 2015a), its lack of efficacy in the MCAO model was surprising. The reason why no observable neuroprotection was obtained for R15 is at present unknown, but it is possible that higher or lower doses of the peptide may be required to provide a neuroprotective effect. As the R15 result was unexpected, it would be of interest in future studies to perform additional dose response studies using a new batch of the peptide in both the permanent and transient MCAO stroke models. It should be mentioned that the R15 peptide batch used in the
initial animal study was confirmed to be neuroprotective in the \textit{in vitro} excitotoxicity model (A/Prof Meloni, personal communication).

Despite the finding of a lack of efficacy following R15 treatment, the results for R12, R18 and protamine, which reduced infarct volume by 12.8\%, 20.5\% and 22.5\% respectively, were in line with a previous study demonstrating that R9 in the same model, dose and administration time-point, reduced infarct volume by 20\% (Meloni et al., 2015a). Interestingly, the results obtained in the two studies indicate that R9 is more neuroprotective than R12 despite its shorter polymer length. A possible explanation for the superior efficacy of the R9 peptide could be due to its likely higher stability and longer serum half-life, as it was synthesised using D-isoform arginine residues, which are known to provide greater resistance to proteolytic degradation, rather than peptides synthesised using the L-isoform (Weinstock et al., 2012). Therefore, based on the potential superior potency of D-isoform peptides, further studies in our laboratory are exploring the relative efficacies of D- and L- isoform poly-arginine peptides in stroke and other ischaemic brain injury models (e.g. global cerebral ischaemia, perinatal hypoxia-ischaemia). To this end, recent studies in A/Prof Meloni’s laboratory using a global cerebral ischaemia model have indicated that R18D is a more potent neuroprotective agent than R18 (personal communication).

Results from this thesis demonstrated for the first time the neuroprotective properties of protamine in \textit{in vitro} and \textit{in vivo} stroke injury models, and its ability to reduce glutamic acid induced neuronal intracellular calcium influx (Chapter 7). These findings are not surprising considering the high arginine content of protamine, and the previous studies in our laboratory confirming the neuroprotective and calcium influx inhibitory properties of other arginine-rich and poly-arginine peptides. However, despite protamine’s neuroprotective properties and its current clinical use, there are several reasons why it was not selected as a lead neuroprotective peptide. Although protamine is regarded as a relatively safe drug with a wide therapeutic index, it can be associated with severe anaphylactic reactions and pulmonary hypotension in a small proportion of patients who are usually allergic to fish (Horrow, 1985; DeLucia et al., 1993). Interestingly, it is for these reasons that the poly-arginine peptide R15 has been evaluated as a safer alternative to protamine as a
heparin reversal agent (Byun et al., 1999; Li et al., 2015). In view of these considerations, and since the neuroprotection provided by protamine in the permanent MCAO models was not significantly different to the neuroprotection provided by R18, the latter peptide was therefore selected as the lead neuroprotective agent. Interestingly, and in line with the known properties of protamine, a slight and reversible reduction in blood pressure was observed during the intravenous administration of the peptide, while a similar effect was not observed for the other poly-arginine peptides (data not shown).

**Neuroprotective dose responsiveness of R18 in permanent MCAO stroke model**

Following the initial permanent MCAO study, and the selection of R18 as the lead peptide, a subsequent study examined the dose responsiveness of R18 at a 60-minute post-occlusion treatment time point. Importantly, the neuroprotective peptide TAT-NR2B9c, which is also known as NA-1, was included in the study as a positive control and benchmark. The NA-1 peptide has undergone rigorous pre-clinical assessment in rodents and non-human primate stroke models (Table 2 Chapter 2; Cook et al., 2012). In addition, administration of NA-1 after endovascular aneurysm surgery in humans is reported to reduce the number, but not volume, of ischaemic lesions (Hill et al., 2012). As a result of positive outcomes in stroke studies assessing NA-1 neuroprotective efficacy, two clinical phase III studies of NA-1 in acute ischaemic stroke are either being planned or underway (ESCAPE-NA1 and FRONTIER; www.strokecenter.org).

The results from this study revealed that R18 reduced infarct volume at the 100, 300 and 1000 nmol/kg doses by 19.7%, 12.0% and 24%, respectively. By contrast, NA-1 had little impact in reducing infarct volume at the doses examined (4.8%, 6.8% and 7.0% for the 100, 300 and 1000 nmol/kg dose, respectively; Chapter 5). In addition, treatment with the R18 peptide, and to a lesser extent NA-1, resulted in some functional recovery as assessed by behavioural tests, but not to statistically significant levels.

These findings further confirmed the neuroprotective properties of R18 and extended its therapeutic time window to 60 minutes after stroke onset, as well as
demonstrating greater efficacy compared to NA-1 in a severe ischaemic stroke model.

**Assessment of arginine-tryptophan peptides in permanent MCAO stroke model**

The presence of other amino acids in arginine-containing peptides has been shown to influence the degree of peptide neuroprotection (Meloni et al., 2015ab). Therefore, the primary aim of this study was to identify and assess a potentially more efficacious arginine-rich peptide, compared to R18, in the permanent MCAO rat stroke model (Chapter 7).

In order to identify a potentially more effective peptide compared to R18, studies performed in A/Prof Meloni’s laboratory screened several arginine-rich peptides, including a cyclic poly-arginine peptide (R12-c), R22 and arginine-tryptophan containing peptides. The initial aim of the study was to assess whether peptide cyclisation, further increasing poly-arginine peptide length (i.e. beyond R18), or the addition of tryptophan residues, would significantly enhance peptide neuroprotective efficacy in the *in vitro* excitotoxicity injury model. Despite showing strong neuroprotective effects, the R12-c and R22 peptides did not appear to be significantly more efficacious than R18. On the other hand, the addition of tryptophan residues significantly improved peptide neuroprotective properties. In particular the peptide R12W8a, composed of 12 arginine and 8 tryptophan residues, displayed the greatest efficacy, with a potency 10 times higher than R18 in the excitotoxicity model. For this reason, R12W8a was selected for further assessment in the permanent MCAO rat stroke model. Likely explanations for the increased neuroprotective potency of tryptophan containing peptides are provided in the Discussion section of the manuscript in Chapter 7.

While the R12W8a peptide was highly neuroprotective *in vitro*, it caused severe hypotension and cyanotic side effects when administered intravenously at relatively low doses of 100 and 300 nmol/kg (data not shown). In this regard, it appears its ability to induce side effects when administered to rats is linked with its increased neuroprotective potency. It is possible that, based on the potential of R12W8a to antagonise plasma membrane ion channel receptor levels/function, the cardiac
depressive and vasodilatory effects induced by the peptide are the result of altered calcium influx and impaired contractility in myocardial and vascular smooth muscle cells (Horrow, 1985; DeLucia et al., 1993). In addition, it is possible that the cyanosis is caused by peptide electrostatic interactions with the lung surfactant phospholipid molecule, phosphatidylglycerol (-1 net charge), leading to increased surface tension of fluids within alveoli, collapse of alveoli, and consequently a reduced transfer of gases between the blood and alveolar air.

Due to the adverse side effects of R12W8a when administered at the 100 and 300 nmol/kg doses, a 30 nmol/kg dose, which did not induce any obvious adverse effects, was used to assess the in vivo efficacy of the peptide. The study revealed that R12W8a did not provide any significant neuroprotection following permanent MCAO, unlike the R18 peptide at the 30 nmol/kg dose, which significantly reduced infarct volume and cerebral oedema. One explanation for the lack of neuroprotection for R12W8a when administered in vivo, is that due to its improved endocytic or membrane traversing properties it is rapidly taken up by vascular tissue (e.g. endothelial cells, smooth muscle cells) and other organs (e.g. liver, kidney) and thereby severely limiting its access to the brain.

As a consequence of these findings with R12W8a, it is likely that if this or more potent arginine-rich peptides are to be developed, a more direct CNS route of delivery (e.g. intranasal or intracerebral artery) that avoids systemic distribution and potential side effects of the peptide may need to be considered.

Assessment of R18 in permanent MCAO stroke model, when administered 120 minutes after stroke onset

Following the positive results obtained for R18 when administered intravenously 60 minutes after permanent MCAO, the treatment administration time point was extended to 120 minutes (Chapter 8). The rationale for this study was to further investigate the therapeutic time window for R18 in the permanent MCAO rat model. In contrast to the previous studies, R18 did not significantly reduce infarct volume when administered 120 minutes after stroke onset at the dose of 1000 nmol/kg. Interestingly however, R18 significantly reduced severity of cerebral oedema (by
21%) and improved some behavioural outcomes, specifically neurological assessment score and rota-rod performance.

These findings suggest that while R18 has a limited ability to reduce infarct volume when administered 120 minutes after stroke onset in the permanent MCAO model used in this study, it still has the capacity to reduce other damaging effects of stroke.

**Neuroprotective dose responsiveness of R18 in transient MCAO stroke model**

An additional aim of this project was to assess the efficacy of R18 in a stroke model incorporating reperfusion-induced damage. Animal stroke models that incorporate cerebral reperfusion are particularly relevant in the clinical setting due to tPA thrombolysis therapy and more recently the application of thrombectomy for ischaemic stroke. While successful recanalisation and reperfusion provides the best opportunity to salvage vulnerable brain tissue following stroke, it can also contribute to brain injury through several different mechanisms (Warach and Latour, 2004; Kalogeris et al., 2012). For example, reperfusion of previously ischaemic cerebral tissue can damage vascular endothelium, cause excessive production of reactive oxygen species and stimulate the production of inflammatory cytokines (Warach and Latour, 2004; Kalogeris et al., 2012). These damaging processes, in addition to increasing neuronal death, can promote the formation of cerebral oedema and in severe cases may result in haemorrhagic transformation or intracerebral haemorrhage, characteristically occurring 24 to 36 hours after stroke (Khatri et al., 2007).

Similar to the permanent MCAO study, R18 dose-dependently reduced infarct volume. For example, at the 30, 100, 300 and 1000 nmol/kg doses, R18 reduced infarct volume by 9.6%, 12.2%, 24.8% and 35.1% respectively. The NA-1 peptide, which was also used as a positive control in the study, was less effective and reduced lesion volume by 7.0%, 16.5%, 16.6% and 26.1% respectively, for the corresponding doses. In addition, R18 significantly reduced cerebral oedema at three doses (100, 300 and 1000 nmol/kg), while NA-1 only significantly reduced swelling at the highest dose of 1000 nmol/kg (Chapter 6). Several of the R18 and NA-1 treatment groups also showed statistically significant improvement in at least one functional
parameter of the adhesive tape test, and a positive trend in the neurological assessment and rota-rod.

Taken together, the results of the study confirm that R18 is effective at reducing brain injury and cerebral oedema, and improving functional outcomes in a stroke model that is associated with reperfusion-induced damage. Moreover, as demonstrated in the permanent MCAO stroke model, R18 appeared to be more effective than the NA-1. The effectiveness of R18, in a stroke model associated with cerebral reperfusion, is evidence that the peptide has the potential to further improve outcomes in stroke patients undergoing endovascular recanalisation therapies.

Interestingly, both R18 and NA-1 treated animals displayed some positive behavioural improvement when treated at the lowest doses (30 nmol/kg), even when infarct volume was not significantly reduced. This result suggests that at low concentration the peptides may have a positive effect on synaptic connectivity and/or plasticity that improves function, as has been recently demonstrated for the closely related NA-1 peptide TAT-HA-NR2B9c (Zhou et al., 2015).

Assessment of R18 in the transient MCAO stroke model, when administered 120 minutes after stroke onset

Following the positive results with R18 in the transient MCAO stroke model, efficacy of the peptide was assessed when administered at a later time point (120 minutes) and following a longer duration of MCAO (180 minutes) (Chapter 8). The study revealed that R18 when administered at the 1000 nmol/kg dose did not significantly reduce infarct volume. Similarly, the NA-1 also did not reduce infarct volume. However, R18 treatment was associated with a reduction in cerebral oedema and a significant improvement in time to remove the adhesive tape from the left paw.

Intra-arterial R18 treatment at time of reperfusion in transient MCAO stroke model

A final aim of this thesis was to assess the effectiveness of R18 when administered intra-arterially immediately after reperfusion in the transient MCAO stroke model (Chapter 8). The intra-arterial administration of a neuroprotective agent has recently
become clinically relevant with the application of endovascular catheters used during mechanical thrombectomy. This study used a transient MCAO model consisting of a 120-minute occlusion period, administration of peptide immediately following reperfusion (i.e. retraction of the intraluminal suture), and a reduced peptide dose (100 nmol/kg). Results revealed that neither R18 nor the NA-1 significantly reduced infarct volume, although a modest positive trend was observed. As in previous studies, R18 reduced cerebral oedema. In addition, R18 and to a lesser extent NA-1 treated animals, displayed improvements in some functional outcomes.

Significance of lack of efficacy of delayed (120 minutes) post-stroke treatment with R18 and NA-1 after permanent and transient MCAO

The inability of R18 and NA-1 to significantly reduce infarct volume when administered 120 minutes after permanent or transient MCAO in rats, does not necessarily indicate that the peptide will be ineffective when administered at the same time point after stroke onset in humans. However, when attempting to extrapolate the results in the rat models to humans, differences in metabolism, physiology and anatomy between the two species need to be taken into consideration. For example, in the setting of stroke, there is evidence that brain infarct development occurs more rapidly in rats than in humans (Saita et al., 2004; Andreollo et al., 2012; Sengupta, 2013). However, of even more relevance is knowledge of the extent of potentially salvageable penumbral tissue after stroke and at the time of treatment administration.

While not extensively studied, available evidence suggests infarct volume development in rats following stroke is animal strain and model dependent. In a study by Wetterling et al. (2016), it was demonstrated that in rats subjected to a transient MCAO the presence of penumbral tissue, before incorporation into the core, can vary from between 1 and 6 hours. In studies using permanent MCAO in rats, Bratane et al. (2011) showed the presence of penumbral tissue up to 2-hours after occlusion, while McCabe et al. (2009) reported the absence of penumbral tissue by 60 minutes after stroke onset.
In addition, the negative results obtained with NA-1 when administered at 2-hours after stroke onset are in line with other studies in stroke models using rodents when the peptide is administered more than 60 minutes after MCAO. For example, NA-1 failed to show neuroprotective effects when administered intravenously after 120 minutes from stroke onset in a permanent MCAO (Soriano et al., 2008; Kleinschnitz et al., 2016).

Therefore, the inability of R18 and NA-1 peptides to reduce infarct volume when administered 2 hour post-stroke, after permanent and transient MCAO, may simply reflect the lack of potentially salvageable penumbral tissue. In support of this, and considering the similarities between non-human primates and humans, a previous study by Cook et al., (2012) demonstrated neuroprotective efficacy of NA-1 when administered 3 hours after stroke onset in macaques subjected to a 3.5-hour MCAO.

Cerebral collateral circulation and infarct volume variability

Experimental variability in animal stroke models is a well-known limitation for preclinical stroke studies (Coyle and Jokelainen, 1982; Cuccione et al., 2016). In this instance, the presence of collateral/accessory circulations between the main cerebral vessels may affect the degree of brain injury by providing additional blood supply to the ischaemic area during MCAO, and thereby resulting in smaller or variable infarct volumes.

In an attempt to identify animals that may return a small infarct volume due to good collateral cerebral circulation following MCAO, during the last animal study (Chapter 8) two laser Doppler probes were utilised to measure cerebral blood. Cuccione et al. (2016) demonstrated the usefulness of this approach to identify animals with good collateral circulation following transient MCAO in rats. The procedure involves using one laser Doppler probe to detect the cerebral perfusion in the territory of the middle cerebral artery (MCA), and a second probe to detect the level of collateral blood flow at the border zone between the anterior cerebral artery (ACA) and MCA territories. A failure to register a significant perfusion deficit (at least 15% change from baseline) by the second probe after MCAO is indicative of the presence of collateral communicating anastomosis between the ACA and the
MCA. However, in this study the results did not reveal any obvious correlation in a drop in cerebral blood flow registered by the second probe and associated infarct volumes (Chapter 8; Appendix).

While the exact reason for the discrepancy between the present findings obtained and those reported by Cuccione et al. (2016) are not known, improving experimental consistency and finding additional tools to exclude animals that are likely to generate a smaller than anticipated infarct volume following MCAO, remains an important objective in future studies.

Limitations of present study

While the results from this study provide strong evidence for the neuroprotective properties of poly-arginine and arginine-rich peptides in rat MCAO stroke models, the limitations of these studies need to be acknowledged.

It is likely that some aspects of the experimental design of the animal studies such as stroke severity, the high variability associated with behavioural outcomes after stroke, and the acute 24 hour endpoint, coupled with the relatively small animal numbers, explain why improvements in functional outcomes were modest and often did not reach statistical significance. Therefore, to overcome these limitations and provide further confirmation of the ability of arginine-rich peptides to improve functional outcomes, additional studies with extended experimental end-points and a larger number of animals, are required. It may also be useful to assess the peptides in animal stroke models that are more amenable for the assessment of functional outcomes following stroke (e.g Endothelin-1 stroke model; Soleman et al., 2010). In addition, extended experimental end-points would also confirm whether the histological neuroprotection provided by arginine-rich peptides is maintained long-term.

This thesis only studied the neuroprotective effects of the peptides in one species of animal (Sprague Dawley rats) and in healthy adult males, while additional factors like age and the presence of comorbidities (e.g. hypertension, diabetes, obesity) were not investigated. Age and comorbidities are particularly relevant to stroke, as
increased age and the presence of comorbidities are risk factors for stroke. It is also recommended (STAIR) that studies are performed in different strains of animal of the same species, as genetic differences between strains can influence the infarct size (Braeuninger and Kleinschnitz, 2009). In addition, following positive results in rodent stroke models, assessment of neuroprotective agents should be tested in higher-order gyrencephalic species (e.g. dogs, sheep, pigs, non-human primates) with a brain and neuro-cortical pathways more similar to humans. It is also important that positive results can be replicated in at least one other independent laboratory (Fisher et al., 2009).

In future studies it would also be useful to assess the time course of ischaemic tissue progression in the stroke models used, in particular in relation to the time of treatment administration and extent of potential salvageable penumbral tissue. This could be achieved with the use of MRI (Magnetic Resonance Imaging), as has been demonstrated previously in animal studies (McCabe et al., 2009; Bratane et al., 2011; Wetterling et al., 2016).

Therefore, while the present study has undertaken the initial steps in the neuroprotective assessment of a poly-arginine peptide, namely R18, it is clear that additional studies as recommended for stroke therapeutics (STAIR) are now required.

**Summary of important findings of study and future directions**

The findings presented in this thesis have further confirmed the neuroprotective properties of poly-arginine peptides (R12 and R18) and an arginine-rich peptide (protamine) following MCAO stroke in the rat. Although additional studies are required, as outlined above, to evaluate more fully the potential of R18 as a neuroprotective therapy in stroke, it would appear that arginine-rich peptides represent a promising new class of neuroprotective agent that warrants further evaluation in other animal models.

Importantly, concomitant studies conducted in A/Prof Meloni’s laboratory are also showing neuroprotective efficacy for R18 in global cerebral ischaemia and neonatal
hypoxia ischaemic models (data not shown). Therefore, based on the results obtained in this thesis, and other ongoing studies in our laboratory and others (Meloni et al., 2015b; Marshall et al 2015), it would appear that arginine-rich peptides have great potential as a treatment for stroke and other forms of acute brain injury, and possibly even for chronic neurological disorders.
References


Appendix
Figure A1. Linear regression analysis between infarct volume and cerebral blood flow deficit after MCAO, as detected by laser Doppler Probe 1 and 2.