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by

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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.

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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

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Meloni B., Cross J., Brookers L., Clark V., **Milani D.,** Knuckey W. 2014. *Poly-arginine peptides are potent stroke neuroprotective agents.* Poster presentation at: The 25th annual scientific meeting of the Stroke Society of Australasia; Hamilton Island, Australia.

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Milani D., Meloni B., Anderton R., Knuckey N. 2015. *Neuroprotective potential of poly-arginine peptides in permanent focal cerebral ischaemia model.* Poster presentation at: The symposium of Western Australian Neurosciences (SWAN); Perth, Australia.

Meloni B., Edwards A., Cross J., **Milani D.,** Anderton R., Knuckey N. 2015. *Characterisation of the neuroprotective properties of poly-arginine peptides using an in vitro glutamic acid excitotoxicity stroke model.* Poster presentation at: The 26th annual scientific meeting of the Stroke Society of Australasia; Melbourne, Australia.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ACA	Anterior cerebral artery
AF	Atrial fibrillation
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
ARRIVE	Animal research: reporting of in vivo experiments
ATP	Adenosine triphosphate
BBB	Blood brain barrier
Ca	Calcium
CCA	Common carotid artery
CNS	Central nervous system
CO	Carbon oxide
CPP	Cell penetrating peptide
CT	Computed tomography
ECA	External carotid artery
ERK	Extracellular signal-regulated kinases
HEK293	Human embryonic kidney 293 cells
ICA	Internal carotid artery
ICU	Intensive care unit
IV	Intravenous
JIP-1	c-Jun N-terminal kinase interacting protein-1
JNK1	c-Jun N-terminal protein kinase 1
KA	Kainic acid
LDL	Low density lipoprotein
MAPK	Mitogen-activated protein kinase
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
MMPs	Matrix metalloproteinases
MRI	Magnetic resonance imaging
NFKB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	N-methyl-D-aspartic acid
NO	Nitric oxide

nNOS	Neuronal nitric oxide synthase
NR2B	N-methyl-D-aspartate receptor subtype 2B
O	Oxygen
PCA	Posterior cerebral artery
pMCAO	Permanent middle cerebral artery occlusion
PSD-95	Postsynaptic density protein 95
Ptm	Protamine
R	Arginine
ROS	Reactive oxygen species
SCU	Stroke care unit
SD	Sprague Dawley
STAIR	Stroke therapy academic industry roundtable
tMCAO	Transient middle cerebral artery occlusion
TNF	Tumor necrosis factor
tPA	Tissue plasminogen activator
TAT	Transcriptional activator protein
TIA	Transient ischemic attack
TTC	2,3,5-triphenyltetrazolium chloride
W	Tryptophan
Zn	Zinc

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 stoke 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).

Risk factor	Evidence
Increasing age	The incidence of stroke increases dramatically after age 65
Gender	Males show an incidence rate of 1.25 compared to women
Genetic factors	A positive family history of cerebrovascular disease is considered a risk factor for stroke
Transient ischaemic attack (TIA)	Patients presenting with a TIA have a risk of stroke estimated at around 4% per year
Prior stroke	Patients with prior stroke are at higher risk of having a subsequent stroke
Ethnicity	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

Table 1.2 Modifiable risk factors for ischaemic stroke (adapted from Goldstein et al., 2006).

Risk factor	Evidence
Carotid atherotromboembolia	Complications of atheroma, such as thrombosis or embolism, are the most common causes of ischaemia and cerebral infarction
Hypertension	High blood pressure is the most important modifiable risk factor with proven correlation with cardiovascular and cerebrovascular complications
Atrial fibrillation (AF)	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
Cigarette smoking	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
Diabetes mellitus	Individuals with diabetes mellitus are at proven increased risk of developing stroke
Hyperhomocysteinaemia	Studies have shown a strong association between stroke and high plasma levels of homocysteine
Alcohol abuse	Alcohol abuse raises blood pressure and increases the risk of stroke
Dyslipidaemia	Data demonstrate a direct correlation between increased total and LDL cholesterol levels and the incidence of extracranial carotid atherosclerosis
Obesity and inactivity	Obesity and a lack of physical activity are major risk factors for cardiovascular diseases, including stroke
Haemostasis and other blood disorders	Studies have demonstrated the role of high levels of fibrinogen and a high red blood cell counts as risk factors for stroke

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).

Affect artery	Symptoms
Anterior cerebral artery (ACA)	Mutism onset, lower limb paresis, hemiparesis, motor aphasia, apraxia, sphincter incontinence, grasping, unilateral sensory deficit, mood disorders, confusional state and hemineglect
Middle cerebral artery (MCA)	Hemianopia, lower limb paresis, motor aphasia, Broca's aphasia, Wernicke's aphraxia, premotor syndrome of Luria, unilateral sensory deficit, confusional state and hemineglect
Posterior cerebral artery (PCA)	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
Cerebellar artery territories	Nystagmus, vertigo, dysmetria, ataxia, lateropulsion, postural instability and dysarthria, paralysis of the V and VII cranial nerves
Infarct of the brainstem	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, hemianaestesia of the face and hemisoma anaesthesia

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).

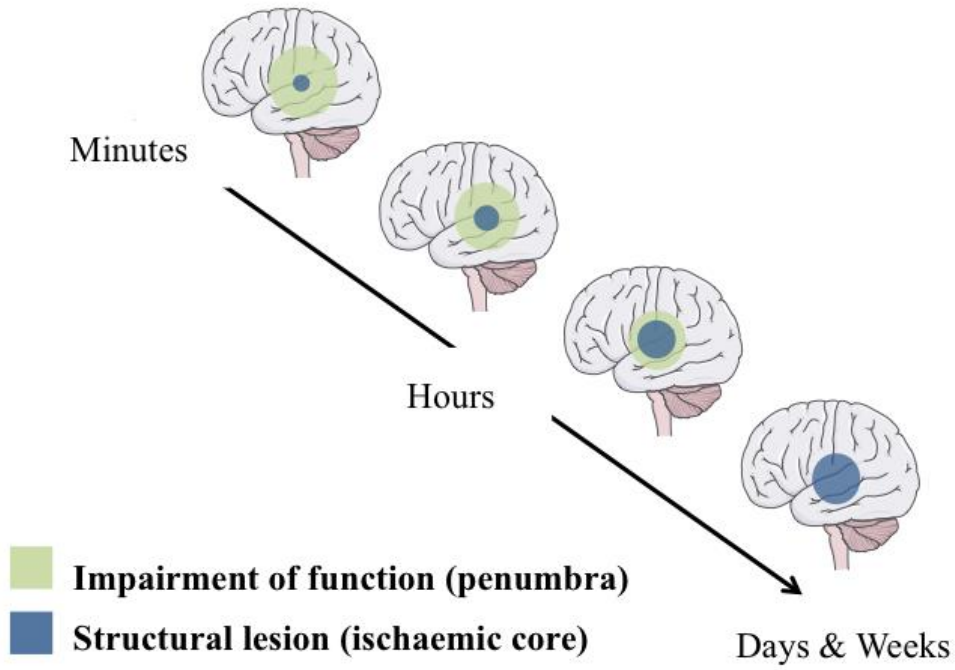

Figure 1.1 Brain showing the central ischaemic core surrounded by penumbra. Since the onset of stroke, the progression of core and penumbra is a progressive process.

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

(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).

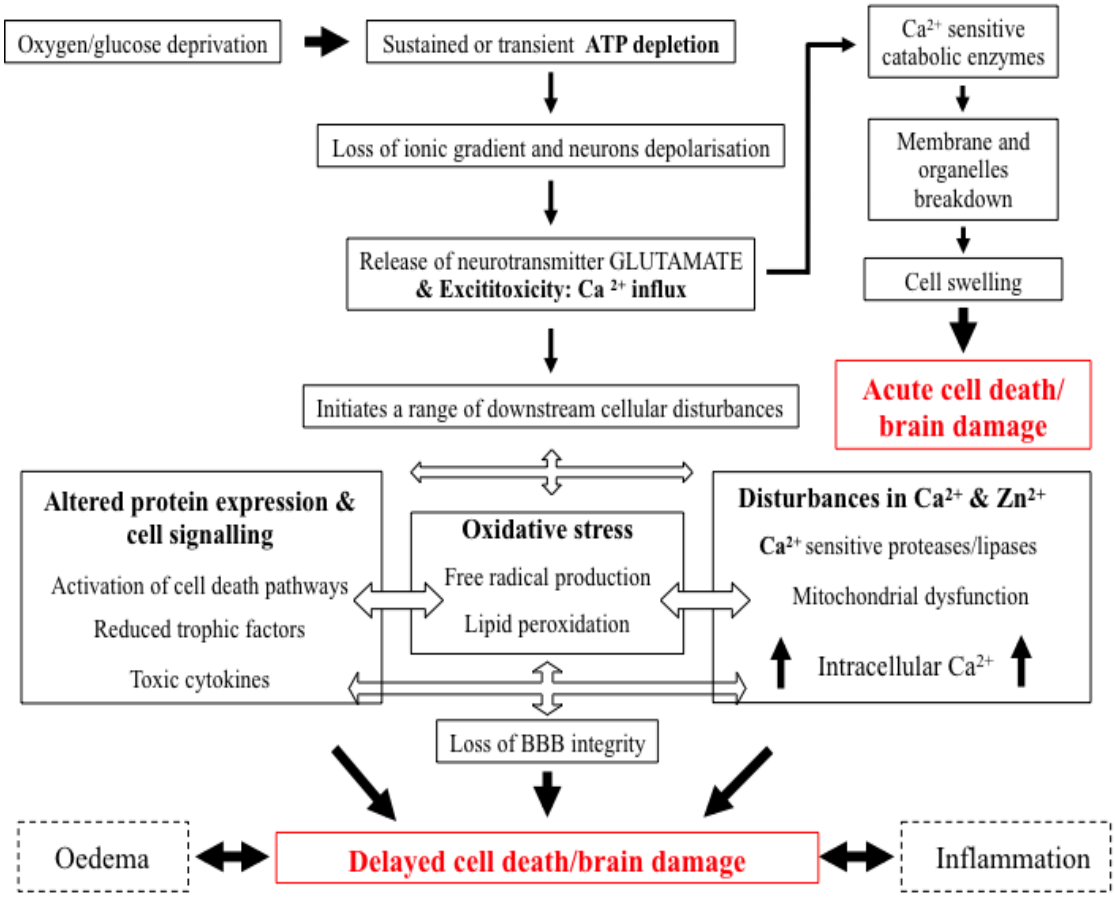

Figure 1.2 Summary of the major events associated with the ischaemic cascade and neuronal cell death. Excitotoxicity and calcium overload cause the induction of several ionic, biochemical and cellular cascades.

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., 2008; Henninger and Fisher, 2016).

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 JNKI-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 JNKI-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 ($NR2B_{CT}$-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.

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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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Signed…… Signed……

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The original version of this thesis includes a published article on this page.

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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 material 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.

Peptide	Sequence*	Arginine residues	Charge at pH 7
R12	H-RRRRRRRRRRRR-OH	12	12
R15	H-RRRRRRRRRRRRRRR-OH	15	15
R18	H-RRRRRRRRRRRRRRRRRR-OH	18	18
R12W8a	H-WWRRRRWWRRRRWWRRRRWW	12	12
TAT-NR2B9c (NA-1)	H-GRKKRRQRRR-KLSSIESDV-OH	6	7

* 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.

Peptide	Sequence*	Arginine residues	Charge at pH7
Ptm sulphate	Mixture of protamine peptides Ptm 1 – 4	≈21	21
Ptm 1	PRRRRRSSSRPIRRRRRPRASRRRRRGGRRRR	21	21
Ptm 2	PRRRRSSRRPVRRRRRPRVSRRRRRGGRRRR	21	21
Ptm 3	PRRRRSSSRPVRRRRRPRVSRRRRRGGRRRR	20	20
Ptm 4	PRRRRASRRIRRRRRPRVSRRRRRGGRRRR	21	21
Ptm 5	PRRRRSSSRPVRRRRRPRVSRRRRRRGGRRRR	21	21
LMWP	VSRRRRRRGGRRRR	10	10

* 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). Ptm sulphate is likely to consist of Ptm 1 – 4 as the sulphate salt. LMWP: low molecular weight protamine. P = proline, R = arginine, S = serine, I = isoleucine, A = alanine, G = glycine, V = valine.

In vivo stroke models

Animal ethics approval

All animal surgical procedures and behavioural studies were approved by the University of Western Australia Animal Ethics Committee and follow the guidelines outlined in the *Australian Code for the Care and Use of Animal for Scientific Purposes* and the *National Health and Medical Research Council of Australia*. In the design of these studies, every effort was made to minimise the amount of animal suffering and follow both the STAIR (Fisher, 2011) and ARRIVE (Kilkenny et al., 2010) guidelines as appropriate.

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% O_2 and 70% of N_2O, 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 (pO_2, pCO_2), 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).

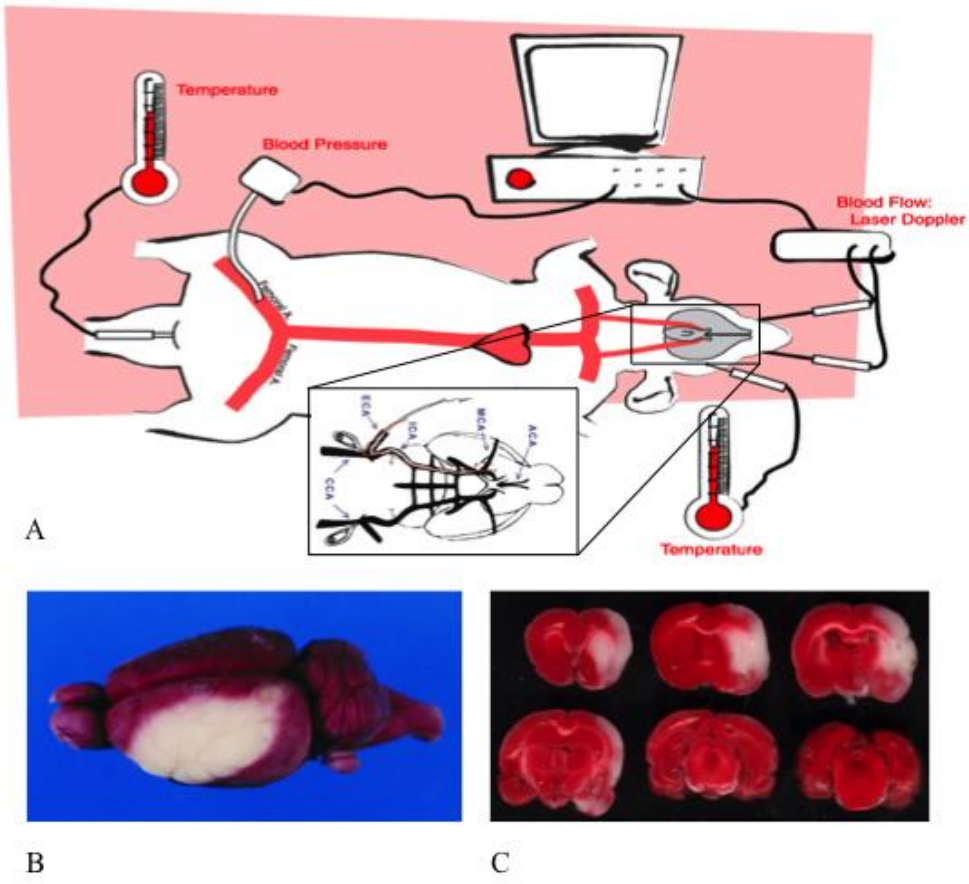


Figure 3.1 Intraluminal filament MCAO model in the rat. (A) An intraluminal filament was advanced into the internal carotid artery until it blocked the MCA, which was confirmed by a laser Doppler. Blood pressure was monitored and temperature maintained at 37°C during surgical procedures. (B) & (C) Entire 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.

Score	Neurological grading scale post-surgery
0	No deficits
1	Flexed forepaw
2	Inability to resist lateral push
3	Circling
4	Agitated circling
5	Stupor

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

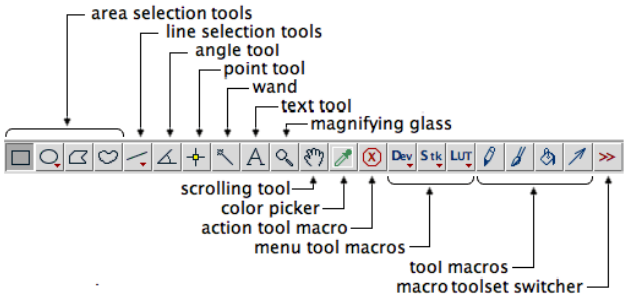
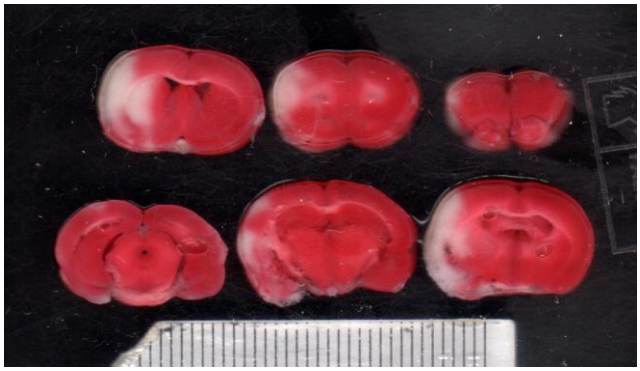
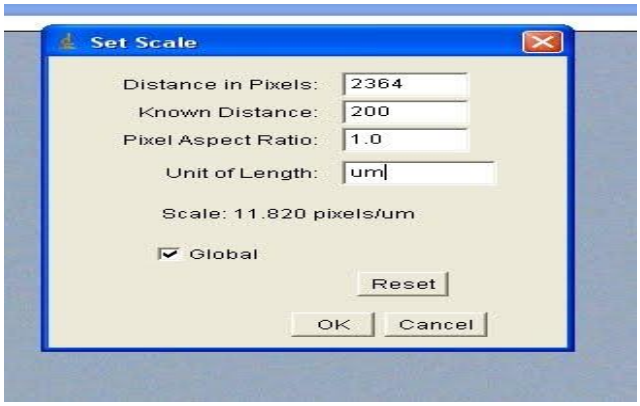
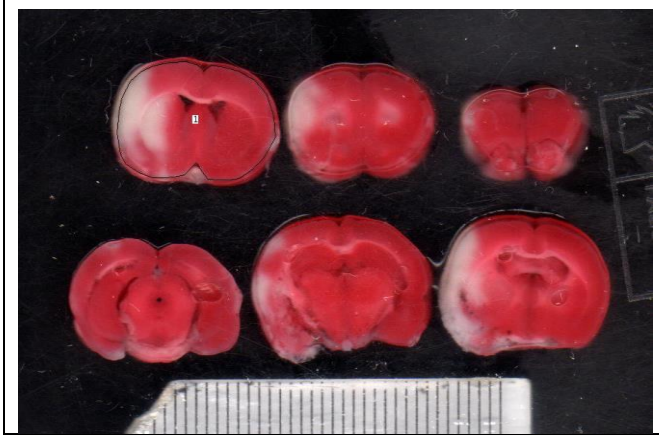
Tissue sectioning and triphenyl tetrazolium chloride (TTC) staining

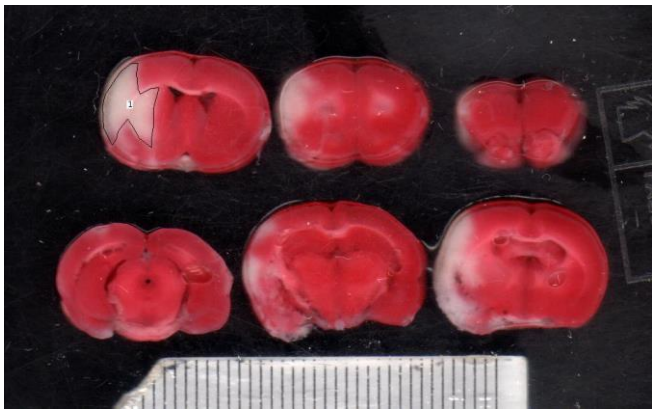
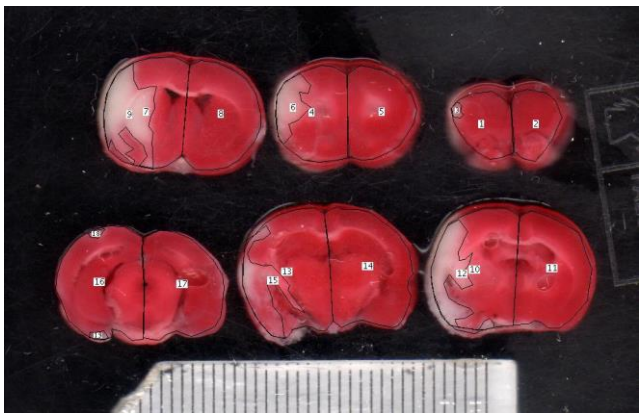
Twenty-four hours post-stroke animals were euthanised by lethal intraperitoneal injection of pentobarbitone (325 mg of pentobarbitone sodium in 1 ml solution). Infarct volume measurement was performed by brain slicing and TTC (triphenyl tetrazolium chloride) staining. The brain was carefully removed 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.

 *ImageJ toolbar*	1) Open ImageJ.
 *Rat brain in 2 mm coronal sections*	2) Click on File → open the image of interest. Check the spatial calibration and set the correct pixel size.
 *Set scale window in ImageJ*	3) 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.
	4) 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.

Entire perimeter of the brain	
 *Perimeter of the infarct area*	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.
 *Example of infarct volume analysis using ImageJ*	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.

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.

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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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Vince Clark: 3%

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Ryan Anderton: 2%

Neville Knuckey: 2%

Signed…… Signed……

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RESEARCH ARTICLE Open Access

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

Diego Milani[2,3,4], Vince W. Clark[1,2,3], Jane L. Cross[1,2,3], Ryan S. Anderton[3,4], Neville W. Knuckey[1,2,3] and Bruno P. Meloni[1,2,3*]

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 resides). 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

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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

	Saline (N = 12)	R12 (N = 9)	R15 (N = 8)	R18 (N = 8)
PaO_2 (mmHg)	115.10 ± 33.51	124.30 ± 18.40	112.80 ± 16.96	120.60 ± 20.34
$PaCO_2$ (mmHg)	42.92 ± 5.82	46.00 ± 4.21	39.38 ± 5.20	44.25 ± 7.74
pH	7.44 ± 0.09	7.33 ± 0.08	7.31 ± 0.09	7.42 ± 0.08
Glucose (mmol/L)	7.74 ± 1.27	7.42 ± 1.06	7.03 ± 1.11	7.13 ± 1.08
Blood pressure (mmHg)	89.00 ± 8.44	78.44 ± 6.98	88.00 ± 9.97	79.63 ± 12.65
Body temperature (°C)	37.48 ± 0.18	37.58 ± 0.13	37.46 ± 0.21	37.51 ± 0.06

PaO_2, $PaCO_2$, pH, blood pressure and glucose measured before MCAO. Body temperature data represent average over 2 h post-surgery monitoring period. Data are mean ± SD

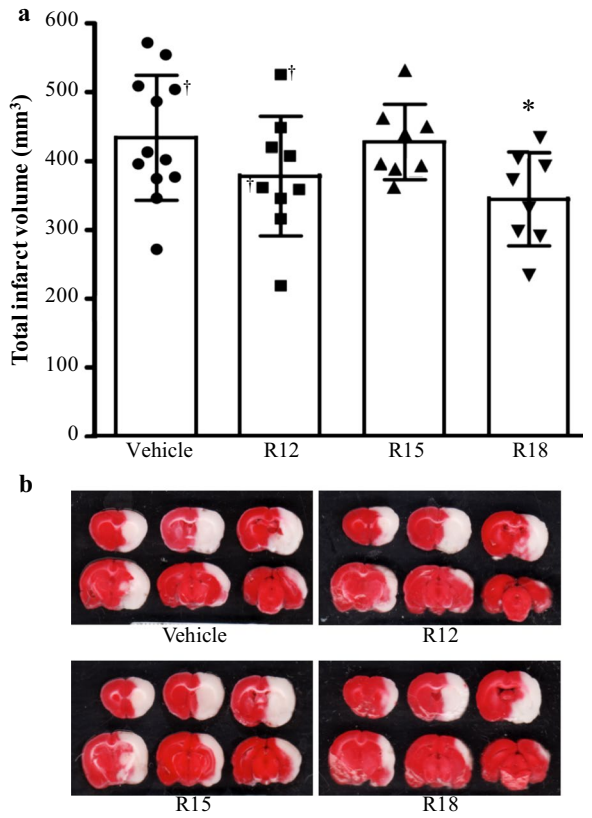

Fig. 1 Infarct volume measurements and coronal brain slices 24 h after permanent MCAO. Treatments were administered intravenously (saline vehicle or peptide 1000 nmol/kg; in 600 µl volume over 6 min) 30 min after MCAO. **a** Values are mean ± SD. *$P < 0.05$ when compared to the vehicle control group. †Denotes animals that died the following day after surgery, before the 24 h post-MCAO end-point, but whose infarct volume was measured nonetheless. **b** Representative TTC coronal brain slices from vehicle and peptide treated animals

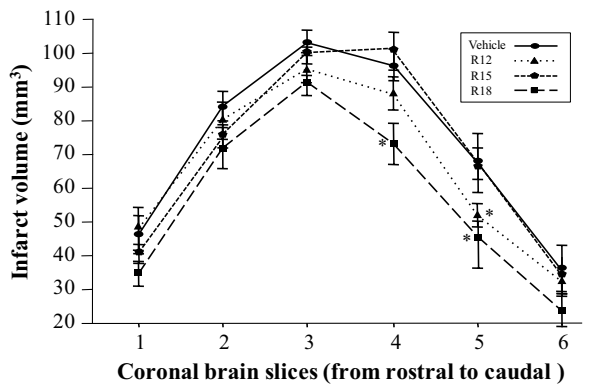

Fig. 2 Infarct volume analysis in coronal brain slices (1–6 from rostral to caudal). The R18 peptide significantly reduced injury in brain slices 4 and 5, and the R12 peptide significantly reduced brain injury in slice 5. Values are mean ± SD; *$P < 0.05$ when compared to the vehicle control group

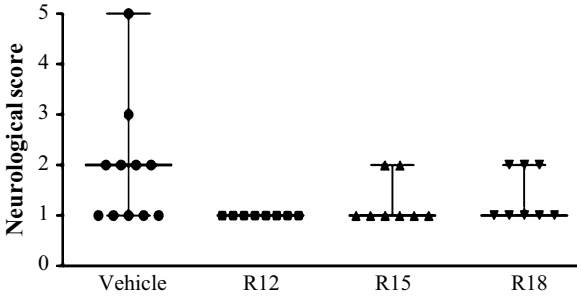

Fig. 3 Neurological grading scores 24 h after permanent MCAO (0 = no deficit, 4 = major deficit) for saline (vehicle) and peptide (R12, R15, R18; 1000 nmol/kg) treatment groups. Assessment was performed immediately before euthanasia. Lines on graph indicate range and median for neurological scores

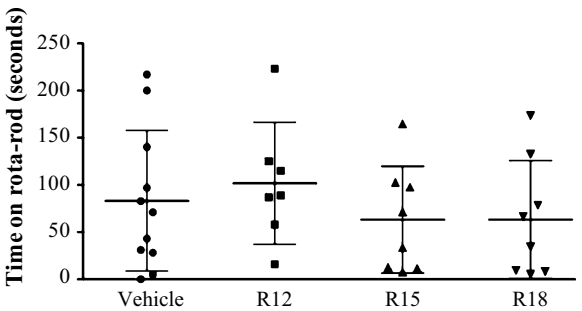

Fig. 4 Rota-rod performance 24 h after permanent MCAO for saline (vehicle) and peptide (R12, R15, R18; 1000 nmol/kg) treatment groups. Results for this test were highly variable within groups and no significant differences were detected. Average time healthy pre-surgery animals remained on rota-rod was 78 s (data not shown). Values 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

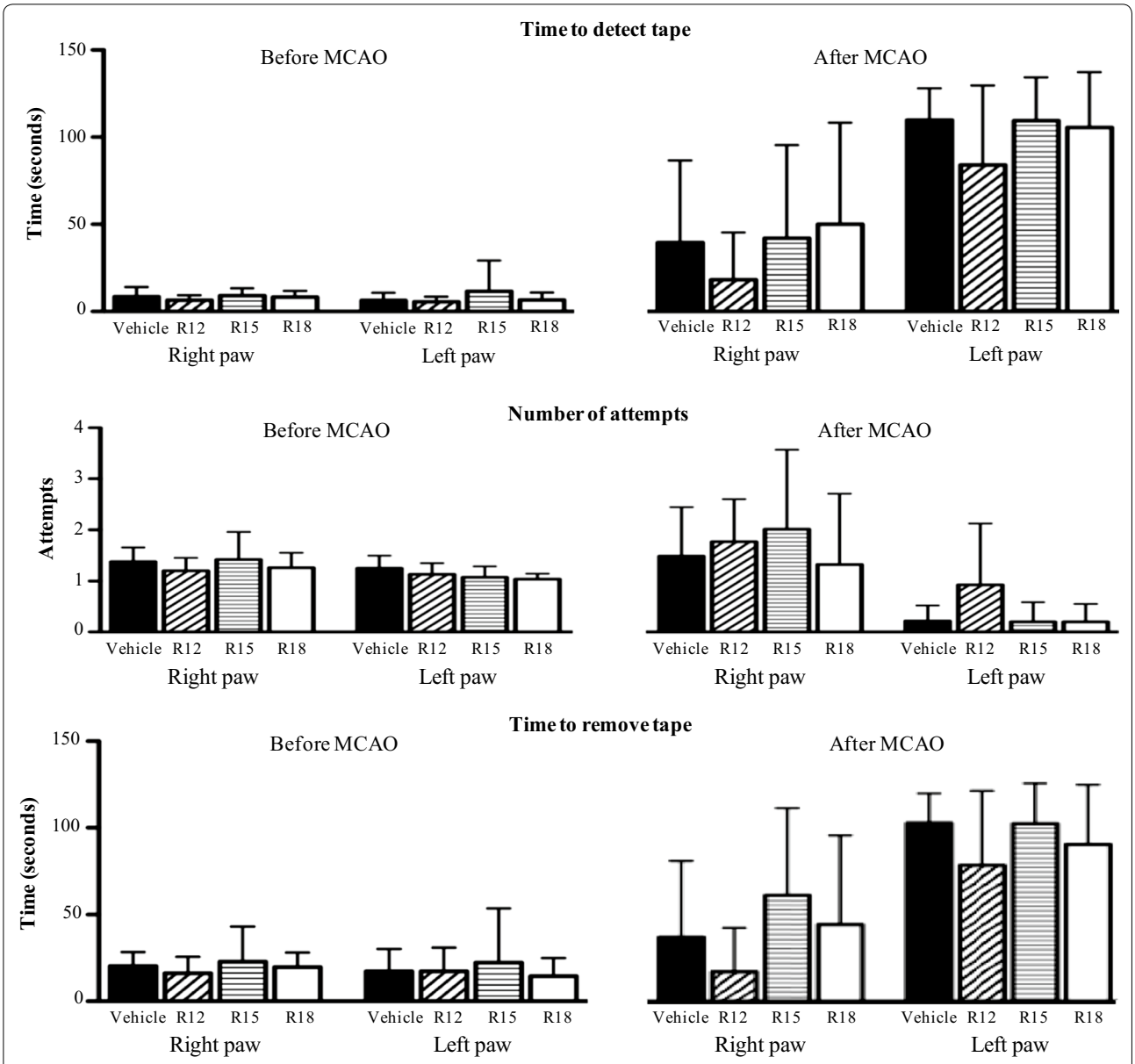

Fig. 5 Functional assessment measurements using adhesive tape removal test before and 24 h after MCAO for saline (vehicle) and peptide (R12, R15, R18; 1000 nmol/kg) treatment groups. Post-MCAO assessment was performed immediately before euthanasia. No treatment significantly improved adhesive tape detection or removal times for the *left* or *right paw*. Values are mean ± SD; N = 11 for vehicle, N = 7 for R12, N = 8 for R15 and N = 8 for R18. Maximum time allowed for adhesive tape removal was 120 s

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

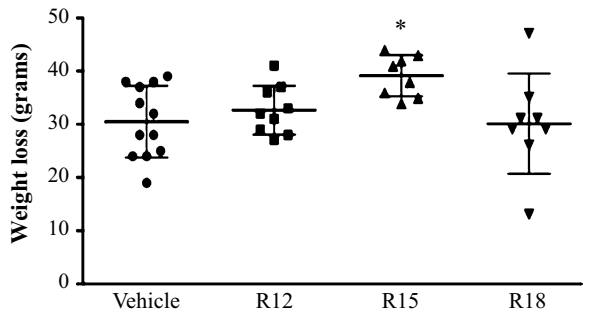

Fig. 6 Weight loss at 24 h after permanent MCAO for saline (vehicle) and peptide (R12, R15, R18; 1000 nmol/kg) treatment groups. Values are mean ± SD; *$P < 0.05$ when compared to the vehicle control group

surprising that the R15 peptide did not have any affect on infarct volume reduction, despite showing comparable neuroprotective efficacy to R18 when assessed in an in vitro neuronal glutamate excitotoxicity model [6]. The reason why no observable neuroprotection was obtained for R15 is at present unknown, but it is possible that a higher or lower dose may be more effective than the dose used in the current study. 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 hypothesised that these peptides have the capacity to inhibit calcium influx by causing the internalisation of cell surface structures such as ion channels and thereby reduce the toxic neuronal calcium entry that occurs after excitotoxicity and cerebral ischemia. We have speculated that due to the cell penetrating properties of arginine-rich peptides, including putative "neuroprotective peptides" fused to the arginine-rich carrier peptide TAT, ion channel receptor internalisation occurs during neuronal endocytic uptake of the peptides [6, 7]. Evidence that supports 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 surface expression of neuronal ion channels [11, 13, 18]; and (3) can induce the endocytic internalisation of epidermal growth factor receptor and tumour necrosis factor receptors in HeLa cells [19].

In support of the poly-arginine neuroprotective findings in the present study, a recent report [8] has confirmed the neuroprotective properties of poly-arginine 7 (R7) containing peptides and other arginine-rich peptides (TAT and TATNR2B9c) in an in vivo retinal ganglion NMDA excitotoxicity model. Moreover, the study also provides evidence for an additional neuroprotective mechanism associated with maintenance of mitochondrial function and integrity.

Studies in our laboratory to confirm peptide-induced internalisation of cell surface receptors and other neuroprotective mechanisms are in progress. While we have demonstrated that arginine-rich peptides have the capacity to reduce excitotoxic calcium influx, it will be important to obtain a more comprehensive understanding of peptide neuroprotective mechanism of action. Nevertheless 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 neuroprotective properties of poly-arginine peptides [5–9], highlights 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-RRRRRRRRRRRRRRR-OH) and R18 H-RRRRRRRRRRRRRRRRRR-OH) peptides used in the study were synthesised by China Peptides (Shanghai, China). The peptides were HPLC purified to >94 % purity. All 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.

Rat permanent middle cerebral artery occlusion procedure

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*. The experimental procedure for performing the permanent middle cerebral artery occlusion (MCAO) stroke model

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 (PaO_2, $PaCO_2$, 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-fours 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

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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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Signed…… Signed……

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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-YRKKRRQRRR-KLSSIESDV-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 anesthesia 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 (pO_2, pCO_2), 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 (Physitemp 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 anesthetic 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 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 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

Table 1: Physiological parameters (mean ± SD).

Parameter	Experimental groups						
	Vehicle (saline)	R18 (nmol/kg)			TAT-NR2B9c (nmol/kg)		
		100	300	1000	100	300	1000
PaO_2, before MCAO	117.19 ± 22.44	114.66 ± 11.34	115.16 ± 13.79	124.16 ± 14.35	109.33 ± 9.04	117.83 ± 16.55	112.83 ± 12.93
$PaCO_2$, before MCAO	38.33 ± 2.94	39 ± 3.57	39.5 ± 3.50	42.68 ± 6.17	43.66 ± 8.93	39.5 ± 5.78	39.83 ± 3.74
pH, before MCAO	7.36 ± 0.05	7.42 ± 0.09	7.37 ± 0.04	7.44 ± 0.06	7.38 ± 0.04	7.45 ± 0.04	7.39 ± 0.06
Glucose (mmol/L), before MCAO	8.0 ± 1.37	8.15 ± 0.68	7.81 ± 0.96	8.11 ± 1.00	7.21 ± 1.31	7.71 ± 0.81	7.50 ± 1.73
BP (mmHg), average during surgery	87.33 ± 4.17	88.33 ± 7.06	84 ± 5.13	91.6 ± 5.44	91 ± 3.09	83.66 ± 6.34	88 ± 3.68
Temperature (°C), average 2 h after surgery	37.51 ± 0.16	37.37 ± 0.25	37.46 ± 0.29	37.46 ± 0.21	37.43 ± 0.25	37.53 ± 0.17	37.52 ± 0.21

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%, $P = 0.045$).

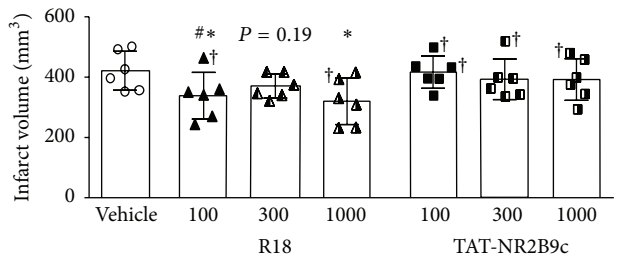

Figure 1: Infarct volume measurements 24 hours after permanent MCAO. Treatments were administered intravenously (saline vehicle or R18 and TAT-NR2B9c peptide at 100, 300, or 1000 nmol/kg; 600 μL volume over 6 min) 60 minutes after MCAO. Values are mean ± SD. $^*P < 0.05$ when compared to the vehicle control group and $^\#P < 0.05$ when compared to the TAT-NR2B9c 100 nmol/kg group. † denotes animals that died several hours before the 24-hour post-MCAO study end-point but were still included in the final infarct volume analysis.

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

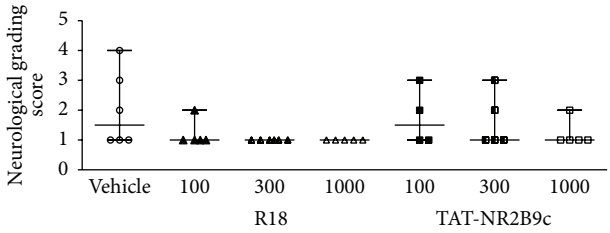

Figure 2: Neurological grading scores 24 hours after permanent MCAO (0 = no deficit, 5 = major deficit) for saline (vehicle) and peptide (R18 and TAT-NR2B9c at 100, 300, or 1000 nmol/kg) treatment groups. Lines on graph indicate range and median for neurological scores.

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 (YRKKRRQRRR) 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

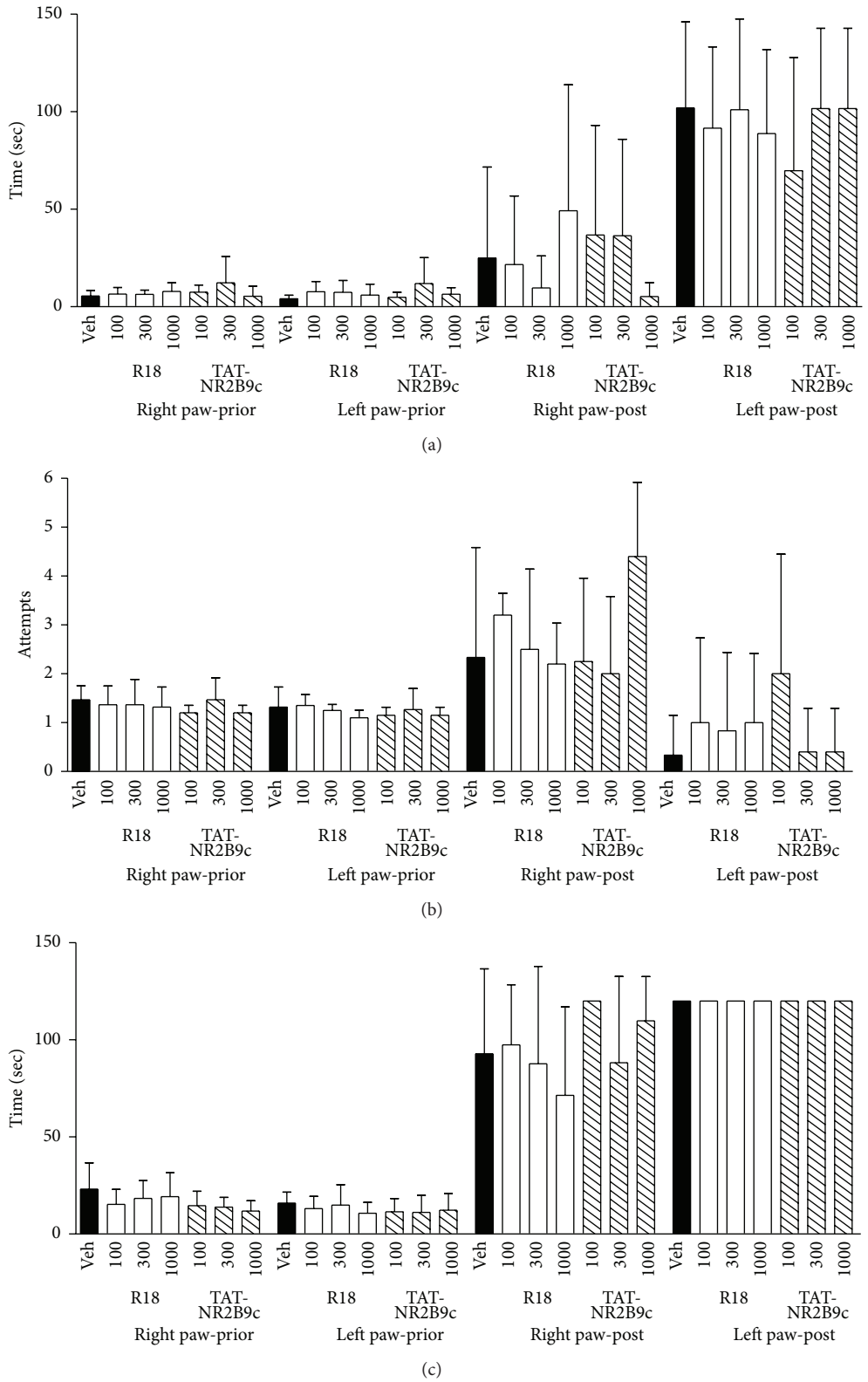

Figure 3: Functional assessment measurements using adhesive tape removal test before and 24 hours after MCAO for saline (vehicle) and peptide (R18 and TAT-NR2B9c at 100, 300, or 1000 nmol/kg) treatment groups. (a) Time to detect tape. (b) Number of attempts to remove tape. (c) Time to remove tape. Values are mean ± SD. Maximum time allowed for adhesive tape removal was 120 seconds.

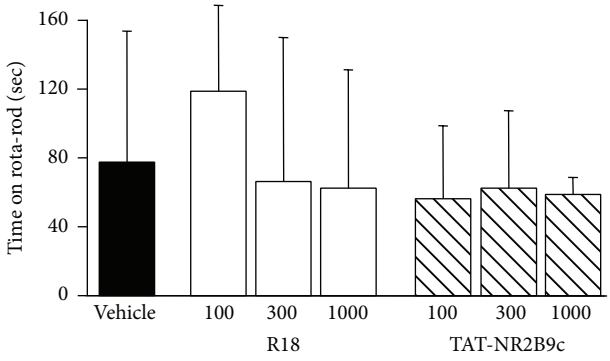

Figure 4: Rota-rod performance 24 hours after permanent MCAO for saline (vehicle) and peptide (R18 and TAT-NR2B9c at 100, 300, or 1000 nmol/kg) treatment groups. Values are mean ± SD.

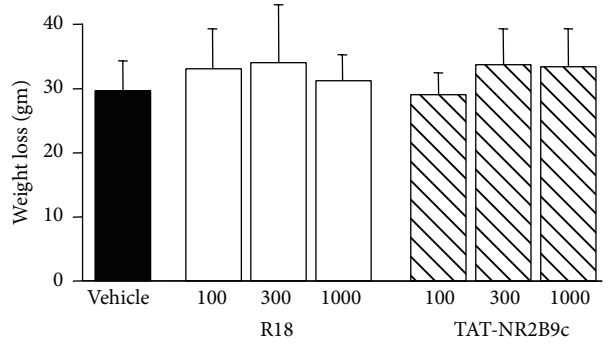

Figure 5: Weight loss at 24 hours after permanent MCAO for saline (vehicle) and peptide (R18 and TAT-NR2B9c at 100, 300, or 1000 nmol/kg) treatment groups. Values are mean ± SD.

mitochondria and reduce mitochondrial respiration, membrane potential, and levels of reactive oxygen species [49]. It is also interesting to note that Marshall et al. [49] found that polyarginine peptides taken up by retinal ganglion cells are localised within small spherical cytoplasmic structures, which the authors suggested to be mitochondria. Also of interest are recent studies surrounding the arginine-rich Borna disease viral mitochondrial-targeting protein, X. The full-length protein X and X-derived peptide fused to a cell penetrating peptide display neuronal, axonal, and mitochondrial protective properties [50, 51].

Another mechanism whereby arginine-rich peptides exert a neuroprotective effect may be related to the ability of polyarginine and arginine-rich peptides to inhibit the proteolytic activity of proprotein convertases (e.g., furin and PC4 [52, 53]), cathepsin C [54], and the proteasome [55, 56], an effect that may be beneficial following brain ischaemia. For example, furin is ubiquitously expressed calcium-dependent convertase responsible for the activation of membrane bound proteins, including metalloproteinases, which are known to have adverse effects on the blood brain barrier following stroke [57]. Similarly, treatments known to inhibit the proteasome which is responsible for the degradation of short-lived cytosolic proteins are known to reduce brain injury in stroke [58, 59].

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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Chapter 6

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

Published: Neuroscience Research

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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Chapter 7

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

Published: NeuroMolecular Medicine

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

Bruno P. Meloni [1,2,3] , Diego Milani [1,2,4] , Jane L. Cross [1,2,3] , Vince W. Clark [1,2,3] , Adam B. Edwards [1,2,4] , Ryan S. Anderton [1,4] , David J. Blacker [1,3,5] , Neville W. Knuckey [1,2,3]

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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

Published: Brain Research Bulletin

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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Milani, D., Cross, J. L., Anderton, R. S., Blacker, D. J., Knuckey, N. W., & Meloni, B. P. (2017). 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. *Brain Research Bulletin, 135*, 62-68. doi:10.1016/j.brainresbull.2017.09.012

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: $R^2 = 0.01$).

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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 *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 *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 *in vitro* and *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.

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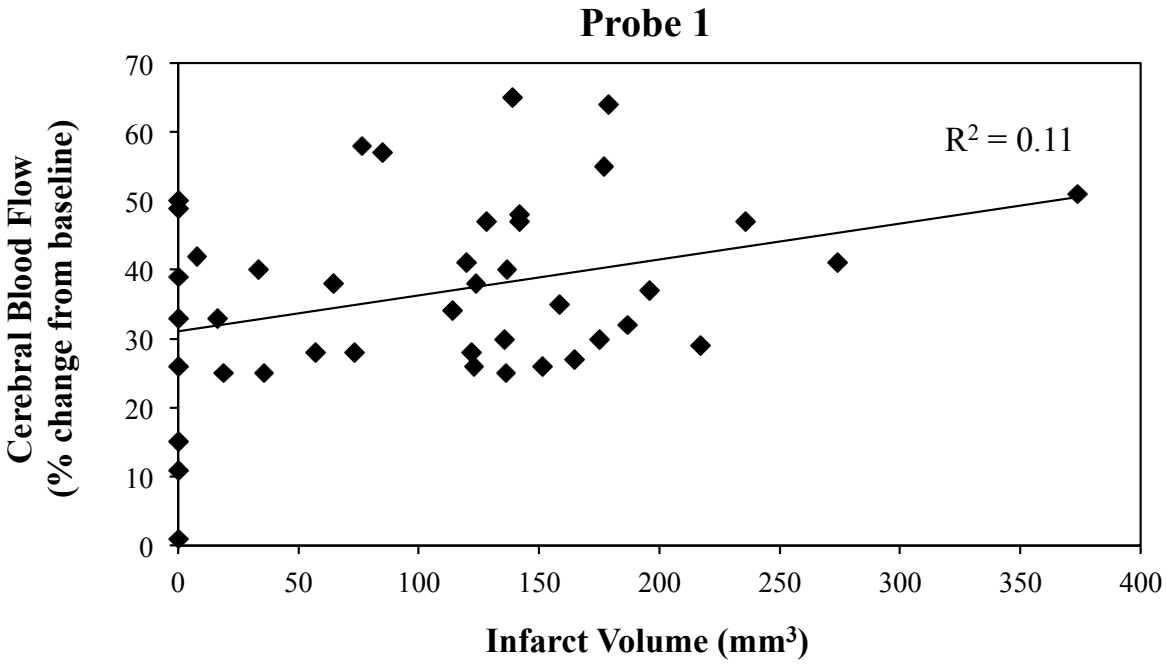
Appendix



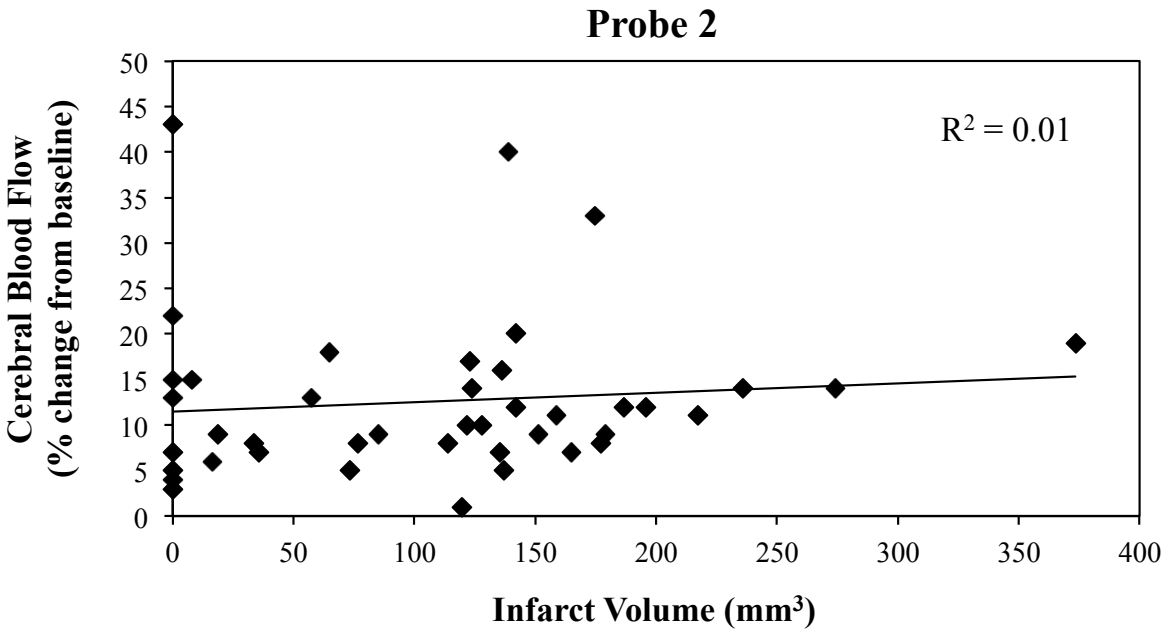


Figure A1. Linear regression analysis between infarct volume and cerebral blood flow deficit after MCAO, as detected by laser Doppler Probe 1 and 2.