Examining the neuroprotective efficacy of arginine-rich peptides in a traumatic brain injury model

Li Shan Chiu
B.MedSc. Pharmacology (Hons)

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Approval #: RA/3/100/1434
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The work described in this thesis was funded by grants from the Brain Foundation (Australia), Neurotrauma Research Program grant scheme, and the Insurance Commission of Western Australia.

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Date: January 31, 2019
Abstract

Traumatic brain injury (TBI) is a clinical condition leaving survivors with lifelong functional impairments and a diminished quality of life. Currently, only preventative, surgical, and rehabilitative interventions exist, and there is an urgent requirement for a pharmacological neuroprotective agent to protect the brain from secondary injury. Cationic arginine-rich peptides (CARPs) are a recently identified class of compound with neuroprotective properties. The Meloni laboratory has demonstrated that CARPs can not only mitigate the excitotoxic effects of calcium influx, but they also have the potential to target multiple aspects of TBI secondary injury, including preserving mitochondrial function and modulating inflammation. The CARP, poly-arginine-18 (R18; 18-mer of arginine), has demonstrated in vitro and in vivo neuroprotective efficacy in models of stroke. Due to the pathophysiological similarities between stroke and TBI, a central hypothesis of this thesis was that R18 could be of similar benefit in TBI. This thesis describes the in vitro and in vivo experimental studies that were performed in order to explore this hypothesis.

An in vitro comparison between R18 and other CARPs examined in TBI models (COG1410 and APP96-110) confirmed that the poly-arginine peptide had superior neuroprotective efficacy. R18 had a greater capacity to protect rat-derived primary cortical neurons from excitotoxic injury and decrease neuronal intracellular calcium influx induced by glutamic acid. A subsequent in vivo study in a rat model of impact-acceleration TBI demonstrated that R18 and COG1410 at a 300 nmol/kg dose could reduce the extent of axonal injury and provide positive trends for improving functional recovery. Based on the in vitro and in vivo findings, it was concluded that R18 had greater neuroprotective potential than other CARPs.
This neuroprotective function was further explored in male Long-Evans rats subjected to TBI, where R18 and its D-enantiomer (R18D) were compared at a 1000 nmol/kg dose. It was demonstrated that R18D could improve sensorimotor and vestibulomotor recovery to a greater extent than R18, and that both peptides significantly reduced GFAP protein levels in the brain; the latter result indicating the peptides have immunomodulatory properties after TBI.

The R18D peptide was then examined at a low (100 nmol/kg) and high (1000 nmol/kg) dose in male Sprague-Dawley rats where it was demonstrated that axonal injury could be reduced with a high dose of R18D, while both doses provided positive trends for recovery in learning and memory, and vestibulomotor function. Given these results, R18D serum pharmacokinetics and tissue biodistribution studies in healthy, male Sprague-Dawley rats were conducted to determine whether the positive effects may be due to R18D. These studies revealed that free R18D peptide had a serum half-life of less than five minutes and only a small proportion of the injected peptide was able to cross the blood brain barrier and accumulate in the brain.

The findings in this thesis have further confirmed the neuroprotective properties of poly-arginine R18 peptides, and have provided justification for further investigation into the application of R18 and other CARPs in neurotrauma. However, although positive effects were observed in these exploratory studies, additional studies in different TBI models and investigating peptide mechanism of action are required.
Acknowledgements

The research done for this thesis was supported by an Australian Government Research Training Program (RTP) Scholarship kindly provided by Mr Jim Litis, and grants from the Neurotrauma Research Program of Western Australia, Insurance Commission of Western Australia, Brain Foundation (Australia), and the Perron Institute for Neurological and Translational Science. I would also like to acknowledge Australian taxpayers, especially West Australians who accrued traffic infringements and may have inadvertently become philanthropists.

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Long live Nibbles.
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Co-author signatures and date:

January 31, 2019       January 31, 2019       January 31, 2019

Student signature:       

Date: January 31, 2019

I, **Prof Norman Palmer** certify that the student’s statements regarding their contribution to each of the works listed above are correct.

As all co-authors’ signatures could not be obtained, I hereby authorise inclusion of the co-authored work in the thesis.

Coordinating supervisor signature:       

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# Table of contents

**Thesis declaration** ........................................................................................................... ii  
**Abstract** ........................................................................................................................... iii  
**Acknowledgements** ........................................................................................................ v  
**Authorship declaration: co-authored publications** ......................................................... viii  
**Publications** .................................................................................................................. x  
**Conferences and presentations** ..................................................................................... xi  
**Awards and grants** ........................................................................................................... xiii  
**Contents** ....................................................................................................................... xiv  
**List of figures** .................................................................................................................. xx  
**List of tables** ................................................................................................................... xxii  
**List of abbreviation** ....................................................................................................... xxiii

## Chapter One

**General introduction** ...................................................................................................... 1  

### 1.1 Background  .............................................................................................................. 2

### 1.2 Epidemiology  ............................................................................................................ 2  
  1.2.1 Incidence and prevalence in high-income countries ............................................ 3  
  1.2.2 Incidence and prevalence in low- and middle-income countries ..................... 5  
  1.2.3 Risk factors and aetiology .................................................................................. 5

### 1.3 Impact of TBI on patients and the community .............................................................. 8  
  1.3.1 Individual impact .................................................................................................. 8  
  1.3.2 Societal impact ..................................................................................................... 10  
  1.3.3 Economic impact .................................................................................................. 11

### 1.4 TBI diagnosis ............................................................................................................. 12

### 1.5 TBI biomechanics and pathophysiology .................................................................. 14  
  1.5.1 Mechanics of TBI ................................................................................................ 14  
  1.5.2 Primary injury ....................................................................................................... 15  
  1.5.3 Secondary injury .................................................................................................. 17

### 1.6 Current interventions ................................................................................................ 19  
  1.6.1 Preventative measures ......................................................................................... 19  
  1.6.2 Neurosurgical measures ..................................................................................... 21  
  1.6.3 Rehabilitation ...................................................................................................... 22

### 1.7 Pharmacological therapies for TBI ............................................................................ 23
Chapter Two

The neuroprotective potential of arginine-rich peptides for the acute treatment of traumatic brain injury ................................................................. 54

Conclusion ........................................................................................................... 55

References ........................................................................................................... 55

Chapter Three

Pharmacological approaches to treating traumatic brain injury: a case for arginine-rich peptides ............................................................. 57

Abstract ............................................................................................................. 58

Introduction ....................................................................................................... 58

TBI pathophysiology .......................................................................................... 59

Excitotoxicity .................................................................................................... 59

Mitochondrial disturbances .............................................................................. 59

Inflammatory response ..................................................................................... 61

Matric metalloproteinases and cerebral oedema ............................................. 62

Past pharmacological approaches to treating TBI ....................................... 62

Towards a peptide-based therapeutic ............................................................. 63

Cyclosporin A .................................................................................................... 63

Conopeptides .................................................................................................. 63

EPO-derivatives ............................................................................................... 64

Pituitary adenylate cyclase activating polypeptide ....................................... 65

Other endogenous neurotrophic protein derivatives ....................................... 65

Nogo extracellular peptide 1 – 40 ................................................................ 66

APP96-110 ........................................................................................................ 66

APOE-derivatives .............................................................................................. 66

Other TAT-fused peptides ............................................................................... 67

A case for arginine-rich peptides ................................................................... 67

Past approaches with peptides containing arginine residues ..................... 67

TAT and neuroprotective peptides fused to TAT ........................................... 68

Arginine-rich peptide interactions with cell-surface structures ................... 68

Arginine-rich peptide interactions with mitochondria ................................. 69
Other potential neuroprotective actions of arginine-rich peptides .................. 69
Designing an arginine-rich therapeutic for TBI ........................................... 70
Efficacy of arginine-rich peptides in stroke models .................................... 70
Conclusion .................................................................................................. 70
References ................................................................................................. 70

Chapter Four

General materials and methods ................................................................... 78
4.1 Peptides used in this project ................................................................... 79
4.2 Primary cortical neuronal cultures ......................................................... 79
4.3 Glutamic acid excitotoxicity model and peptide treatments ................. 80
  4.3.1 Neuronal viability assessment .............................................................. 81
4.4 Animals and husbandry ....................................................................... 81
4.5 Traumatic brain injury model and peptide administration .................. 82
  4.5.1 Anaesthesia and surgical preparation .................................................. 82
  4.5.2 Weight-drop impact-acceleration injury model .................................. 82
  4.5.3 Treatment administration ................................................................ 84
4.6 Post-surgical animal care and monitoring ............................................. 84
4.7 Functional assessments ........................................................................ 85
  4.7.1 Barnes maze test ........................................................................... 85
  4.7.2 Adhesive tape removal test .............................................................. 86
  4.7.3 Rotarod test ................................................................................... 87
4.8 Tissue processing .................................................................................. 87
4.9 Histological assessment for brain injury ............................................... 88
  4.9.1 Nissl staining .................................................................................. 88
  4.9.2 Bielschowsky’s silver staining ......................................................... 89
4.10 Statistical analysis ............................................................................... 90
4.11 References ......................................................................................... 91

Chapter Five

Assessment of R18, COG1410, and APP96-110 in excitotoxicity and traumatic
brain injury .................................................................................................. 93
Abstract ...................................................................................................... 94
Introduction ................................................................................................. 94
Chapter Six

Poly-arginine peptide R18D reduces neuroinflammation and functional deficits following traumatic brain injury in the Long-Evans rat

Abstract

Introduction

Methods

Peptides used in this study

Traumatic brain injury model and peptide administration

Post-surgical animal care and monitoring

Functional assessments

Protein extraction and Western blotting

Statistical Analysis

Results

Functional assessments

Western blot analysis for neuroinflammatory response

Discussion

Limitations

References
8.2.1 Poly-arginine peptide R18 is more effective than COG1410 and APP96-110 peptides at protecting cultured cortical neurons from glutamic acid excitotoxicity. 148
8.2.2 Poly-arginine peptides can improve functional recovery in male rats subjected to TBI .................................................. 150
8.2.3 Poly-arginine peptide R18D has limited distribution in the brain of healthy, male Sprague-Dawley rats ................................................ 151
8.2.4 R18 reduces the extent of axonal injury in the corpus callosum of male Sprague-Dawley rats subjected to TBI ......................................... 153
8.2.5 R18D reduces the neuroinflammatory response in male Long-Evans subjected to TBI .............................................................................................................. 155

8.3 Future directions .................................................................................................................. 156

8.3.1 Assessing peptide efficacy using different methods of injury induction ........ 156
8.3.2 Assessing peptide efficacy in phylogenetically higher animal TBI models .... 158
8.3.3 Alternative neurological and behavioural assessments after TBI .......... 159
8.3.4 Extending end-of-experiment time-point after TBI ................................. 160
8.3.5 Peptide mechanism of action ................................................................. 161
8.3.6 Pharmacological profiling of poly-arginine peptides .............................. 162

8.4 Concluding remarks ......................................................................................................... 164

8.5 References .......................................................................................................................... 166
List of figures

Chapter One

Figure 1.1 The major secondary pathophysiological events of TBI

Chapter Three

Figure 1 Secondary injury cascade following TBI and proposed intervention sites of neuroprotective peptides

Chapter Four

Figure 4.1 Marmarou’s weight drop model of TBI
Figure 4.2 Barnes maze configuration
Figure 4.3 Male Sprague-Dawley rat demonstrating pawplacement of adhesive tape
Figure 4.4 Bielschowsky’s silver staining and axonal injury grading criteria

Chapter Five

Figure 1 Peptide dose response in a glutamic acid neuronal excitotoxicity model
Figure 2 Intracellular calcium assessment after glutamic acid exposure
Figure 3 Adhesive tape test measurements at post-injury days 1 – 4
Figure 4 Rotarod measurements at post-injury days 1 – 4
Figure 5 Barnes maze measurements at post-injury days 1 – 4
Figure 6 Axonal injury grades of each treatment group on post-injury day 4
Figure 7 Representative images of axonal injury grading criteria on a 5-point scale
Figure 8 Number of hippocampal neurons with a normal appearance

Chapter Six

Figure 1 Percentage change from baseline performance on adhesive tape test
Figure 2 Percentage change from baseline performance on rotarod
Figure 3 Percentage change from baseline performance on Barnes maze
Figure 4 Western blot analysis for neuroinflammatory response in brain lysates
Chapter Seven

**Figure 1** Learning and memory functional recovery on the Barnes maze at post-injury days 1 and 3

**Figure 2** Vestibulomotor functional recovery on the rotarod at post-injury days 1 and 3

**Figure 3** Grading of corpus callosal axonal injury as determined by Bielschowsky’s silver stain

**Figure 4** Detection of free R18D in healthy rat serum by HPLC

**Figure 5** Time activity curve of $^{18}$F-R18D intravenously injected in healthy rats

**Figure 6** Biodistribution of $^{18}$F-R18D in a representative animal across time-points
# List of tables

## Chapter One

**Table 1.1** Classification of TBI severity using GCS and Westmead PTA scale

## Chapter Three

**Table 1** Studies using peptides in animal TBI models

## Chapter Four

**Table 4.1** Summary of peptides used in this thesis

## Chapter Five

**Table 1** Summary of peptides used in study  
**Table 2** Summary of animal deaths following TBI

## Chapter Seven

**Table 7.1** Percentage of injected dose distributed in major organs
List of abbreviations

ADME \textit{(pharmacokinetics)} absorption, distribution, metabolism, and excretion

AMPA $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA Analysis of variance

APP Amyloid precursor protein

ATP Adenosine triphosphate

BBB Blood brain barrier

CARPs Cationic arginine-rich peptides

CCI Controlled cortical impact

CNS Central nervous system

CPP Cell-penetrating peptide

CSF Cerebrospinal fluid

DAI Diffuse axonal injury

DIV Day \textit{in vitro}

DMEM Dulbecco's Modified Eagle's Medium

EAA Excitatory amino acid

EPO Erythropoietin

FPI Fluid percussion injury

GCS Glasgow coma scale

GFAP Glial acidic fibrillary protein

GOS-E Glasgow outcome-scale – extended

HIE Hypoxic-ischaemic encephalopathy

HPLC High-performance liquid chromatography

HRP Horseradish peroxidase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>HSPG</td>
<td>Heparan sulfate proteoglycans</td>
</tr>
<tr>
<td>I/A</td>
<td>Impact-acceleration</td>
</tr>
<tr>
<td>ICV</td>
<td>Intracerebroventricular</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IM</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>LC/MS</td>
<td>Liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>MCAO</td>
<td>Middle cerebral artery occlusion</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MPTP</td>
<td>Mitochondrial permeability transition pore</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MTS</td>
<td>$3-(4,5\text{-dimethylthiazol}-2\text{-yl})-5-(3\text{-carboxymethoxyphenyl})-2\text{-}(4\text{-sulphophenyl})-2\text{H}-\text{tetrazolium}$</td>
</tr>
<tr>
<td>NADPH</td>
<td>Reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NCX</td>
<td>Sodium-calcium exchanger</td>
</tr>
<tr>
<td>NF-κβ</td>
<td>Nuclear factor kappa beta</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-aspartic acid or N-Methyl-D-aspartate</td>
</tr>
<tr>
<td>NMDAR</td>
<td>NMDA receptor</td>
</tr>
<tr>
<td>NOX</td>
<td>NADPH oxidase</td>
</tr>
<tr>
<td>PACAP</td>
<td>Pituitary adenylate cyclase-activating peptide</td>
</tr>
<tr>
<td>PBBI</td>
<td>Penetrating ballistic-like brain injury</td>
</tr>
<tr>
<td>PET/CT</td>
<td>Positron emission tomography–computed tomography</td>
</tr>
<tr>
<td>PID</td>
<td>Post-injury day</td>
</tr>
<tr>
<td>PTA</td>
<td>Post-traumatic amnesia</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Arginine amino acid</td>
<td>R</td>
</tr>
<tr>
<td>Poly-arginine-18 peptide</td>
<td>R18</td>
</tr>
<tr>
<td>Radioimmunoprecipitation assay</td>
<td>RIPA</td>
</tr>
<tr>
<td>Reactive oxygen species</td>
<td>ROS</td>
</tr>
<tr>
<td>Subcutaneous</td>
<td>SC</td>
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<tr>
<td>Spinal cord injury</td>
<td>SCI</td>
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<tr>
<td>Standard error of the mean</td>
<td>SEM</td>
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<tr>
<td>Superoxide dismutase</td>
<td>SOD</td>
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<tr>
<td>Traumatic brain injury</td>
<td>TBI</td>
</tr>
<tr>
<td>Toll-like receptor</td>
<td>TLR</td>
</tr>
<tr>
<td>Tumour necrosis factor alpha</td>
<td>TNFα</td>
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<tr>
<td>Voltage-dependent anion channels</td>
<td>VDAC</td>
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<td>Voltage-dependent calcium channels</td>
<td>VDCC</td>
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Chapter 1

General introduction
1.1 Background

Traumatic brain injury (TBI) is broadly defined as encompassing any insult to the brain resulting from external mechanical forces. It is a significant but latent public health and socio-economic concern of developed countries throughout the world. The subsequent brain damage is complex, resulting in a spectrum of symptoms and disabilities that can last for the remainder of the individual’s lifetime. Despite its devastating impact, there is no clinically available neuroprotective treatment for TBI. Current practices only aim to either prevent, or indirectly minimise the inflicted damage to the brain. Recent studies have shown that cationic arginine-rich peptides (CARPs) may have potential therapeutic benefit following injuries to the brain, including TBI. The primary aim of this thesis was to examine the effectiveness of CARPs at minimising brain injury and improving functional outcomes, using a model of TBI in rats.

1.2 Epidemiology

Often described as a “hidden epidemic”, TBI persists as a major cause of death and disability worldwide. Quantifying TBI in populations is difficult and often unreliable due to a host of factors such as varying definitions, under-reporting, and incomplete datasets on incidence and outcome (Carroll, Cochran, Guse, & Wang, 2012; Roozenbeek, Maas, & Menon, 2013). Moreover, variations in diagnostic methods, standard of care in hospitals, and healthcare systems make comparisons of TBI epidemiology between countries challenging (Faul & Coronado, 2015; Kou, Hou, Sun, & Chu, 2015). In low-middle income countries, data available for comprehensive research into TBI epidemiology is scarce. Furthermore, other confounding factors such as infectious diseases, lack of resources, and insufficient healthcare infrastructure contribute to the gap
in knowledge of TBI epidemiology in these populations (Hofman, Primack, Keusch, & Hrynkw, 2005; Puvanachandra & Hyder, 2009).

1.2.1 Incidence and prevalence in high-income countries

In Australia, recent studies into TBI epidemiology are lacking, although it is believed that incidences are higher in rural areas than urban (Harradine et al., 2004; Woodward, Dorsch, & Simpson, 1984). Over 12 months in 1987 and 1988, reported incidence rates varied from 100 to 322 new cases per 100,000 of the population in South Australia and New South Wales (Hillier, Hiller, & Metzer, 1997; Tate, McDonald, & Lulham, 1998), the former of which was notably high when compared to similar studies from the United States and Europe at that time. In the North Coast region of New South Wales, the greatest portion of hospital admissions for head trauma were categorised as mild injuries, whereas the incidence of severe TBI was estimated to only be 12 new cases per 100,000 persons in a year (Tate et al., 1998). Over the years, hospital admissions for mild TBI and concussions appears to be on the rise, with one study noting a 38.9% increase from 443 in 2002 – 2003 to 621 out of 100,000 persons in 2010 – 2011 (Finch, Clapperton, & McCrory, 2013). Incidence rates are even higher in paediatric cases of TBI, with mild TBI accounting for almost 90% of admissions (Crowe, Babl, Anderson, & Catroppa, 2009).

Similar to the early Australian study, a more recent study in New Zealand found that TBI incidence was higher in rural populations than urban. They also reported a much higher total incidence rate (790 new cases per 100,000 persons per year), however this included patients of all ages across the country (Feigin et al., 2013). In Europe, most incidence rate reports before 2010 range between 150 to 300 incidents per 100,000 persons per year for
all severities, averaging about 243 incidents per 100,000 (Tagliaferri, Compagnone, Korsic, Servadei, & Kraus, 2006). Similar to trends in Australia, there seems to be an overall increase in incidence. After 2010, one cross-sectional analysis across 24 European countries averaged 280.5 new cases per 100,000 persons per year (Majdan et al., 2016), whereas a 2015 review reported 262 new cases per 100,000 persons per year (W. Peeters et al., 2015). Likewise, an increase in TBI incidence was also seen in the United States. Between 2002 and 2006, the annual occurrence of TBI was estimated to be 576.8 per 100,000 (Faul, Xu, Wald, & Coronado, 2010), increasing to 823.7 per 100,000 of the population in 2010 (Centers for Disease Control and Prevention, 2015). With regards to milder forms of TBI, a recent review estimates that the annual incidence of mild TBI and concussions in the United States is around 500 per 100,000 persons (Voss, Connolly, Schwab, & Scher, 2015). Conversely, a more recent study in an Ontarian population found a decreased TBI incidence rate over an eight-year span (Fu, Jing, Fu, & Cusimano, 2016).

Increasing TBI rates are particularly evident in military personnel, especially since the commencement of Operations Enduring Freedom and Iraqi Freedom in 2001 and 2003, respectively. From 2000 to 2011, the incidence rate of TBI more than doubled from 720.3 to 1,811.4 new cases per 100,000 service members across all components of the Army, Air Force, Navy, and Marine Corps (Centers for Disease Control and Prevention, National Institutes of Health, Department Of Defence, & The Department of Veterans’ Affairs Leadership Panel, 2013), representing an estimated 20% of all defence personnel in these wars (Swanson et al., 2017). Although the number of reported incidents have since slowly decreased, there was a reported total number of 327,299 personnel who have sustained some type of TBI as of June 5, 2015 (Fischer, 2015).
1.2.2 Incidence and prevalence in low- and middle-income countries

TBI is quickly becoming an increasing concern for low-middle income countries, particularly those undergoing rapid economic development (Puvanachandra & Hyder, 2009). Unfortunately, due to more pressing health concerns such as infectious diseases and inadequate provision of high-quality medical and health care (Hofman et al., 2005), few studies reliably address the true burden of disease.

The incidence of TBI in Asia is reportedly on the rise (S. Peeters et al., 2017; Wu et al., 2008). Despite an estimated annual occurrence of 160 new cases per 100,000 of the population in India (Gururaj, 2002), the real figures are invariably much higher due to the number of individuals with mild and/or sub-concussive TBI who go untreated. In the Middle East, the median annual number of new cases per capita is an estimated 45 per 100,000 persons (El-Menyar, Mekkodathil, Al-Thani, Consunji, & Latifi, 2017), around 2.5% of which are cases of repetitive TBI (Fakharian et al., 2016). Higher still are figures from African nations. The cumulative incidence of admissions for TBI in Uganda is 89 per 100,000 persons in a year (Tran et al., 2015), compared to 316 per 100,000 persons in Johannesburg (Nell & Brown, 1991). TBI occurrence is also reported to be particularly high in regions of South America, with one study suggesting 360 new cases per 100,000 persons per year in Brazil (De Andrade, Marino, Ciquini, Eberval Gadelha Figueiredo, & André Guelman Machado, 2001).

1.2.3 Risk factors and aetiology

There has been a recent shift in the epidemiological patterns of TBI (Roozenbeek et al., 2013), particularly in more developed countries with an aging population. Generally,
motor vehicle accidents, falls, and assaults are the three main causes of TBI. Mild TBI is often sustained by those who play contact sports or are military personnel, but the types of head injuries encountered on the battlefield can be much more complex (Ling & Ecklund, 2011).

Much like other developed countries (Faul et al., 2010; Koskinen & Alaranta, 2008), the TBI demographic in Australia is skewed towards males (Harradine et al., 2004; Hillier et al., 1997; Myburgh et al., 2008), who over-represent females by as much as three-fold in hospital admissions. Despite higher incidence rates in rural populations compared to urban, both exhibit similar demographics (Harradine et al., 2004). Younger adults within the 20- to 40-year old age bracket, and those who have had some secondary education but did not complete year 12 are also more likely to be hospital-treated for TBI (Harradine et al., 2004). Additionally, older individuals over the age of 65 in Australia are at an increasing risk of sustaining a TBI (Harvey & Close, 2012) – a pattern that has been echoed by other studies in both the United States (Faul et al., 2010) and Europe (W. Peeters et al., 2015).

Motor vehicle-related injuries, including accidents involving pedestrians, account for the greatest proportion of severe TBIs in Australia, followed by falls and assault (Harradine et al., 2004; Hillier et al., 1997; Myburgh et al., 2008; Tate et al., 1998). In the United States, road trauma caused the greatest proportion of TBI deaths, but were only the second leading cause of TBI to falls (Faul et al., 2010). Motor vehicle accidents also contributed to a large number of TBI incidents in Europe and, particularly for some earlier studies, often eclipsed falls as the most common cause of TBI (Majdan et al., 2016; W. Peeters et al., 2015; Tagliaferri et al., 2006). However, the relative contribution of motor vehicle accidents to mortality varied across countries (Majdan et al., 2016). Motor vehicle-related
accidents are a major contributor to civilian death in low-middle income countries, especially for pedestrians who share multipurpose roads and streets with other fast-moving vehicles (Naci, Chisholm, & Baker, 2009; Nantulya & Reich, 2003; Wu et al., 2008).

Due to an aging population in developed high-income countries, the incidence of fall-related TBIs is increasing (Faul et al., 2010; Harvey & Close, 2012; W. Peeters et al., 2015). An overwhelming proportion of TBIs among older adults in Australia are due to falls from slipping, tripping or stumbling, or as a result of colliding with another person, generally at home (Harvey & Close, 2012). Falls are also responsible for the majority of TBIs among younger children (Crowe et al., 2009; Faul et al., 2010; Feigin et al., 2013), some of which are the result of sport-related activities (Crowe et al., 2009; Selassie et al., 2013). Also of note is a Victorian study, which found that severe TBIs resulting from self-harm was most commonly caused by intentional falls from great heights (Beck, Bray, Cameron, Cooper, & Gabbe, 2016).

Individuals involved in the military are a group with risks unique from civilians of high-income countries. Although the use of Kevlar armour has greatly reduced penetrating head injuries, it still leaves soldiers susceptible to non-penetrating, concussive forces from falls, motor vehicle impacts, and blast explosions (Lew, Poole, Alvarez, & Moore, 2005; Okie, 2005). The latter is a common cause of TBI in U.S. military soldiers (Ling & Ecklund, 2011; Okie, 2005), a considerable majority of which report co-morbidities such as post-traumatic stress disorder, pain, depression, anxiety, and suicidal ideation (Bryan & Clemans, 2013; Hoge et al., 2008; Lew et al., 2005; Okie, 2005; M. B. Stein et al., 2015; Taylor et al., 2012). Recent studies have also found that active involvement in the military can increase the risk of sustaining a TBI post-deployment (Regasa, Michael
Thomas, Gill, Marion, & Ivins, 2016), especially as the result of motor vehicle accidents (Bullman et al., 2017; Carlson et al., 2016).

1.3 Impact of TBI on patients and the community

Survivors of TBI often suffer from lifelong cognitive, behavioural, and communicative deficits (Bruce et al., 2015; Theadom et al., 2018). Such injuries are also believed to be a risk factor for the later development of anxiety and depressive disorders (Nicholl & LaFrance, 2009; Osborn, Mathias, Fairweather-Schmidt, & Anstey, 2017; Ponsford, Spitz, Cromarty, Gifford, & Attwood, 2013), and neurodegenerative diseases such as chronic traumatic encephalopathy, Alzheimer’s and Parkinson’s diseases, and motor neuron disease (Sundman, Hall, & Chen, 2014; Vincent, Roebuck-Spencer, & Cernich, 2014). Additionally, the impact of TBI on survivors also negatively affects families, communities, and the economy.

1.3.1 Individual impact

For individuals who survive a TBI, life dramatically changes. Survivors often experience impairments to both their mental health and cognitive function, while physical impairments are more common following severe injuries. TBI sequelae vary in nature and severity, however, worse outcomes are generally associated with more severe injuries (Novack & Alderson, 2000). Patient recovery is greatly dependent on their rehabilitative environment, availability of resources, and support from their community (Lu, Gary, Neimeier, Ward, & Lapane, 2012; Tomberg, Toomela, Ennok, & Tikk, 2007).
Depression and anxiety disorders are particularly common following TBI (Bombardier et al., 2010; Silver, Kramer, Greenwald, & Weissman, 2001), and their incidence is considerably higher in TBI survivors than the general population (Scholten et al., 2016), many of whom are novel cases (Whelan-Goodinson, Ponsford, Johnston, & Grant, 2009) that can manifest up to a year or longer after the injury (Lavoie et al., 2017; Van der Horn, de Koning, Scheenen, Spikman, & van der Naalt, 2018). Moreover, cognitive difficulties are prominent in patients who have sustained moderate-severe TBI. While long-term intellectual impairments occur after TBI, further deterioration over years does not appear to be significant (Wood & Rutterford, 2006). TBIs sustained in children also have negative outcomes. Although gradual improvements were seen in children with mild-moderate TBI, those who suffered a severe injury early in childhood (3 – 7 years old) demonstrated a more plateaued IQ trajectory (Anderson, Catroppa, Morse, Haritou, & Rosenfeld, 2005).

Somatic complaints are also commonly associated with TBI. The development of epilepsy following TBI is well-established (Annegers & Coan, 2000; D’ambrosio & Perucca, 2004; Lowenstein, 2009), and patients of moderate-severe TBI often report fatigue and problems with balance and memory (Ponsford et al., 2013). Headaches, dizziness, and impairments to a host of cognitive domains are also long-term consequences of TBI (Fagerholm, Hellyer, Scott, Leech, & Sharp, 2015; Ponsford et al., 2014). Moreover, patients and their families often report negative personality and behaviour changes (Levack, Kayes, & Fadyl, 2010), which have been shown to correlate with dysexecutive function and emotional distress (Roscigno & Swanson, 2011; Weddell & Wood, 2016, 2018; Whiffin, Ellis-Hill, Bailey, Jarrett, & Hutchinson, 2017). In addition to the neurological and psychological symptoms, older patients who have sustained a TBI later on in life report problems with their metabolic and endocrine
systems (Breed, Flanagan, & Watson, 2004). Collectively, some of these complaints are part of what has been called “post-concussion syndrome”, however the direct link between TBI and post-concussion syndrome has been disputed (Smith-Seemiller, Fow, Kant, & Franzen, 2003; Wäljas et al., 2015).

1.3.2 Societal impact

Prognosis is often poor for patients who have sustained a moderate-severe TBI, and ongoing, long-term care and support is required (Gagnon, Lin, & Stergiou-Kita, 2016). Predictably, this results in an additional burden to family members of patients affected by a TBI, which is considerably greater in lower-income households (Gagnon et al., 2016; Nabors, Seacat, & Rosenthal, 2002). For example, even at six months post-injury, caregivers can experience increased distress in their lives resulting from less time to themselves, changes in sleep pattern, financial and relationship difficulties, increased anxiety, and less privacy (Marsh, Kersel, Havill, & Sleigh, 2002). Similar concerns were also echoed by caregivers of TBI-affected military service members (Carlozzi et al., 2016). Additionally, the age and educational level of the caregiver also determines whether needs of the TBI patient would be adequately met (Nabors et al., 2002).

The perceived personality and behavioural changes in TBI patients hinders their ability to re-integrate into society (May et al., 2017) and return to work (Cuthbert et al., 2015; Gabbe et al., 2016). The associated feelings of hopelessness, depression, and anxiety (Cuthbert et al., 2015), plus negative attitudes and a lack of wider understanding from the community (Fortune & Wen, 1999) result in diminished quality of life for the patient. Moreover, TBI patients of minority populations tend to have poorer recovery and community integration, even when controlled for age, education, income, and injury.
severity (Gary, Arango-Lasprilla, & Stevens, 2009; Sander et al., 2009). Patient outcome is also negatively affected if the individual is from a lower-income country, with greater instances of death and disability (De Silva et al., 2008).

Paediatric patients also experience difficulties integrating into society (Ganesalingam et al., 2011; Meadows et al., 2017; Yeates et al., 2013), but this may be improved if patient recovery occurs in a positive family environment (Yeates, Taylor, Walz, Stancin, & Wade, 2010). However, recovery in social functioning is often not substantial, potentially worsening over longer periods of time (Yeates et al., 2004). There are also findings that support a causal link between childhood TBI and criminal behaviour later on in life (Timonen et al., 2002; Williams, Cordan, Mewse, Tonks, & Burgess, 2010). The prevalence of TBI is remarkably high among persons with substance abuse issues (Corrigan, Bogner, & Holloman, 2012), and within the prison population of Australia (Schofield et al., 2006) and other developed countries (Hughes et al., 2015; Turkstra, Jones, & Toler, 2003). Although most studies focus on the male population, there are suggestions that proclivity for criminal behaviour may also exist in female TBI patients (O’Sullivan, Glorney, Sterr, Oddy, & da Silva Ramos, 2015).

1.3.3 Economic impact

The costs for TBI vary depending on the population studied, injury severity, and economic climate. Although the economic impact of TBI is considerably greater in low-middle income countries, the burden is still significant in developed countries (Dismuke, Walker, & Egede, 2015; Hyder, Wunderlich, Puvanachandra, Gururaj, & Kobusingye, 2007). In Australia, the total annual cost of TBI was estimated to be $8.6 billion which appears to be substantially more than workplace injuries with a similar causal mechanism,
and other neurological conditions such as dementia, bipolar disorder, multiple sclerosis, muscular dystrophy, and cerebral palsy (Deloitte Access Economics, 2009). The greatest portion of this cost is borne by the individual, which becomes a greater burden for those at a socioeconomic disadvantage due to poorer functional and return-to-work outcomes (Gabbe et al., 2016). Even in milder cases of TBI such as concussion, this consequently results in productivity loss (Silverberg, Panenka, & Iverson, 2018) that is detrimental to the economy. Additionally, the resulting disabilities and care needs of TBI patients places extensive dependence on the healthcare system. In particular, patients with combat-related TBI tend have concomitant diagnoses of psychiatric illness and/or pain. These co-morbidities greatly increase the median annual cost per patient (Taylor et al., 2012). For geriatric TBI patients, re-hospitalisation is a major driving factor in costs as a result of increased risk of complications and prolonged recovery (Thompson et al., 2012).

### 1.4 TBI diagnosis

The complex nature and severity of TBI means that symptoms vary considerably between individuals. In Australia, injury severity is assessed by the length of coma and post-traumatic amnesia (PTA) using the Glasgow Coma Scale (GCS) (Teasdale & Jennett, 1974) and Westmead PTA scale (Ponsford et al., 2004), respectively. The GCS score indicates the depth and duration of a coma or impaired consciousness as assessed by three aspects: eye opening, verbal performance, and motor responsiveness (Teasdale & Jennett, 1974). In conjunction with the Westmead PTA scale, the severity of a suspected head injury in an individual patient may be classified as shown in Table 1.1. Addition of the Westmead PTA scale to assessing injury severity better discriminates between patients with milder TBI. This avoids discharge of those with an apparently normal level of
consciousness as determined by the GCS (Meares et al., 2015), since not all patients with GCS 15 return to normal cognitive function.

Table 1.1 Classification of TBI severity using the Glasgow coma scale (GCS) and Westmead post-traumatic amnesia scale (PTA). Adapted from Jagnoor & Cameron, 2014.

<table>
<thead>
<tr>
<th>Severity</th>
<th>Initial GCS</th>
<th>PTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>13 – 15</td>
<td>&lt; 1 hour</td>
</tr>
<tr>
<td>Moderate</td>
<td>9 – 12</td>
<td>1 – 24 hours</td>
</tr>
<tr>
<td>Severe</td>
<td>3 – 8</td>
<td>1 – 7 days</td>
</tr>
<tr>
<td>Very severe</td>
<td>–</td>
<td>1 – 4 weeks</td>
</tr>
<tr>
<td>Extremely severe</td>
<td>–</td>
<td>&gt; 4 weeks</td>
</tr>
</tbody>
</table>

Clinical observations may also be supplemented with neuroimaging techniques such as computed tomography (CT) and magnetic resonance imaging (MRI) to detect structural changes in the brain (Bruce et al., 2015; Håberg et al., 2014). Currently, CT is generally accepted as the standard method for initial triaging of patients presenting with suspected TBI since it is adequately sensitive, and does not require screening patients for ferromagnetic material (Bruce et al., 2015; Wintermark et al., 2015). However, if a patient’s CT image does not show changes, it is recommended that an MRI be performed since it is more sensitive in detecting all stages of epidural and subdural haematomas (Wintermark et al., 2015). Although each imaging technique has its strengths, no one imaging modality is suitable for all patients due to the heterogeneity of TBI pathology (Amyot et al., 2015).
1.5 **TBI biomechanics and pathophysiology**

TBI is initiated by an external mechanical force. The manner in which the brain reacts to the force is determined by the mechanical nature of the force and structural characteristics of the brain and surrounding tissue. The events following TBI are an amalgamation of highly complex processes and is usually described in terms of primary and secondary phases of injury, although there is much overlap. Generally, primary injury encompasses damage to the brain parenchyma from the initial mechanical force at the time of trauma, as a result of shearing, tearing, or stretching forces (Loane & Faden, 2011). This leads to secondary injury, which includes the biochemical, metabolic, and cellular changes that occur in response to the primary event (Loane & Faden, 2011; Werner & Engelhard, 2007). Together, the extent of these injuries is collectively responsible for the ultimate severity of cognitive, motor, and other neurological sequelae experienced by TBI survivors (Walker & Tesco, 2013).

1.5.1 **Mechanics of TBI**

The conditions in which a TBI occurs is unique for every scenario, and understanding the link between the physical input and mechanical response is critical for developing clinically relevant models for study, and identifying key therapeutic targets in the pathology. Mechanical loading in TBI can be categorised in myriad ways, but broadly described as static or dynamic. *Static loading* occurs as a result of slowly applied direct loads where there is physical contact between the head and another object. Crush injuries to the head, although relatively uncommon (LaPlaca, Simon, Prado, & Cullen, 2007; Ommaya & Gennarelli, 1974), is one such example. *Dynamic loading* is far more common and occurs when the head moves independently in response to the impacting
force. This type of loading is more complex, and can thus be further described as either impact or impulsive loading. Dynamic impact loading is a direct force where there is very short contact between the head and another object, such as a blunt force strike to the head. Dynamic impulsive loading is indirect i.e. there is no contact, and the head moves in response to pressure waves, such as those constituting blast injuries. These types of forces that act on the brain may cause linear, rotational, and angular movements of acceleration-deceleration which are responsible for the primary injury (LaPlaca et al., 2007; Meaney, Morrison, & Dale Bass, 2014).

1.5.2 Primary injury

Primary injury largely encompasses the mechanical response to injury, and is usually distinguished from the biochemical response which comprises the major component of secondary injury. Contusions, blood-brain barrier (BBB) damage, and diffuse axonal injury (DAI) are some of the major events of the primary injury phase. The point at which structural and physiological failure occurs as a result of the mechanical insult is known as tissue tolerance (LaPlaca et al., 2007). Focal cerebral contusions are lesions commonly found after TBI and includes damage to both blood vessels and the surrounding parenchyma. These lesions can form in response to the coup-contrecoup movements of the brain against the skull during acceleration-deceleration, or be indicative of the impact location (Monson, Converse, & Manley, 2018).

The mechanical forces that impact the brain may also damage the cytoskeletal integrity of cells comprising the BBB and can persist for years after TBI (Hay, Johnson, Young, Smith, & Stewart, 2015). Tearing of blood vessels and endothelial membrane disruption are acute consequences of the mechanical insult (Başkaya, Muralikrishna Rao, Doğan,
Donaldson, & Dempsey, 1997), and contribute to the loss of vascular endothelial cell tight-junction proteins responsible for maintaining BBB integrity (Andrews, Lutton, Merkel, Razmpour, & Ramirez, 2016). Even in milder forms of experimental TBI, there is evidence for BBB compromise (Johnson et al., 2018).

DAI is another common and important feature of all TBI severities that forms in response to a mechanical insult. It can acutely manifest as a loss of consciousness, and may persist as coma and/or cognitive dysfunction. The symptoms associated with DAI are reliant upon the severity and extent of the injury, especially in the brainstem (Davceva, Basheska, & Balazic, 2015; Hume Adams et al., 1989; Johnson, Stewart, & Smith, 2013). DAI encompasses a range of effects, from primary mechanical shearing of axons, to secondary axonal degeneration involving axonal transport interruption, swelling, and proteolysis. The major mechanism by which DAI arises, is rotational acceleration-deceleration of the unrestrained head (Davceva et al., 2015; Gennarelli et al., 1982; Johnson et al., 2013), which damages axons within white matter regions of the brain. The complete severance of axons is followed by its retraction into a bulb, a process known as primary axotomy. Secondary axotomy is the impairment of axoplasmic transport usually associated with secondary injury, induced by cytoskeletal disruption and activation of proteolytic enzymes (Davceva et al., 2015; Johnson et al., 2013). The release of glutamate from damaged neurons into the extracellular space further exacerbates injury to axons and surrounding tissue by altering calcium homeostasis and creating oxidative stress (Frati et al., 2017; Johnson et al., 2013).
1.5.3 Secondary injury

There is chronological overlap between primary and secondary injury phases. However, secondary injury generally refers to the chemical responses to mechanical impact, and a more detailed discussion of secondary injury can be found in Chapter 3. Pathological processes such as excitotoxicity, mitochondrial disturbances, altered cell-signalling, proteolytic enzyme activation, and oxidative stress lead to other downstream cascades associated with cerebral oedema, inflammation, and cell death (Figure 1.1).

**Figure 1.1** The major secondary pathophysiological events in TBI. MMPs: matrix metalloproteinases, MPTPs: mitochondrial permeability transition pores, ROS: reactive oxygen species. Adapted from Chiu, Anderton, Knuckey, & Meloni, 2017.

Excitotoxicity is a prominent neurodamaging event initiated in the acute stages after TBI. The sudden depolarisation of nerve cells leads to an excessive release of excitatory amino acids, particularly glutamate, accumulating in the extracellular space (Faden, Demediuk, Panter, & Vink, 1989) and can persist for several days after TBI (Baker, Moulton,
MacMillan, & Shedden, 1993; Bullock et al., 2006; Chamoun, Suki, Gopinath, Goodman, & Robertson, 2010). Increased extracellular concentrations of glutamate results in the toxic influx of ions such as sodium and calcium into neurons and other cells affected by excitotoxicity, which has detrimental downstream effects on cellular function.

High intracellular calcium is detrimental to neuronal bioenergetics due to its effects on mitochondria. Mitochondrial uptake of calcium initially acts to buffer intracellular calcium. However, when excessive uptake occurs as a result of excitotoxicity, mitochondria generate excessive reactive oxygen species (ROS), and ATP production via the electron transport chain is reduced. This can result in loss of transmembrane potential and induce mitochondrial membrane permeability (Brustovetsky et al., 2003; Dubinsky & Levi, 1998) eventually leading to neuronal cell death. Clinically, ATP demand is critically high in injured patients, thus depleted levels in the body can be detrimental to patient outcomes (Andriessen, Jacobs, & Vos, 2010; Mazzeo, Beat, Singh, & Bullock, 2009).

Moreover, the resultant oxidative stress from dysfunctional mitochondria has been argued to negatively influence the inflammatory cascade (Stoiber, Obermayer, Steinbacher, & Krautgartner, 2015). ROS production from mitochondria can enhance neutrophil infiltration by activating the superoxide generating enzyme NADPH oxidase 2 (NOX2) (Vorobjeva et al., 2017), eliciting a response which positively feeds back to ROS levels (Lozano et al., 2015). Interestingly, markers indicative of oxidative stress also suggest that this phenomenon is more prominent in males than females (Bayir et al., 2004). Ultimately, excitotoxicity, mitochondrial disruption, and dysregulation of the inflammatory response contribute to neuronal and glial cell death and manifests as poorer cognitive outcomes in patients (Walker & Tesco, 2013).
The pathological processes of secondary injury are often targeted when developing neuroprotective therapeutics, since primary injury is only amenable to preventative measures. However, the complex nature of TBI pathophysiology has hindered such progress. Proposed neuroprotective agents are often designed to target single aspects of the injury cascade, and many have failed in phase II/III clinical trials (Tolias & Bullock, 2004). Recently, there has been increased recognition that combination therapies or multi-targeted drugs may have a greater likelihood of achieving neuroprotective efficacy at the clinical level (Anighoro, Bajorath, & Rastelli, 2014; Kline, Leary, Radabaugh, Cheng, & Bondi, 2016; Margulies et al., 2016; Van der Schyf & Youdim, 2009).

### 1.6 Current interventions

Despite major research efforts over decades, there are no clinically effective neuroprotective treatments to minimise brain injury following a TBI. Given the devastating consequences, the development of an effective neuroprotective agent is an urgent priority. In the meantime, various preventative, surgical, and rehabilitative interventions have been developed to minimise the overall occurrence of TBI, its impact on patients, and improve the trajectory of patient recovery.

#### 1.6.1 Preventative measures

Motor vehicle accidents were previously the leading cause of TBI, and its dramatic decrease over time has been attributed to road-safety awareness programs and initiatives. Unfortunately, this success is not universal, as low-middle income countries have seen increased use of motor vehicles, while infrastructure and road safety measures remain
inadequate (Maas et al., 2017). Driver education and safety programs in Australia vary between states and territories, but are overall quite intensive. Whether these have any long-term impact in reducing road fatalities across the nation is yet to be comprehensively evaluated (Senserrick, 2007). Although, the Prevent Alcohol and Risk-Related Trauma in Youth (P.A.R.T.Y) program found attrition over time from the initial success in improving the safety perception of driving in youths (Gunn et al., 2018). Closer to home, Western Australia has implemented an ambitious road safety strategy (Towards Zero 2008 – 2010) in response to worsening road death casualties (Corben, Logan, Fanciulli, Farley, & Cameron, 2010) so that policy recommendations can be made to improve road safety.

The implementation of protective headwear in sports has long been a topic of controversy. Severe head injuries are common in equestrian (Bixby-Hammett, 1983) and winter sports (Yamazaki et al., 2015) due to the propensity for collisions with the surrounding environment. The introduction of protective headwear is now standard in these sports due to a reduction in the number of severe TBIs (McIntosh et al., 2011). However, the efficacy of headwear in the many codes of football has been inconclusive (Abernethy & Bleakley, 2007; Benson, Hamilton, Meeuwisse, McCrory, & Dvorak, 2009; McIntosh et al., 2011). This is further complicated by findings that athletes wearing protective headwear are more likely to risk aggressive play due to a false sense of safety (Barnes, Rumbold, & Olusoga, 2017; Finch, McIntosh, & McCrory, 2001; Menger, Menger, & Nanda, 2016). In the Australian Football League and codes of rugby, protective headwear is not mandatory due to poor attitudes towards its use and inconclusive evidence for efficacy in reducing the consequences of a TBI (Finch et al., 2001; Finch, McIntosh, McCrory, & Zazryn, 2003; McIntosh et al., 2009).
Although TBI can affect any individual at any age, children, adolescents, and the elderly are particularly vulnerable (Maas et al., 2017). Non-accidental trauma is increasingly becoming recognised as a significant cause of TBI in young children, especially infants (Australian Institute of Health and Welfare, 2018; Davies, Coats, Fisher, Lawrence, & Lecky, 2015). Since awareness has increased and family risk factors have been identified, local programmes have been implemented to either remove children from abusive households or educate parents on how to reduce child maltreatment (Australian Institute of Health and Welfare, 2018; M. Chen & Chan, 2016). Prevention strategies are also needed for the elderly who are prone to falls. Physical exercise that aims to strengthen and condition lower-limb muscles have been shown to reduce the risk of falls, and thus TBI in the elderly (Burton et al., 2015; Ishigaki, Ramos, Carvalho, & Lunardi, 2014; Sherrington et al., 2017). Moreover, a multifaceted approach that includes a review of their medical history, medication, and nutrition, in addition to home hazard assessment and modification could further reduce the rate of falls (Goodwin et al., 2014; Palvanen et al., 2014).

1.6.2 Neurosurgical measures

It is believed that since the 1800s, TBI mortality has decreased by almost 50% to a rate of about 35% in 1990 (S. C. Stein, Georgoff, Meghan, Mizra, & Sonnad, 2010) and remaining steady since (Morrison et al., 2016; S. C. Stein et al., 2010). These improvements are largely due to regular updates by The Brain Trauma Foundation on recommendations and guidelines for the neurosurgical management of severe TBI (Carney et al., 2017). Decompressive craniectomy and cerebrospinal fluid drainage have both been introduced into the guidelines to relieve intracranial pressure, whereas recommendations on hypothermia, nutrition, and infection prophylaxis were revised due
to conflicting evidence of their benefits (Carney et al., 2017). Thresholds for intracranial pressure, cerebral perfusion pressure, and advanced cerebral monitoring regimens have also been revised so that it best directs treatment for the patient, thus maximising beneficial outcomes (Carney et al., 2017).

In low-middle income countries and rural areas, treatment is usually directed by clinical assessment alone since more recent technology is often unavailable and costly to patients (Kuo et al., 2017). This also means that patients presenting to an emergency department in such a setting are disadvantaged due to longer wait times for surgical intervention, or not receiving adequate medical attention in an appropriate timeframe (Kuo et al., 2017; Upadhyayula, Yue, Yang, Birk, & Ciacci, 2018). Mortality is often consequently poor, with one recent study in Sri Lanka reporting survival in only 46% of patients at six months after sustaining a moderate-severe TBI (Samanamalee et al., 2018).

### 1.6.3 Rehabilitation

The goal of rehabilitation is to help patients regain as much of their physical and cognitive abilities prior to the injury. Because most survivors of TBI are currently left with life-long health issues, it is arguably more appropriate to manage it as a chronic disease rather than a curable injury (Masel & DeWitt, 2010). Since TBI sequelae spans more than just neurological complaints (Murphy & Carmine, 2012), an interdisciplinary approach to rehabilitation is more effective (Khan, Baguley, & Cameron, 2003; Martino et al., 2015). Although deficiencies in motor and self-care skills seem to be the most amenable to inpatient rehabilitation (McLafferty et al., 2016), cognitive rehabilitation tends to dominate recent TBI rehabilitation research as an added benefit to physiotherapy. The use of non-invasive transcranial brain stimulation has demonstrated improved recovery in
motor and balance dysfunction (Martino et al., 2015), and cognition (Neville et al., 2015). The use of virtual-reality has also shown some efficacy in improving recovery for both adult (Cuthbert et al., 2014; Robitaille et al., 2017) and paediatric patients (Shen, Johnson, Chen, & Xiang, 2018).

Following acute rehabilitation, community re-integration is also crucial to maximising patient recovery. This requires adequate long-term support from family, education, and counselling (Khan et al., 2003), in addition to quality, accessible community support services. Multidisciplinary rehabilitation services that aid community re-integration include occupational therapy, speech and language pathology, physiotherapy, social work, and neuropsychology (Fleming, Tooth, Hassell, & Chan, 1999). Although this approach to rehabilitation generally achieves positive outcomes for patients (Powell, Heslin, & Greenwood, 2002), other factors such as age, disability level, and cognition post-injury influence the extent of community re-integration (Fleming et al., 1999).

1.7 Pharmacological therapies for TBI

1.7.1 Treating the neurological sequelae of TBI

Although there are currently no proven neuroprotective pharmacological agents, treating the neurological sequelae associated with TBI has demonstrated some clinical benefits in improving aspects of patient quality of life. However, there lacks a consensus on evidence-based guidelines for these treatments (Warden et al., 2006). Methylphenidate has long been used to improve deficits in attention and information processing in paediatric (Hornyak, Nelson, & Hurvitz, 1997) and adult (Johansson, Wentzel, Andréll, Mannheimer, & Rönnbäck, 2015; Johansson, Wentzel, Andréll, Rönnbäck, &
Mannheimer, 2018) cases of TBI. Anti-epileptic medications such as Levetiracetam have demonstrated efficacy in experimental (Browning et al., 2016; Caudle, Lu, Mountney, Shear, & Tortella, 2016; Kochanek et al., 2018) and clinical (Nita & Hahn, 2016; Patanwala, Kurita, & Truong, 2016) TBI, the latter particularly as a prophylaxis for seizures which are commonly reported post-injury.

For neuropsychiatric disorders such as depression and anxiety that frequently manifest after TBI, medications such as selective serotonin re-uptake inhibitors have been recommended despite some trials finding these agents to be no more effective than placebo (Jorge, Acion, Burin, & Robinson, 2016; Rapoport et al., 2010). However, serotonin re-uptake inhibitors have had some success when prescribed for post-traumatic stress disorder (Vasterling, Jacob, & Rasmusson, 2017; Yue et al., 2017). Other medications such as melatonin (Grima et al., 2018), amphetamines (Hornstein, Lennihan, Seliger, Lichtman, & Schroeder, 1996), and cannabinoids (Mechoulam, Panikashvili, & Shohami, 2002) have also garnered (albeit controversial) interest as treatments for the neurological sequelae associated with TBI.

1.7.2 Neuroprotective approaches to treating TBI

Although secondary injury is most amenable to neuroprotective strategies, its multifaceted nature has made it a challenging barrier to overcome in terms of developing an effective pharmacological therapeutic. A multitude of experimental pharmacological agents have demonstrated pre-clinical promise, though isolated targeting of the complex injury cascade may be a reason why none have successfully translated to human TBI (McConeghy, Hatton, Hughes, & Cook, 2012; Narayan et al., 2002). Early pharmacological strategies aimed to reduce the effects of excitotoxicity with the use of
glutamate, calcium channel, and NMDA antagonists; however undesirable off-target effects have limited their potential (Ikonomidou & Turski, 2002; Muir, 2006). Cyclosporin A and its associated analogues were extensively studied for its ability to alleviate the effects of mitochondrial dysfunction (Crompton, 1999; Mbye, Singh, Carrico, Saatman, & Hall, 2009; Sullivan, Thompson, & Scheff, 1999). Despite reaching clinical trials that demonstrated its safety and lack of toxicity (Mazzeo, Brophy, et al., 2009), further rigorous pre-clinical studies concluded a lack of beneficial effects (Dixon et al., 2016). The use of other molecules such as steroids and hormones as multi-targeted pharmacotherapies also elicited detrimental side-effects or had no benefit (McConeghy et al., 2012; Nichol et al., 2015; D. G. Stein, 2015) despite demonstrating reductions in oedema, and neurotrophic, anti-apoptotic, and anti-inflammatory effects.

Although there is the option for combined therapies, an extraordinary amount of work is required for rigorous evaluation of their efficacy and safety (Margulies et al., 2016). Thus, an alternative therapeutic approach is required. Peptides are naturally occurring compounds with low immunogenicity and toxicity, and high specificity and affinity for target molecules (Craik, Fairlie, Liras, & Price, 2013; Otvos & Wade, 2014). These characteristics make them a valuable agent for therapeutic discovery, with many having been assessed for their neuroprotective efficacy in TBI experimental models (Chiu et al., 2017). Those that have progressed to Phase I clinical trials, such as PACAP38 (Doberer et al., 2007) and CN-105 (Guptill et al., 2016), have demonstrated safety and tolerability, and are interestingly both cationic and arginine-rich.
1.7.3 *Cationic arginine-rich peptides (CARPs)*

Cationic arginine-rich peptides (CARPs) appear to have diverse neuroprotective properties, and have thus been assessed for their ability to mitigate ROS production (Dai et al., 2011; Szeto, 2006; Szeto et al., 2011), reduce inflammation (Laskowitz et al., 2017; Laskowitz, Fillit, Yeung, Toku, & Vitek, 2006), and protect cells from excitotoxic damage (Y. Chen, Brennan-Minnella, Sheth, El-Benna, & Swanson, 2015). The Meloni laboratory has discovered that CARP neuroprotective efficacy increases with increasing arginine content a positive charge and that peptides comprised solely of arginine residues are highly neuroprotective, with peak efficacy plateauing with poly-arginine-15 to -18 (Meloni et al., 2015). The lead CARP in the Meloni laboratory, poly-arginine-18 (R18), has demonstrated efficacy in various animal models of stroke (Meloni et al., 2015, 2017, Milani et al., 2017a, 2017b, 2018; Milani, Clark, et al., 2016; Milani, Knuckey, Anderton, Cross, & Meloni, 2016) and hypoxic ischaemic encephalopathy (Edwards, Cross, Anderton, Knuckey, & Meloni, 2018), which share pathophysiological similarities with TBI; hence R18 also represents a potential neuroprotective therapeutic for TBI.
1.8 Statement of aims

Due to the lack of an effective treatment for TBI, this thesis sought to examine the neuroprotective efficacy of several CARPs in a TBI model. In particular, the lead CARP in the Meloni laboratory, poly-arginine-18 (R18), was assessed alongside its D-enantiomer peptide (R18D). Additionally, studies examining R18D serum pharmacokinetics and tissue distribution in healthy rats were also conducted. The findings from this thesis will pave the way for further exploration into the neuroprotective potential of cationic arginine-rich peptides in TBI.

Specifically, the project for this thesis will aim to:

**Aim 1.** Establish a rat model of impact-acceleration TBI.

**Aim 2.** Compare the *in vitro* and *in vivo* neuroprotective efficacy of poly-arginine peptide R18 with other experimental neuroprotective peptides for TBI.

**Aim 3.** Compare the efficacy of L- and D-enantiomers of the poly-arginine peptide R18 in the established rat model of TBI.

**Aim 4.** Determine potential dose-effects of the poly-arginine peptide R18 in the established rat model of TBI.

**Aim 5.** Investigate R18D serum pharmacokinetics and tissue distribution in healthy, uninjured rats.
1.9 References


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Chapter 2

The neuroprotective potential of arginine-rich peptides for the acute treatment of traumatic brain injury

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The neuroprotective potential of arginine-rich peptides for the acute treatment of traumatic brain injury

Traumatic brain injury (TBI) encompasses any insult to the brain resulting from external mechanical forces. The subsequent brain damage is complex, spanning a spectrum of symptoms and disabilities. TBI is a significant, but under-appreciated public health and economic concern burdening developed countries. With only preventative and rehabilitative measures currently in place, there is a dire need to develop a therapeutic that preserves brain tissue in the acute stages following TBI. Recent research has highlighted the neuroprotective properties of arginine-rich peptides in vitro following neuronal excitotoxicity and in vivo following stroke. Therefore, based on the in vitro and in vivo neuroprotective actions of arginine-rich peptides these molecules are promising therapeutics to reduce acute TBI.

Brain trauma is a major cause of morbidity and mortality in populations worldwide, usually as a consequence of road traffic accidents, falls, street violence and contact sports. Although this affliction perpetuates developed society, the occurrence of TBI-related hospitalizations is greatest in young adult males [1]. Children, adolescents and adults aged 75 or over are also more susceptible to head trauma because of physical activity, and a risk of falls [1]. More recently, TBI has become a concern for active military personnel, who may be exposed to blast waves and other combat-related traumatic events [2].

Survivors of TBI often suffer from lifelong cognitive, physical, behavioral and communicative deficits that negatively affect families, communities and the economy. Furthermore, TBI sufferers are at a higher risk of developing anxiety and depressive disorders [3], and neurodegenerative diseases such as chronic traumatic encephalopathy, Alzheimer’s disease, Parkinson’s disease and amyotrophic lateral sclerosis [4].

Current strategies to minimize the impact of TBI focus on preventative measures, acute neuro-critical care and neuro-restorative practices. However, targeting the secondary neuro-damaging processes that follow the primary insult provides an additional and potentially more effective, treatment to complement existing interventions. Therefore, any acute neuroprotective treatment strategy that maximizes preservation of brain tissue provides the best opportunity to improve outcomes following TBI.

Of the multitude of experimental drugs studied for treating TBI, a number have shown preclinical neuroprotective efficacy and advanced to clinical trials. From there, the drug trials have returned neutral, negative or contradictory outcomes. While there may be several reasons why a pharmacological neuroprotective agent has failed clinically, one possibility is that the therapy specifically targets one of the many pathophysiology events activated in the brain following TBI.

Many approaches aimed at mitigating secondary injury after TBI have been directed at attenuating excitotoxicity, a neuro-damaging process caused by the uncontrolled release of the neurotransmitter glutamate into the extracellular space. Clinical studies of glutamate receptor antagonists that target excitotoxicity such as dexamabolin, selfotel and magnesium, reported no statistically significant effects on Glasgow Outcome Score or mortality [5]. Another neuroprotective strategy is to block ion channels, thereby reducing the toxic intracellular influx of calcium and other ions into neurons, and the subsequent production of reactive oxygen species, and activation of calpains and endonucleases [6]. Unfortunately, clinical trials of calcium-channel blockers such as nimodipine and nicardipine have only shown an ability to decrease mortality or severe disability by reducing the onset of vasospasm in a small subset of TBI patients suffering a subarachnoid hemorrhage [5], making this type of medication unsuitable for the general TBI populace. The hormones erythropoietin (Clinicaltrials.gov Identifier: NCT00987454) and progesterone (Clinicaltrials.gov Identifier: NCT00822900), and the immunosuppressive peptide cyclosporine A (CsA) (Clinicaltrials.gov Identifier: NCT02496975) are other therapeutics that have undergone recent clinical trials after demonstrating preclinical efficacy. However, it appears that these agents may be of limited benefit following TBI [5,7].

The jaded nuance felt from the failure of the large number of TBI neuroprotection clinical trials beckons a shift in perspective. Although there have been recent calls for combined therapies of existing drugs, this direction may be ill-advised, as the additive effects could complicate pharmacokinetic interactions to the detriment of the patient [8]. Given the lack of success with previous neuroprotective agents for TBI, and the challenges combined therapy presents, an alternative therapeutic approach involves the use of cationic arginine-rich cell-penetrating peptides (CPP). Cationic CPPs fused to different neuroprotective peptides have demonstrated efficacy in numerous acute brain injury models including stroke, perinatal hypoxia-ischemia, epilepsy and TBI. The most commonly used cationic CPP is the HIV-derived TAT peptide (GRKKRRQRRR), which allows delivery of fused cargos (e.g. peptides, proteins, drugs) into the brain and neurons without any apparent toxicity in preclinical studies. TAT-mediated uptake into the brain is attributable to its composition of the basic amino acids lysine (K) and especially arginine (R), which confer a positive charge to the peptide [9]. Interactions between the cationic TAT peptide and anionic cell surface structures induces endocytosis and/or membrane transduction resulting in the transport of TAT and its cargo across the blood–brain barrier and cell membranes.
However, of even greater potential significance was the demonstration that the TAT peptide itself possessed neuroprotective properties and that other cationic arginine-rich CPPs displayed considerably greater neuroprotective efficacy than the TAT peptide, following in vitro excitotoxicity [10]. Furthermore, studies revealed that arginine residues were the critical elements for neuroprotection, with peptide efficacy increasing with arginine content [11]. In addition, arginine-rich peptides were shown to be neuroprotective in a rat stroke model and also capable of reducing neuronal calcium influx following glutamate excitotoxicity [11]. Finally, mounting evidence suggests that the neuroprotective actions of TAT-fused neuroprotective peptides is largely mediated by the arginine residues and to a lesser extent lysine and tryptophan residues within the TAT and/or cargo peptide [12].

Unlike previously explored therapeutics, arginine-rich peptides are not believed to act as direct glutamate receptor and/or calcium channel antagonists. Rather, it has been proposed that at least one mechanism by which these arginine-rich peptides exert their neuroprotective effects is by inducing the internalization of neuronal cell surface structures, as a result of endocytosis [10,12,13], thereby reducing the effects of excitotoxicity and its down-stream neuro-damaging signaling pathways. Additionally, poly-arginine and other cationic arginine-rich peptides are potent inhibitors of proprotein convertase enzymes, including furin [14], a ubiquitously expressed calcium-dependent convertase involved in both physiological and pathological processes such as matrix metalloproteinase activation.

Moreover, once internalized, arginine-rich peptides may target mitochondria [15] and exert beneficial actions on mitochondrial membranes, thus behaving as a multipotent therapeutic with both extracellular and intracellular neuroprotective mechanisms. In targeting the mitochondria, cationic arginine-containing peptides have been demonstrated to reduce reactive oxygen species production, preserve ATP synthesis, inhibit membrane permeability transition, limit calcium influx and prevent cytochrome c release [16,17].

Interestingly, the neuroprotective efficacy of peptides containing arginine and lysine residues such as those derived from apolipoprotein E (APOE) and the amyloid precursor protein (APP) has been investigated in TBI and other brain injury models. These peptides have demonstrated reduced axonal, ischemic and hypoxic injury, in addition to anti-inflammatory effects and functional improvements in animal models of TBI [18–22]. Although these studies hypothesize that these peptides are neuroprotective through other mechanisms, their arginine content and cationic charge makes a compelling argument for a neuroprotective effect similar to that of arginine-rich peptides. Furthermore, it should be noted that in vitro comparisons between these and other peptides containing more arginine residues, found that those derived from APOE and APP were considerably less neuroprotective (unpublished observation).

A TAT-fused peptide, known as TAT-NR2B9c (also known as NA-1) has recently demonstrated preclinical efficacy in rodent and non-human primate stroke models, a disease which shares many pathophysiological similarities with TBI [23]. Although TBI is more complex and heterogeneous than stroke, many neuroprotective therapeutics trialed for TBI were initially investigating for stroke, so it stands to reason that the neuroprotective potential of arginine-rich peptides may also extend to TBI. Moreover, substantial safety and efficacy in the administration of TAT-NR2B9c in humans and non-human primates has been established [24]. To this end, studies in our laboratory have shown that a poly-arginine-18 peptide is more neuroprotective than TAT-NR2B9c in both in vitro [11] and in vivo (unpublished observation) stroke models. Therefore, based on the TAT-NR2B9c, and APOE- and APP-derived peptide studies, plus the superior efficacy of poly-arginine peptides compared with the three aforementioned peptides, it is possible that poly-arginine, or other arginine-rich peptides, may be effective agents following TBI. However, the extent to which arginine-rich peptides have the capacity to influence pathological processes known to exacerbate the negative outcomes of TBI, such as intracranial hypertension, cerebrovascular dysautoregulation, and neuro-biochemical modifications, is yet to be determined.

Conclusions

A collective assessment of previous neuroprotection studies has emphasized the need to reconsider our perspective when developing a therapeutic for TBI. From a pharmacotherapy viewpoint, arginine-rich peptides represent a new and exciting class of neuroprotective agents, which have the capacity to target the secondary damaging events of traumatic injury within the central nervous system. Not only do arginine-rich peptides appear to reduce excitotoxicity, they also have the ability to attenuate mitochondrial dysfunction and inhibit extracellular matrix metalloproteinase activation, thereby assisting the viability of the neurovascular unit within the TBI affected brain. Furthermore, the recent preclinical arginine-rich peptide studies in stroke, a condition that shares many pathophysiological similarities to TBI, provides promise that this class of peptide will be effective following trauma to the brain.

Financial and competing interests disclosure

BP Meloni is a named inventor of several patent applications regarding the use of arginine-rich peptides as neuroprotective agents. NW Knuckey is a named inventor of several patent applications regarding the use of arginine-rich peptides as neuroprotective agents. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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Li Shan Chiu
Western Australian Neuroscience Research Institute, Nedlands, Australia
Centre for Neur muscular and Neurological Disorders, The University of Western Australia, Nedlands, Australia
http://orcid.org/0000-0002-5601-7739

Ryan S. Anderton
Western Australian Neuroscience Research Institute, Nedlands, Australia
Centre for Neuromuscular and Neurological Disorders, The University of Western Australia, Nedlands, Australia
School of Heath Sciences, The University Notre Dame Australia, Fremantle, Western Australia, Australia
http://orcid.org/0000-0003-0568-9794

Neville W. Knuckey
Western Australian Neuroscience Research Institute, Nedlands, Australia
Centre for Neuromuscular and Neurological Disorders, The University of Western Australia, Nedlands, Australia
Department of Neurosurgery, Sir Charles Gairdner Hospital, QEII Medical Centre, Nedlands, Western Australia, Australia
bruno.meloni@wanri.uwa.edu.au

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Chapter 3

Peptide pharmacological approaches to treating traumatic brain injury: a case for arginine-rich peptides

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Peptide Pharmacological Approaches to Treating Traumatic Brain Injury: a Case for Arginine-Rich Peptides

Li Shan Chiu 1,2 • Ryan S. Anderton 1,2,4,5 • Neville W. Knuckey 1,3 • Bruno P. Meloni 1,3

Abstract Traumatic brain injury (TBI) has a devastating effect on victims and their families, and has profound negative societal and economic impacts, a situation that is further compounded by the lack of effective treatments to minimise injury after TBI. The current strategy for managing TBI is partly through preventative measures and partly through surgical and rehabilitative interventions. Secondary brain damage remains the principal focus for the development of a neuroprotective therapeutic. However, the complexity of TBI pathophysiology has meant that single-action pharmacological agents have been largely unsuccessful in combatting the associated brain injury cascades, while combination therapies to date have proved equally ineffective. Peptides have recently emerged as promising lead agents for the treatment of TBI, especially those rich in the cationic amino acid, arginine. Having been shown to lessen the impact of ischaemic stroke in animal models, there are reasonable grounds to believe that arginine-rich peptides may have neuroprotective therapeutic potential in TBI. Here, we review a range of peptides previously examined as therapeutic agents for TBI. In particular, we focus on cationic arginine-rich peptides – a new class of agents that growing evidence suggests acts through multiple neuroprotective mechanisms.

Keywords Traumatic brain injury • Neuroprotection • Peptide • Arginine

Introduction

Traumatic brain injury (TBI) remains a major cause of morbidity and mortality worldwide. While TBI can occur at any age, it is especially prevalent in young adults. Motor vehicle accidents, falls, and violence account for the majority of TBIs [1–3]. More recently, TBI has also become an increasing concern for active military personnel who are at risk of damaging blast waves from explosions [4]. For those that do survive the initial injury, physical and cognitive disabilities follow, impairing the individual’s ability to learn, function socially, and return to their previous work [5, 6]. Although some patients show improvements in the short term, these disabilities persist for the vast majority [7], often declining and adversely impacting their quality of life [5–9]. Aside from the well-documented short- and long-term physical and cognitive consequences, TBI can predispose individuals to other neurological conditions such as Alzheimer’s and Parkinson’s diseases, chronic traumatic encephalopathy, and amyotrophic lateral sclerosis [10], as well as anxiety and depressive disorders [11].

Current interventions for TBI mainly consist of preventative measures focusing on reducing the risk of traumatic injury, and neurosurgery and rehabilitation to improve functional recovery [12, 13]. Previous investigations into potential pharmacological strategies for TBI have all failed in the “bench-to-bedside” transition, and the development of effective...
neuroprotective therapies remains paramount, as they have the potential to minimise secondary brain injury and improve patient functional outcomes. Recent evidence from preclinical studies in rats indicates that cationic arginine-rich peptides may have potent neuroprotective properties, suggesting that these peptide-derived therapies may provide an avenue for neuroprotection post-TBI [14]. As we acquire more knowledge about the crucial events in TBI pathophysiology, pharmacological strategies will emerge to better target the critical deleterious changes that occur in the brain following TBI. To this end, it appears that arginine-rich peptides operate through several neuroprotective mechanisms of action, and therefore may be effective in protecting neurons during damaging events, including TBI.

This review focuses on a number of peptides that have demonstrated neuroprotective capabilities (Table 1), and discusses their development from conception through to application in in vitro and/or in vivo neuronal injury models, as well as discussing possible mechanisms of action, and highlighting areas for future research. In particular, we highlight the potential of arginine-rich peptides as a new class of neuroprotective agent in TBI.

TBI Pathophysiology

The pathophysiology of TBI is multifactorial and highly complex and still only partly elucidated. Consequently, this lack of clarity has hindered the development of effective therapies. The pathophysiological progression of TBI consists of two stages, beginning with the primary injury, which is initiated by a mechanical force that damages the brain parenchyma. Secondary injury then ensues, involving a series of damaging events such as excitotoxic cascades, oxidative stress, mitochondrial dysfunction, and inflammatory processes (Fig. 1) that follow in response to the primary insult, and occurs over the course of hours to days following TBI. Since the primary injury is only amenable to preventative measures, the target of therapeutic investigations for TBI exclusively focuses on the processes involved in secondary brain injury. Below is a description of TBI pathophysiology that is not intended as exhaustive, but rather highlights some of the more common targets for pharmacological intervention.

Excitotoxicity

A prominent acute and ongoing neurodamaging event that occurs after TBI is excitotoxicity, where excitatory amino acids (EAA), particularly glutamate, are rapidly released in high concentrations into the extracellular space and cerebrospinal fluid (CSF) (Fig. 1) [42, 43]. Enhanced exocytosis, damage to cerebral structures, and impairment of glutamate transporters lead to post-traumatic surges in glutamate concentrations after TBI [44]. Excessive accumulation of glutamate in the synaptic space triggers over-stimulation of ionotropic and metabotropic glutamate receptors, leading to an excessive influx of intracellular calcium, eventually culminating in excitotoxic injury. Neurons, oligodendrocytes, and astrocytes all express glutamate receptors and are therefore susceptible to excitotoxic injury, with neurons being the most sensitive.

The three main ionotropic glutamate receptor subtypes implicated in excitotoxic cell death are the N-methyl-D-aspartate (NMDA), 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) propionate (AMPA), and kainate receptors. However, it is generally agreed that the NMDA subtype is the most significant, owing to its high calcium permeability [45]. Over-stimulation of these receptors leads to fluxes in calcium, sodium, and potassium, which all play an important pathological role in excitotoxic injury. The stimulation of metabotropic glutamate receptors, which are linked to G-protein activation of phospholipid C, can cause a further increase in intracellular calcium via inositol triphosphate (IP3)-mediated calcium release from the endoplasmic reticulum. In addition, the over-stimulation of glutamate receptors can trigger the secondary activation of voltage-gated ion channels (e.g. Na+, Ca2+, K+), Gq (G-protein subunit that activates phospholipase C), and downstream signalling cascades, and activation of a number of kinases (e.g. mitogen-activated protein kinase) that further exacerbate disturbances in ion cellular homeostasis [46]. While intracellular calcium serves as a second messenger mediating a wide range of cellular responses, it is generally accepted to be the major driving force in the deleterious excitotoxic cascades that occur after trauma to the brain. In the event of injury, this includes activating destructive enzymes such as calpains and phospholipases, and causing metabolic disturbances that lead to the formation of reactive oxygen species (ROS) and oxidative stress, eventuating in cell death via the activation of one or more cell death pathways [47].

Mitochondrial Disturbances

In the absence of injury, mitochondria primarily function to produce ATP through oxidative phosphorylation. Upon injury, the accumulation of intracellular calcium induces mitochondrial permeability transition (MPT) by activating the associated pores (MPTP; mitochondrial permeability transition pore) bridging the inner and outer mitochondrial membranes (Fig. 1), which become increasingly permeable to molecules up to 1.5 kDa. This is followed by a loss in membrane potential, swelling, architectural breakdown, release of pro-apoptotic factors (cytochrome c and apoptosis-inducing factor), inhibition of ATP synthesis, and ROS production, leading to oxidative stress and eventual neuronal cell death [48–50]. The MPTP is a protein complex comprised of three major elements, adenine nucleotide translocase...
<table>
<thead>
<tr>
<th>Peptide information</th>
<th>TBI model/s</th>
<th>Dose (nmol/kg)</th>
<th>Treatment regimen/s</th>
<th>Neuroprotection/other</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CsA</strong></td>
<td>I/A, rat (SD)</td>
<td>8315</td>
<td>Infused into cisterna magna, 30 min post-TBI</td>
<td>Yes, reduced AI</td>
<td>[15]</td>
</tr>
<tr>
<td>ALLVT(Abu)(Ser)LVLA</td>
<td>CCI, rat (SD)</td>
<td>16,630</td>
<td>IP, 15 min post-TBI</td>
<td>Yes, attenuated mitochondrial dysfunction</td>
<td>[16]</td>
</tr>
<tr>
<td>Charge 0</td>
<td>I/A, rat (SD)</td>
<td>8315</td>
<td>Infused into cisterna magna, 30 min pre-TBI</td>
<td>Yes</td>
<td>[17]</td>
</tr>
<tr>
<td>CCI and FPI, rat (SD)</td>
<td>832, 4157, 8315, 16,630, 33,261</td>
<td>IP; 15 min and subsequent 24 h post-TBI; IP; 16,630 nmol/kg at either 15 min, 1, 6, 24 h post-TBI, and subsequent 24 h after first dose</td>
<td>Yes at 15 min, 1, 24 h post-TBI; histological No, at 6 h</td>
<td>[18]</td>
<td></td>
</tr>
<tr>
<td><strong>SNX-111</strong></td>
<td>CCI, rat (SD)</td>
<td>16,630, then 3742 or 8315 nmol/kg/d</td>
<td>Initial IP at 15 min post-TBI, continuous SC infusion for 7 days</td>
<td>Yes with continuous infusion; histological</td>
<td>[19]</td>
</tr>
<tr>
<td>CKGKGAKCSCRILMYDCRSCRGK</td>
<td>FPI, rat (SD)</td>
<td>8315</td>
<td>IP; 15 min post-TBI, then daily for 28 days</td>
<td>Yes, functional</td>
<td>[20]</td>
</tr>
<tr>
<td>Charge +4.6</td>
<td>FPI, CCI, PBBI, rat (SD)</td>
<td>8315, 16,630</td>
<td>IV; 15 min and 24 h post-TBI</td>
<td>No</td>
<td>[21]</td>
</tr>
<tr>
<td><strong>SNX-185</strong></td>
<td>CCI, rat (SD)</td>
<td>1512</td>
<td>IV; 15 min pre-TBI, 15 min, 1, 2, 4, 6, 10 h post-TBI</td>
<td>Yes, functional</td>
<td>[22]</td>
</tr>
<tr>
<td>CLSPGSSCPTSYNCCRSCNPYSRKC</td>
<td>CCI, rat (SD)</td>
<td>189, 378, 756, 1512, 2268</td>
<td>IV; 756 nmol/kg at 3, 5 and 24 h post-TBI</td>
<td>Yes, functional</td>
<td>[23]</td>
</tr>
<tr>
<td>Charge +2.6</td>
<td>I/A, rat (SD)</td>
<td>2268</td>
<td>IV; 0.05, 0.1, 0.2 nmol</td>
<td>Yes, functional</td>
<td>[24]</td>
</tr>
<tr>
<td><strong>pHBSP</strong></td>
<td>FPI, rat (SD)</td>
<td>0.05, 0.1, 0.2 nmol</td>
<td>ICV (ipsilateral CA2/3 region); 5 min post-TBI</td>
<td>Yes, histological and functional</td>
<td>[25]</td>
</tr>
<tr>
<td>(Pyr)EQLERALNSS</td>
<td>CCI (mTBI) + HS, rat (LE)</td>
<td>23.5</td>
<td>IP; 110 min post-TBI, then every 12 h for 3 days for chronic studies</td>
<td>Yes, histological and functional</td>
<td>[26]</td>
</tr>
<tr>
<td>Charge −1</td>
<td>CCI (mTBI) rat (LE)</td>
<td>23.5</td>
<td>IP; 12 h for 3 days for chronic studies</td>
<td>Yes, histological and functional</td>
<td>[27]</td>
</tr>
<tr>
<td><strong>JM4</strong></td>
<td>CCI, mouse (C57BL/6)</td>
<td>197–246</td>
<td>IP; 15 min, or 3, 9, and 24 h post-TBI</td>
<td>Yes, histological</td>
<td>[28]</td>
</tr>
<tr>
<td>GCAEHCSLNEITVPTDKV</td>
<td>Feeney weight-drop, rat (SD)</td>
<td>0.22 nmol</td>
<td>ICV; immediately pre-TBI</td>
<td>Yes, histological and functional</td>
<td>[29]</td>
</tr>
<tr>
<td>Charge −2</td>
<td>CCI, mouse (C57BL/6)</td>
<td>5</td>
<td>IV; immediately post-TBI</td>
<td>Yes, histological</td>
<td>[30]</td>
</tr>
<tr>
<td><strong>PACAP38</strong></td>
<td>CHI, mouse (Sabra)</td>
<td>303—363</td>
<td>SC; 15 min post-TBI</td>
<td>Yes, histological</td>
<td>[31]</td>
</tr>
<tr>
<td>HSDGIHTDYSRYRQMAVKKYLA</td>
<td>CHI, mouse (Sabra)</td>
<td>303—363</td>
<td>SC; 15 min post-TBI</td>
<td>Yes, attenuated Mac-1 increases</td>
<td>[32]</td>
</tr>
<tr>
<td>AVLGGKRKRYQVRKNNK</td>
<td>CCI, mouse (APP−/−)</td>
<td>0.03 nmol</td>
<td>ICV; 30 min post-TBI</td>
<td>Yes, histological and functional</td>
<td>[33]</td>
</tr>
<tr>
<td>Charge +9.1</td>
<td>F/A, rat (SD)</td>
<td>0.03 nmol</td>
<td>ICV; 30 min post-TBI</td>
<td>Yes, functional</td>
<td>[34]</td>
</tr>
<tr>
<td><strong>NAP</strong></td>
<td>CHI, mouse (C57BL/6 J)</td>
<td>95, 191</td>
<td>IV; 30 min post-TBI</td>
<td>Yes, histological</td>
<td>[35]</td>
</tr>
</tbody>
</table>
situated on the inner membrane, cyclophilin D in the matrix, and the voltage-dependent anion channel (VDAC) located on the outer membrane [51]. Structural changes in the VDAC account for much of the permeabilisation of the mitochondrial outer membrane, bringing about the opening of the MPTP and an influx of cations into the inner membrane space, effectively destroying the tightly regulated membrane potential and normal mitochondrial function [51, 52].

**Inflammatory Response**

In the event of a traumatic injury to the brain, inflammatory responses can occur within minutes of the primary injury, beginning with the activation of microglia [53]. Microglia are the principal immune cells of the brain that acutely respond to tissue disturbances by releasing cytokines into the brain extracellular space (Fig. 1), acting as antigen-presenting cells, and clearing debris [54–56]. Normally, the microglia exist in a quiescent state that is morphologically characterised by a small soma and ramified processes. At the onset of injury, the cells become functionally distinguished as one of two phenotypes, namely phagocytic or antigen-presenting [53, 57].

Although the initial inflammatory response may be beneficial to the damaged brain, longer-term effects can become deleterious when microglial activity is prolonged or poorly regulated. Neuroinflammation within the injured brain has long been considered to intensify the damage sustained following TBI [58]. The cytokines that are released by microglia do not directly cause neuronal cell death, but rather up-regulate the expression of molecules that mediate apoptosis, such as the Fas protein and Fas-ligand, members of the tumour necrosis factor (TNF) family [55, 59]. Additionally, interleukin-1, interleukin-6, and interleukin-8 levels have been found to be elevated in in vitro models of TBI [60, 61], preclinical animal TBI models [62–64], and brain-injured human subjects [65, 66]. Excessive levels of these molecules may lead to metabolic dysfunctions and organ failure [65].

For survivors of TBI, the inflammatory response has been found to persist for over a decade after the initial trauma [55, 67, 68]. Surprisingly, chronic microglial activation is not found in areas of focal brain injury, but are rather more prominent within subcortical structures remote from the site of primary injury, particularly the thalamus and putamen [55]. This phenomenon is also observed in animal models with evidence of chronic astrogliosis for up to a year following TBI [69].
Matrix Metalloproteinases and Cerebral Oedema

Matrix metalloproteinases (MMP) are multifunctional, zinc-dependent endopeptidases involved in the enzymatic cleavage of extracellular matrix proteins and cell surface receptors involved in the regulation of physiological processes such as tissue remodelling, apoptosis, angiogenesis, and cell proliferation and migration. The abnormal activation of MMPs is associated with a number of CNS disorders and is evident following TBI. MMP-2, MMP-3, and MMP-9 have been implicated in contributing to BBB breakdown, cerebral oedema, neuroinflammation, and neural cell death (Fig. 1) [70–75].

The development of cerebral oedema as a consequence of MMP activation and subsequent inflammatory responses (Fig. 1) is a complex and important injury process following TBI. MMP-2, MMP-3, and MMP-9 have been implicated in contributing to BBB breakdown, cerebral oedema, neuroinflammation, and neural cell death (Fig. 1) [70–75].

The stages of oedema are accompanied by BBB disruption. Following injury in mice, the BBB is initially permeable to larger proteins for up to 5 h, before becoming permeable to only smaller molecules, a state that persists for 4 days post-TBI [77].

Past Pharmacological Approaches to Treating TBI

Many attempts have been made to use experimental neuroprotective agents for the treatment of TBI with little or no success. Early experimental neuroprotective agents focused on targeting a single pathophysiological event, such as attenuating excitotoxicity. Dexanabinol, magnesium sulphate, and Selfotel [78] were some of the compounds investigated in this category. Although beneficial outcomes were reported from some of these agents in clinical trials, others had no significant effects [79, 80]. Similarly, other interventions have sought to block calcium ion channels, since cellular homeostatic disruption is a major factor in triggering excitotoxic injury. Originally used to treat high blood pressure, nimodipine and nicardipine are voltage-gated calcium channel blockers with neuroprotective effects in preclinical experiments; however, efficacy has not been confirmed in clinical trials [79].

While the exact reasons for the failure of previous therapeutics in TBI is beyond the scope of this review, several explanations have been proposed. For example, a drug may be beneficial in the acute neurodestructive phases following injury, but persistent antagonistic effects on a receptor or cellular target may hinder neuronal survival [81]. Moreover, the therapeutic agent may only target one neurodamaging event; therefore, any neuroprotective actions are eclipsed by other aspects of the
secondary injury cascade, lending credence to multifunctional drug investigations [82]. It is also possible that the drug has not reached therapeutic levels at the appropriate time following TBI, perhaps due to pharmacokinetic alterations [83]. Alternatively, there may be inherent problems in the way therapeutic efficacy of compounds are assessed, ranging from poor extrapolation of preclinical data, clinical irrelevance, and model variations [17]. Recently, the Operation Brain Trauma Therapy consortium screened a number of small compounds that had reported favourable preclinical outcomes. Of the three therapies, only levetiracetam had beneficial effects [16]. Simvastatin [19] and nicotinamide [15] both had sporadic results which led the consortium to conclude that further exploring their potential was largely not worthwhile [20].

Towards a Peptide-Based Therapeutic

Peptides are rapidly emerging as useful therapeutic agents because they can possess beneficial therapeutic properties such as the ability to cross membranes, high specificity and affinity for target molecules, and low immunogenicity and toxicity [18, 84]. In addition, chemical modifications can be easily introduced into peptides to increase serum and oral stability, and improve pharmacokinetic properties [85]. The global peptide therapeutic market is predicted to increase to US$25.4 billion in 2018 [86, 87].

Given that peptide-based therapeutics have been widely used in the treatment of diabetes, cardiovascular disease, asthma, allergies, cancer, and infectious diseases, the fact that they have been used so sparingly in CNS disorders is striking. Because of the high social and economic burden associated with TBI and stroke alone, these diseases represent an enormous pharmaceutical market segment and a major area of unmet need [21, 87]. Given that a number of peptides have already shown efficacy in preclinical TBI models, it seems logical to suggest that future effective neuroprotective agents may be peptide-based therapeutics. Below, we highlight the peptides that have been assessed in animal TBI models, and discuss their mechanisms of action, with an emphasis on arginine-rich peptides. The outcomes of preclinical TBI studies utilising peptides are summarised in Table 1.

Cyclosporin A

Cyclosporin A (CsA) is a powerful immunosuppressant and calcineurin inhibitor originally derived from an isolate of soil fungi. The peptide is an 11-amino acid cyclic structure (aLLVT-Abu-Sar-LVLA; where a = D-alanine; Abu = L-alpha-aminobutyric acid; Sar = sarcosine) consisting of mostly hydrophobic amino acids such as tyrosine, leucine, and valine. In addition, most of the amino acids in CsA are chemically modified or are atypical amino acids (e.g. D-alanine and sarcosine). CsA is widely used in organ transplantation procedures to prevent rejection, and its neuroprotective properties are attributable to its ability to interact with the mitochondrial protein, cyclophilin D, preventing MPTP opening (Fig. 1) [88, 89]. Evidence suggests that by inhibiting the opening of the MPTP, CsA reduces excitotoxic-mediated mitochondrial calcium uptake and ROS production [22, 90–92]. It has also been suggested that the neuroprotective action of CsA may in part be due to its ability to inhibit calcineurin [22, 23]; however, a study using a non-calcineurin inhibitory analogue of CsA has shown that this is not the case [24].

The positive effects of CsA on mitochondria prompted a preclinical assessment of CsA as a potential therapeutic for acute brain injuries, including TBI. In rats modelling both cortical impact and diffuse axonal injuries (CCI and DAI, respectively), administration of CsA within half an hour of the injury attenuated mitochondrial dysfunction and limited axonal damage [22, 91]. In a subsequent CsA dose-response study, a 16,630 nmol/kg dose was shown to be optimal for neuroprotection when administered up to 24 h after TBI [93]. Later studies demonstrated that a lower dose of CsA (8315 nmol/kg) administered daily after TBI was also neuroprotective and improved functional outcomes (Table 1) [23, 92].

The positive outcomes achieved with CsA in animal studies have led to clinical studies of its efficacy in humans. Early phase studies established a good safety profile for CsA when infused at a dose of 4158 nmol/kg over 24 h [94, 95]. However, no clear evidence of neuroprotective efficacy for CsA in TBI could be demonstrated, mostly due to its powerful immunosuppressant activity having detrimental side effects. Despite this lack of demonstrable clinical efficacy in TBI, there have been renewed studies of CsA (ClinicalTrials.gov Identifiers: NCT01825044, NCT02496975) investigating its pharmacokinetics, other effects on acute administration, and as candidate in combined therapies for TBI [96].

Mixed results from past preclinical trials have recently prompted one group to investigate the efficacy of CsA in different TBI animal models [25]. Doses of 8315 and 16,650 nmol/kg were given at 15 min and 24 h after fluid percussion injury (FPI), CCI, and penetrating ballistic-like brain injury (PBBI) models (Table 1). In the milder FPI and CCI models, CsA had limited beneficial effects, whereas deleterious and toxic effects were observed in the more severe PBBI model, leading the investigators to conclude that there is little reason for further clinical translation for the peptide [25].

Conopeptides

Conotoxins are a class of peptides derived from the venom of sea snails, a subset of which was first utilised as an analgesic for severe and chronic pain before its discovery as a potential neuroprotective agent for TBI. Purposed to act as N-type voltage-dependent calcium channel blockers (VDCC) (Fig. 1), ω-conotoxins have shown to be potently neuroprotective in
preclinical TBI studies but not in subsequent clinical trials. Despite these past failures, studies on the neuroprotective potential of other similar conotoxins continue.

SNX-111 (also Ziconotide; CKGKGAKCSRLMY DCCTGSCRSGKC) is a synthetic derivative of ω-conotoxin from the venom of Conus magus sea snails. It is a highly potent and selective antagonist of VDCCs (Fig. 1), its interactions with which are highly dependent on lysine and arginine residues in the peptide [97]. Following reports of neuroprotective effects in animal models of cerebral ischaemia [98–100], Bowersox and Luther [101] suggested that SNX-111 may be efficacious in other neurological disorders. Subsequent investigations showed that SNX-111 was effective in animal models of TBI, where it was capable of reducing intracellular neuronal calcium accumulation and mitochondrial dysfunction (Table 1) [102, 103]. In another study using a rat DAI model, SNX-111 improved behavioural outcomes when administered 3, 5, and 24 h post-injury (Table 1) [104]. Additionally, when SNX-111 peptide was combined with the brain-penetrating antioxidant U-101033E (known to reduce infarct size in rat models of middle cerebral artery occlusion) following TBI, mitochondrial dysfunction was also significantly reduced [105]. Given the positive outcomes of these preclinical TBI studies, clinical trials were undertaken with a phase I trial showing good safety and tolerability profiles for SNX-111, after a 24-h infusion at a dose of up to 16 nmol/kg/h [26]. Subsequently, a phase II study in 160 head-injured patients was commenced, but was suspended after a higher mortality rate was observed in SNX-111-treated patients (25 %) compared to placebo (15 %) [106].

Despite this setback with SNX-111, research on other conopeptides as potential neuroprotective agents has continued. SNX-185 (CLSPGSCSPSYNCCRPSCPSPYSRKC) is the synthetic form of another ω-conotoxin peptide, derived from the Conus tulipa sea snail. SNX-185 shares a 46 % amino acid homology with SNX-111 and has a similar inhibitory effect on VDCCs (Fig. 1), but greater bioavailability and a longer half-life in the rodent brain following intravenous administration compared to SNX-111, a feature attributed to its greater resistance to proteolytic degradation [107]. In a fluid percussion injury (FPI)-induced focal model of TBI, SNX-185 administration into the ipsilateral CA2–3 subregion of the hippocampus 5 min post-injury increased neuronal survival after 42 days, and improved behavioural outcomes (Table 1) [27]. More recently, in vitro investigations have revealed that SNX-185 increases cell survival in cortical neuronal [28] and astrocytic cultures [108] subjected to mechanical strain and/or a secondary low pH insult. Despite these positive preclinical findings for SNX-185, there have been no further reports examining its neuroprotective capabilities in TBI.

### EPO-Derivatives

Erythropoietin (EPO) is a protein that has potential neuroprotective actions due to its ability to decrease inflammation and neuronal death, and promote neurogenesis [109–112]. However, EPO given either by intravenous infusion or subcutaneous injections after TBI in humans does not improve neurological outcomes, but contributes to raised intracranial pressure and deep vein thrombosis [113, 114]. Given these negative findings, the focus has switched to peptides derived from the EPO in an attempt to circumvent detrimental side effects while still retaining the neuroprotective activities of the full-length protein.

An 11-amino acid peptide (QEQLERALNSS) synthesised from the helix B region of EPO (designated HBSP) has been demonstrated to be non-erythropoietic and capable of reducing the degree of injury in a non-TBI CNS injury model to a molar-equivalent extent compared to EPO [29]. The N-terminal glutamine in the HBSP peptide, however, is prone to cyclisation to pyroglutamate (pHBSP; pyr-EQLERALNSS; pyr = pyroglutamate). Furthermore, pHBSP, unlike EPO, has the added advantage of stability for up to 12 months at room temperature, or 24 months at 4 °C [30]. Having established its cytoprotective activity in a number of non-TBI models of injury [29, 115, 116], pHBSP has been identified as a potential therapeutic neuroprotective agent. In a mild model of TBI (mTBI) induced by CCI, rats intraperitoneally administered 23.5 nmol/kg pHBSP every 12 h for 3 days, beginning at either 1 or 24 h post-injury, improved histological and functional outcomes when compared to rats treated with a scrambled version of the peptide [117]. Even more promising results are reported for pHBSP by the same group in an mTBI model compounded with haemorrhagic hypotension [30].

A peptide (GCAEHCSLNENITVPDTKV) known as JM4 derived from the EPO protein AB loop is non-erythropoietic and shows neuroprotective properties [118]. The presence of two cysteine residues has been identified as a desirable characteristic that enables the peptide to form a stable cyclic structure due to the formation of disulphide bonds. In a mouse CCI TBI model, intraperitoneal administration of JM4 reduced lesion size when given up to 9 h post-injury (Table 1).

The neuroprotective mechanism of EPO-derived peptides such as pHBSP and JM4 is yet to be fully elucidated. Since these peptides are derived from regions of the EPO protein that interact with the EPO receptor, it is believed that their neuroprotective activity is mediated via this receptor, leading to suppression of the inflammatory response (Fig. 1) and stimulated expression of protective molecules such as brain-derived neurotrophic factor [117]. To date, no clinical trials of EPO-derived peptides have been reported, although a patent for the JM4 peptide as a therapeutic for CNS injuries has been filed.
Pituitary Adenylate Cyclase Activating Polypeptide

The pituitary adenylate cyclase activating polypeptide (PACAP) consists of 175 amino acids and is a member of the vasoactive intestinal peptide (VIP)/secretin/glucagon peptide family. It is widely distributed in the central and peripheral nervous systems where it plays an important role in neurogenesis and neuroregeneration, and for these reasons has been investigated for the assessment of its neuroprotective potential [119]. Administration of PACAP in TBI-induced rats via impact acceleration reported therapeutic benefit [120, 121]. Despite this, the native polypeptide does have a number of drawbacks that limit its therapeutic potential, such as poor bioavailability, low tissue absorption, and short biological half-life [122].

More recently, shorter peptides derived from PACAP have emerged. In particular, a 38-amino acid form (PACAP-38: HSDGIFTDSYRKYKQAIVLYAValGKRyRQRVKNK) was found to have beneficial effects on nutrient-deprived PC12 cells, increasing intracellular calcium and potassium levels, and protecting cells from apoptosis [31]. Additionally, suppression of microglial activity was observed using a much shorter, 3-amino acid PACAP-derived peptide (GIF) in cell culture following 2.5 ng/mL lipopolysaccharide exposure [123]. In vivo studies of PACAP-38 have also produced positive results. Early studies demonstrated that intracerebroventricular or intravenous infusion of PACAP-38 could reduce CA1 hippocampal cell death in rats exposed to cerebral global ischaemia [32]. For TBI studies, intracerebroventricular administration of PACAP-38 to rats prior to induction of injury using a modified Feeney weight-drop model improved behavioural outcomes and reduced inflammatory responses and tissue injury (Table 1) [124]. Miyamoto et al. [125] investigated PACAP-38 in a mouse CCI model and suggested that its neuroprotective effects were mediated by enhancing cellular antioxidant activity (Table 1).

There has been speculation as to the mechanisms associated with PACAP-38 peptide-mediated cytoprotection and, more specifically, its anti-apoptotic and anti-inflammatory activities (Fig. 1). It was hypothesised that the neuroprotective activity of PACAP-38 may be attributed to its ability to activate calcium channels, thus increasing intracellular calcium, which combines with nerve growth factor expression to promote neurite outgrowth and cell survival [31]. However, the exact means by which calcium channels are activated are still unknown. PACAP-38 is also believed to attenuate the TLR4/MyD88/NF-κB signalling pathway in microglia and neurons [124], a response that plays a crucial role in inflammatory aspects of TBI pathogenesis. It has been suggested that PACAP-38, by attenuating microglial NADPH oxidase activity and ROS production, reduces microglial pro-inflammatory signalling, and enhances the action of microglia SOD-2 and GPx-1 antioxidants [123, 125].

Intranasal administration delivery of PACAP-38 has been recommended by some, as the peptide binds to blood proteins resulting in reduced effectiveness when administered intravenously [126]. The proven neuroprotective properties of PACAP-38 suggest that the peptide warrants investigation in other areas of CNS injury such as brain ischaemia and spinal cord injury (SCI) [126]. The ability of cyclodextrin-coupled PACAP-38 to target specific areas in the brain has also been explored as a therapeutic option in the treatment of Alzheimer’s disease [127].

Other Endogenous Neurotrophic Protein Derivatives

Neuronal well-being is reliant on endogenous neurotrophic polypeptides and proteins, such as the aforementioned VIP. The neuroprotective and growth-promoting actions of VIP are purported to be mediated by two glia-derived proteins, activity-dependent neurotrophic factor (ADNF) [128] and activity-dependent neuroprotective protein (ADNP) [129]. The latter is essential for brain formation, while the former plays a vital role in protecting neurons from death associated with electrical blockade.

Brenneman and Gozes [128] were the first to isolate an active 14-amino acid portion of the ADNF protein (ADNF-14: VLGGSALLRSIPA) and report its exceptional potency in the femtomolar range in preventing neuronal cell death in electrically blocked spinal neuronal cultures. These researchers commented on the similarity of the peptide’s sequence to those in molecular chaperones that are induced by a variety of cellular stresses. Subsequent studies also reported positive neuroprotective effects of ADNF-14, and a shorter 9-amino acid ADNF peptide (ADNF-9: SALLRSIPA) [130–133] (Table 1). An 8-amino acid ADNP-derived peptide (NAP: NAPVSIPQ) that shares structural and immunological similarities with ADNF-9 also possesses neuroprotective properties [129, 132, 133] (Table 1). Although both ADNF-9 and NAP are thought to be neuroprotective by promoting neurite outgrowth, NAP is considered the preferred lead peptide due to its greater capacity to stimulate production of VIP, allowing interaction with glial cells (Fig. 1), and secretion of proteins that increase cell survival [129].

With respect to neuroprotective efficacy, a single subcutaneous dose of NAP (363 nmol/kg) is reported to reduce brain injury, purportedly by inhibiting the long-term accumulation of TNF-α in a mouse model of closed head injury (CHI) (Table 1) [134–136]. It is also thought that the neuroprotective effects of NAP are mediated by the peptide acting as a chaperone to protect against toxic β-amyloid plaque (Aβ) aggregation [33]. Furthermore, it is also believed that NAP protects against tau hyperphosphorylation [137], an event implicated in both traumatic brain injury and Alzheimer’s disease.

Clinical studies on NAP for TBI have not progressed, although its application in other neurodegenerative diseases has
been investigated. Similar to PACAP-38, NAP was initially developed for intranasal administration and marketed as Davunetide. Although it proved to be safe in human phase 1 studies (ClinicalTrials.gov Identifiers: NCT00404014, NCT00422981, NCT01110720) [138], no further developments for Davunetide have been reported since June 2015.

Nogo Extracellular Peptide 1–40

The myelin-derived neurite outgrowth inhibitor, Nogo protein, is a well-known inhibitor of neurite outgrowth in mature neurons. Of its three isoforms, Nogo-A is the most studied in terms on its effects on the CNS in injury. Nogo-66 is the functional domain common to all three isoforms and forms part of the extracellular loop that binds to the Nogo-66 receptor 1 (NgR1) to transduce growth inhibitory signals [139]. Nogo-66 receptor 1 signalling has been confirmed as a potential neuroprotective target in TBI based on the finding that NgR knockout mice subjected to a CCI have a better cognitive outcome than wild-type mice [139].

A 40-amino acid peptide derived from the Nogo-66 domain (NEP1–40; RIYKGVQAIQKSDEGHFPRAY LESEVAISEELVQKYSNS) has been shown to possess neuroprotective properties in vitro [140] via a mechanism thought to be mediated by the peptide acting as a competitive antagonist for Nogo-NgR binding (Fig. 1). To provide a more effective means of delivery of NEP1–40 across the blood brain barrier (BBB), NEP1–40 was fused to the cationic arginine-rich cell-penetrating peptide (CPP) TAT (named TAT-NEP1–40) [141]. The resultant TAT-NEP1–40 peptide was able to protect cultured PC12 cells from cell death induced by oxygen-glucose deprivation, an effect mediated by the peptide up-regulating Bcl-2, and promoting neurite outgrowth [141].

While the neuroprotective mechanism associated NEP1–40 and TAT-NEP1–40 has centred on their ability to disrupt binding of Nogo to NgR, conflicting evidence for this mechanism has come to light. Hånell et al. [142] reported that genetic deletion and pharmacological inhibition of NgR1 resulted in poorer outcomes in mice following TBI. Although there is potential for the application of NEP1–40 and TAT-NEP1–40 in TBI therapy and related injuries (stroke and SCI), further studies are required to validate the neuroprotective potential of these peptides and better elucidate their mechanism of action.

APP96–110

The amyloid precursor protein (APP) is a ubiquitously expressed transmembrane protein that is particularly concentrated in the synapses of neurons. Increasing levels of APP in the brain have been implicated in the events following TBI for having deleterious effects due to the production of neurotoxic Aβ plaques, that allows for its use as a biomarker [34, 143]. Despite the potentially toxic effects of APP, a derivative of the protein generated from the non-amyloidogenic processing pathway was found to have neuroprotective and neurotrophic properties [35, 36]. Similarly, early studies into the therapeutic potential of secreted forms of APP have revealed that they are protective against calcium-induced neuronal injury, and attenuate the neurotoxicity of Aβ [37]. Further, intracerebroventricular administration of the soluble α form of APP (sAPPα) is neuroprotective in rats with TBI where it reduces neuronal cell loss and axonal injury, as well as improving functional outcome [38] (Table 1). Recently, an APP-derived peptide comprising residues 96 to 110 (APP96–110; NWCKRGRKQCKTHPH) of the heparin binding D1 domain was also shown to improve functional outcomes in mouse TBI models [35, 39] (Table 1).

The mechanism(s) by which APP96–110 exerts its neuroprotective effect is not known, but appears to be related to the heparan sulphate proteoglycan (HSPG) binding abilities of the peptide (Fig. 1). Currently, it has been proposed that the formation of a β-hairpin loop within the peptide as a result of a disulphide bond between cysteine residues 98 and 105 facilitates HSPGs binding and triggers physiological changes such as the promotion of neurite outgrowth [40, 144]. Despite uncertainties regarding the neuroprotective mechanism of APP96–110, research continues on the therapeutic potential of the peptide in TBI [40].

APOE-Derivatives

The 299-amino acid apolipoprotein E (APOE) protein has multiple biological functions, most notably in relation to lipoprotein metabolism and transport. Moreover, APOE is anti-inflammatory and has neuroprotective effects within the CNS. APOE can protect primary mixed neuronal glial cell cultures from NMDA excitotoxicity [145] via mechanisms thought to involve the protein interacting with the low-density lipoprotein receptor-related protein (LRP) receptor [146, 147]. Due to its inability to readily cross the BBB, a major focus has been on developing an APOE-derived peptide that retains the neuroprotective properties of the full-length protein but has improved CNS bioavailability.

The first APOE-derived peptide investigated was COG133 (LRVRLASHLRKLRKLL), generated from amino acids 133–149 of the APOE protein. Initial in vitro investigations with COG133 demonstrated that the peptide was able to protect cultured neurons from NMDA excitotoxicity, but efficacy was slightly less than the APOE protein [148]. In animal models of TBI, intravenous administration of COG133 within 30 min of injury favourably modifies systemic and CNS inflammatory responses [41, 149]. Despite these positive effects, the 30 min therapeutic window is too short for therapeutic use [150] (Table 1). As a consequence, the COG133
peptide was further modified to generate COG1410 (AS-Aib-LRKL-Aib-KRLL; Aib = 2-aminoisobutyric acid).

Compared to COG133, COG1410 has an extended therapeutic time window and can improve histological and functional outcomes following TBI [150–155]. A dosage regimen consisting of five daily intravenous injections of COG1410 at 710 nmol/kg provides a therapeutic time window of 2 h post-injury in rats following FPI (Table 1) [154]. Similarly, the same daily intravenous dose of COG1410 over 3 days improves outcomes after sub-arachnoid haemorrhage [156] and TBI in rats by suppressing MMP-9 activity, thus preserving brain extracellular matrix integrity and reducing BBB disruption [155] (Table 1). Further investigation into the safety and toxicity of COG1410 are underway by the pharmaceutical company Cognosci, Inc. as a preliminary to clinical trials.

As mentioned above, the neuroprotective action of APOE-derived peptides is thought to be related to their interaction with the LRP receptor (Fig. 1). A study by Wang and Gruenstein [147] reported that a 9-mer peptide consisting of a tandem repeat of APOE residues 141–149 (RKLRRKRL) rapidly and irreversibly increases neuronal cytoplasmic calcium. Interestingly, the increase in intracellular calcium was associated with the APOE 9-mer peptide stimulating the synthesis of IP3 mediated by activation of a G-protein [146], suggesting that the calcium originated from the endoplasmic reticulum. Leupold et al. [157] subsequently proposed the involvement of HSPGs in the transport of APOE-derived peptides across the plasma membrane of target cells as an important event associated with neuroprotection.

Other TAT-Fused Peptides

The collapsin response mediator protein 2 (CRMP2) is a highly-expressed, brain-specific protein involved in axonal guidance during development, and pre- and post-synaptic calcium regulation. CRMP2 is also implicated in various neurological disorders and is suspected to play a role in neuronal regeneration. Following injury, CRMP2, as well as CRMP1, and CRMP4 are degraded by calpain [158–161], a process that correlates with neuronal intracellular calcium influx, cell injury, and neurite damage. A 15-amino acid peptide (CBD3: ARSRLAELRGVPRGL) derived from the calcium-binding domain of CRMP2, fused to the CPP TAT (named TAT-CBD3) was demonstrated to have neuroprotective properties following excitotoxic injury in cultured neurons [162–164]. Subsequent studies demonstrated that the TAT-CBD3 also improved outcomes following TBI in mice [162] (Table 1).

It is believed the CBD3 peptide can alter the location and/or function of voltage-gated ion channels (e.g. CaV2.3), glutamate receptors (e.g. NMDA receptor), and calcium transporters (e.g. NCX3) on the plasma membrane (Fig. 1), thereby reducing calcium influx. Consequently, the neuroprotective mechanism of action for TAT-CBD3 is commonly thought to relate to its ability to suppress the excitotoxic influx of calcium. In vitro experiments with CBD3 have shown that, as long as the peptide is fused to the TAT carrier peptide, neuroprotection is achieved [162–164]. Interestingly, the neuroprotective properties of CBD3 are enhanced when the TAT peptide is replaced with the poly-arginine-9 (R9) CPP (R9-CBD3) [164]. While this effect could be due to the enhanced uptake of R9-CBD3, it could equally reflect the properties of the CPP conjugate itself, an effect that might be further enhanced by the arginine content of the cargo peptide [165, 166].

Another TAT-fused peptide (gp91ds-TAT; RKRRQRRRCSTRIRRQRL) developed for its ability to inhibit activity of the plasma membrane-bound enzyme complex NADPH oxidase 2 has been shown to mitigate ROS and improve outcomes following TBI in mice. [167]. However, given the number of arginine residues present in the gp91ds-TAT peptide, it is equally possible that the neuroprotective action of the peptide is not mediated by its proposed inhibitory actions on NADPH oxidase 2, but rather, via the arginine-rich TAT peptide itself, with further enhancement due to the arginine content of gp91ds.

A Case for Arginine-Rich Peptides

Because of past therapeutic failures and the complex pathology of TBI, current thinking has focused on developing pharmacological therapies that target multiple facets of TBI pathophysiology. The National Institutes of Health has encouraged trials of combination therapies in an attempt to explore the potential of combined agents with complementary mechanisms of action [96]. Of the trials that had been funded, many of these failed to demonstrate any significant difference when compared to their respective mono-therapies, with one trial producing poorer outcomes [168]. As an alternative approach, we propose that arginine-rich peptides be considered, given that they showed neuroprotection in preclinical stroke models, and have the potential to target several neurodamaging processes such as excitotoxicity, mitochondrial dysfunction, ROS production, and activation of proteolytic enzymes (Fig. 1) [14]. The identification of a range of peptides rich in the cationic amino acid arginine and, to a lesser extent, lysine, as neuroprotective suggests that arginine-rich peptides may be considered as a new class of neuroprotective agent for assessment in TBI and other acute CNS injuries.

Past Approaches with Peptides Containing Arginine Residues

A range of peptides containing positively charged arginine and/or lysine residues, lending to the peptide’s overall cationic state, have shown to be neuroprotective in TBI. The SNX-111 conopeptide (with 2 arginine and 4 lysine residues), for
example, was highly effective in preclinical studies [106] compared to other drugs at the time. The ability for it to antagonise calcium channels and attenuate mitochondrial dysfunction initially made it an attractive drug candidate for TBI, characteristics which were attributed to its arginine and lysine content [97]. Unfortunately, clinical development of SNX-111 was abandoned following adverse outcomes in a phase 2 TBI study. Other cationic peptides investigated for use in either TBI or other CNS injuries include PACAP38, APP96–110, APOE-derived, and TAT-fused neuroprotective peptides, whereas neutral peptides such as CsA [25] and NAP have been largely discontinued as potential treatments for TBI.

**TAT and Neuroprotective Peptides Fused to TAT**

As the most commonly used CPP for drug delivery, TAT has been widely used in CNS studies due to its ability to traverse the BBB. The TAT peptide (TAT$_{48-57}$: GRKKRRQRRR) was derived from the protein transduction domain (PTD) within the human immunodeficiency virus-type 1 trans-activator of transcription (HIV-TAT) protein [169]. The membrane-traversing properties of TAT and other cationic arginine-rich peptides are attributable to peptide electrostatic interactions with negatively charged plasma membrane moieties, and subsequent uptake by endocytic and/or non-endocytic pathways [169, 170]. Over 30 different putative neuroprotective peptides have been fused to the TAT peptide with the vast majority showing neuroprotection in various animal models associated with CNS disorders, including TBI, stroke, perinatal hypoxic-ischaemia, SCI, and pain. However, several years ago, our laboratory and others reported that the TAT peptide possessed modest neuroprotective properties in its own right [171–173].

Additional studies from our laboratory have demonstrated that the related cationic arginine-rich CPPs, poly-arginine-9 (R9), and penetratin (RQIKIWFQRRMKWKK) are even more neuroprotective than TAT [174]. Further analysis of different poly-arginine and arginine-rich peptides, as well as some previously described TAT-fused neuroprotective peptides has led us to conclude that the mode of action of neuroprotective peptides fused to arginine-rich CPPs is mediated exclusively by the carrier peptide itself, with efficacy being further enhanced by the arginine content of the cargo peptide [165, 166]. On this basis, we have identified arginine content and peptide positive charge as critical factors in determining neuroprotective capacity, a finding supported by an earlier study from Ferrer-Montiel et al. [175] who screened a 6-mer peptide library for the ability to block glutamate-evoked ionic currents in *Xenopus* oocytes, and later by Marshal et al. [176] who screened a range of arginine-rich peptides in a NMDA retinal ganglion cell excitotoxicity model. There is evidence that other amino acid residues can also influence the efficacy of arginine-rich peptides. For example, cationic lysine, highly hydrophobic tryptophan, and disulphide bond-forming cysteine residues appear to increase the level of neuroprotection, while alanine appears to decrease neuroprotection [166, 176].

Our research increasingly supports the view that peptides rich in the cationic amino acid arginine, including poly-arginine peptides and other neuroprotective peptides fused to TAT, represent a new and promising class of neuroprotective agents for the treatment of CNS disorders, including TBI [14, 165, 166]. Furthermore, based on the biological properties of arginine-containing peptides, it is highly likely that this class of peptide operates through several different neuroprotective mechanisms of action, a property that is highly advantageous as neurodegenerative disorders invariably involve multiple neurodamaging processes operating in tandem. A summary of our current understanding of the neuroprotective mechanisms for arginine-rich peptide is provided below.

**Arginine-Rich Peptide Interactions with Cell Surface Structures**

The neuroprotective ability and efficacy of arginine-rich peptides is critically reliant on their arginine content and peptide charge, which correlate with the endocytic or cellular uptake properties of the peptide [166]. These two critical properties, which are attributed to the guanidino chemical head group, are only found with the amino acid arginine [177, 178]. The guanidino head group confers a cationic charge and uniquely allows arginine residues present in arginine-rich peptides to undergo bidentate hydrogen bonding with anionic sulphate, phosphate, or carboxylate moieties on the plasma membrane [179], and thereby induce cellular uptake by endocytic and non-endocytic pathways. Consistent with this critical structural feature of arginine residues, poly-lysine peptides containing cationic lysine residues that can only form monodentate electrostatic bonds with sulphate, phosphate, or carboxylate moieties on the plasma membrane show poor cellular uptake and neuroprotective properties compared to poly-arginine peptides [165].

Our laboratory has demonstrated that arginine-rich peptides are potent inhibitors of glutamate excitotoxic neuronal death with neuroprotective potency increasing with the number of arginine residues [165]. Based on the ability of arginine-rich peptides to reduce excitotoxic calcium influx, we have hypothesised that the peptides down-regulate and/or interfere with plasma membrane glutamate receptors during peptide uptake. Furthermore, we have conjectured that arginine-rich peptides have the capacity to alter the function of other cell surface receptors, ion channels, and transporters during internalisation and/or interaction with the plasma membrane. For example, we have highlighted [165, 166] that many studies with arginine-rich peptides, including putative “neuroprotective peptides” fused to TAT, can interfere with or reduce the expression of cell surface ion channels and
receptors, including NMDAR, VR1, CaV2.2, CaV3.2, and NCX. Investigators studying the neuroprotective properties of conopeptides also noted the significance of arginine (and lysine) residues, particularly in relation to calcium channel inhibition [97]. Moreover, TAT, penetratin, and R9 have been shown to induce the endocytic internalisation of TNF receptors and/or the EGF receptor in HeLa cells [180]. More recently, we have shown that exposure of cortical neurons to R12 (poly-arginine-12) reduces cell surface levels of the NMDA receptor subunit protein, NR2B (unpublished observation). Similarly, the interaction of the APP96–110 peptide with negatively charged cell surface HSPGs structures, which is known to trigger endocytosis, may be necessary for the peptide to exert a neuroprotective effect. Interestingly, HSPGs and LRPs have also been implicated in the uptake of the HIV-1 TAT protein [181], which may partly explain the neuroprotective actions of APOE-derived peptides.

Overall, we believe it is highly likely that arginine-rich peptides have neuroprotective actions that are associated with the suppression of the toxic effects of ion channels and receptors that are activated following TBI and are involved in neurodamaging pathways, such as excitotoxicity, cell death signalling, and inflammation. Notwithstanding the above, it is also possible that the correlation of peptide uptake with neuroprotective efficacy is related to increased intracellular bioavailability of the peptide and hence increased opportunities to positively interact with intracellular targets, such as mitochondria.

**Arginine-Rich Peptide Interactions with Mitochondria**

As discussed previously, mitochondrial disturbances in the form of reduced ATP synthesis, excessive ROS production, release of pro-apoptotic proteins, and opening of the MPTP play a major role in damaging brain tissue following TBI. Arginine-rich peptides are known to target mitochondria [182, 183] primarily due to their cationic property conferred by the incorporation of basic amino acid residues. Moreover, arginine residues within arginine-rich peptides are thought to electrostatically interact with phosphate head groups on the negatively charged mitochondrial inner membrane phospholipid, cardiolipin [184]. Increasing evidence suggests that arginine-rich peptides have a positive effect on mitochondria in terms of maintaining mitochondrial integrity in times of stress, as occurs in the brain following TBI. By modulating the MPTP, arginine-rich peptides are able to rescue cells from oxidative stress as a result of an influx of calcium. Calcium-induced mitochondrial swelling is reduced [185, 186], protecting the mitochondrial cristae architecture. There is also inhibition of complex I activity and ROS production, accelerating ATP recovery, and preventing cytochrome c release.

The Szeto-Schiller (SS) peptides are a class of polycationic peptides that have proven to be incredibly effective in mitigating ROS production [187–189], a capability that may be extended to PACAP-38 and APOE-derived peptides. The main neuroprotective ability of the SS-peptides is purportedly due to their action on mitochondria, protecting the organelle against oxidative damage by inhibiting ROS production, preventing swelling, cytochrome c release, and apoptosis [190, 191]. Like a number of the other peptides discussed, SS-peptides are cationic, containing both arginine and lysine residues, with the arginine residue considered particularly critical for activity. Since oxidative stress is also a product of other forms of injury, it is not surprising that SS-peptides have shown to be efficacious in heart and renal disorders [188, 192, 193]. PACAP-38 reportedly had a similar effect in mice, suppressing cortical damage through antioxidant activity [125]. APOE similarly down-regulates microglial activity, reducing ROS production, and resulting in protection from oxidative stress [148, 150, 194, 195]. Since the cationic state appears crucial in determining the neuroprotective efficacy of peptides in mitigating mitochondrial ROS production, it could be argued that arginine-rich peptides may also provide neuroprotection via the described mechanisms with mitochondria.

**Other Potential Neuroprotective Actions of Arginine-Rich Peptides**

Arginine-rich peptides, in particular poly-arginine peptides, have been shown to inhibit the activity of proteolytic enzymes, such as matrix metalloproteinases (MMPs), the proteasome, and cathepsin C. The activation of proteases, especially matrix metalloproteinases, is known to be implicated in tissue injury following TBI [71, 196]. MMPs are responsible for the degradation of extracellular matrix proteins leading to BBB disruption and cerebral oedema after TBI [70, 71]. MMPs can be activated following cleavage by the proprotein convertase furin, of which arginine-rich CPPs [197] and especially poly-arginine peptides [198] are potent inhibitors. Furin is expressed in the brain and can activate MMP-2, MMP-3, and MMP-14 (also known as MT1-MMP) [199]. Furthermore, MMP-14 activates MMP-2, which in turn along with MMP-3 activates MMP-9 [200, 201]. Importantly, MMP-2, MMP-3, and MMP-9 have all been associated with tissue injury and BBB disruption in TBI [71, 72, 202]. By extension, it would also be reasonable to associate MMP-14 with the deleterious TBI cascade as it is activated in other acute brain injuries such as stroke. Therefore, it is possible that the inhibition of furin by arginine-rich peptides reduces the damaging effects MMPs following TBI.

Arginine-rich peptides may have additional beneficial effects by inhibiting the activity of the intracellular proteasome complex in brain cells affected by TBI [203–205]. Proteasome activity is known to increase following TBI [206, 207], and is thought to be involved in accelerating the degree of axonal
shearing [208]. In addition, inhibition of the ubiquitin-proteasome system may rescue mitochondria from mitophagy [209] and disrupt the NF-κβ pathway, thereby reducing inflammation [210].

Designing an Arginine-Rich Therapeutic for TBI

Axonal injury is a prominent feature across all severities of TBI [211] that produces the worst outcomes in human patients, making it a vital target for therapy. Mechanical stretching of the axon acutely increases intracellular calcium, leading to delayed Wallerian degeneration [212]. Additionally, the influx of calcium has detrimental effects on mitochondria, such as opening the MPTP, ROS production, mitochondrial swelling, and cristae disruption [213, 214]. Mechanoporation has been suggested in the past as a major mechanism associated with intracellular calcium influx, but this is not supported by more recent findings [215]. Together, these insults result in a breakdown of the axon’s structural integrity, eliciting microstructural changes in the white and grey matter [216]. By targeting the plasma membrane and calcium channels/influx, and mitochondria, arginine-rich peptides have great potential to counteract DAI damaging pathways.

To be clinically effective in TBI, arginine-rich peptides need to exhibit several characteristics. Specifically, arginine-rich peptides are very stable, do not require refrigeration for storage, and are easily transported (e.g. protamine sulphate). On this basis, and coupled with the effectiveness of intravenous delivery, arginine-rich peptides have features that make it feasible for them to be administered in the field at the site of the TBI prior to hospital admission, thereby providing a means for early intervention to reduce brain injury and maximise patient outcomes.

Efficacy of Arginine-Rich Peptides in Stroke Models

The similarities between stroke and TBI are well known [217] and have provided the basis for numerous therapeutics developed in a stroke context, and subsequently applied in the treatment of TBI. In our laboratory, arginine-rich peptides, particularly poly-arginine peptides, have been shown to have highly potent neuroprotective effects in various in vitro and in vivo models of ischaemic stroke [165, 173, 174, 218]. Recently, the R18 poly-arginine peptide was shown to be more effective than the highly characterised TAT-fused NR2B9c neuroprotective peptide (TAT-NR2B9c; also known as NA-1) in permanent and transient middle cerebral artery occlusion models of stroke [218]. The TAT-NR2B9c peptide is neuroprotective in a number of rodent [219–224] and non-human primate [225, 226] stroke models, and it reduces ischaemic brain lesions in humans following endovascular aneurysm repair [227]. R18 contains significantly more arginine residues and has a higher cationic charge than TAT-NR2B9c, and we believe that this is the main reason for its enhanced neuroprotective activity compared to TAT-NR2B9c peptide. Based on the efficacy of R18 in experimented stroke, our intention in the near future is to assess the potential of this peptide in a rat model of TBI.

Conclusion

The clinical impact of a lack of an effective pharmacological therapy for such a devastating condition as TBI cannot be overstated. Many previous attempts at therapy have failed, and consequently, a new direction for the development of neuroprotective agents for TBI is urgently needed. Here, we have drawn attention to evidence that suggests arginine-rich peptides represent a new class of neuroprotective agent and have argued that they should be considered in the context of neuroprotective therapeutic drug development for TBI. Adding to this, past studies using TAT-fused and other cationic peptides as a potential neuroprotective agent for TBI have produced some promising results that warrant additional investigation. This class of peptides have established efficacy in other brain injury models such as stroke, suggesting that they may be equally efficacious in TBI. Importantly, it appears that the arginine residues are especially critical in allowing these peptides to cross the BBB and be internalised by neurons and other neural cells. Upon being internalised, these peptides appear to act via multiple mechanisms, namely, maintaining mitochondrial integrity, reducing destruction of the brain extracellular matrix, and preserving BBB by inhibiting MMPs. Given these findings, there is growing evidence that arginine-rich peptides, including poly-arginine peptides such as R18, warrant consideration as neuroprotective agents for TBI.

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Compliance with Ethical Standards

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

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Chapter 4

General materials and methods
In addition to the information provided in manuscripts (Chapters 5, 6, and 7), many of the materials and methods used in this thesis are also provided below.

4.1 Peptides used in this project

Details of the peptides used in this project are provided in Table 4.1. Peptides were synthesised by Mimotopes (Australia) and purified by high performance liquid chromatography to at least 98%. For the \textit{in vitro} and animal studies, peptides were prepared as a 500 µM stock solution in water for irrigation (Baxter, Australia), and in 0.9% sodium chloride for injection (Pfizer, Australia), respectively. Reconstituted peptides were stored at -20°C until use.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence*</th>
<th>Number of arginine residues</th>
<th>Net charge at pH 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>R18</td>
<td>H-RRR...RRR-RRRRRRRRRR-OH</td>
<td>18</td>
<td>+18</td>
</tr>
<tr>
<td>R18D</td>
<td>H-rrrrrrrrrrrrrrrrr-OH</td>
<td>18</td>
<td>+18</td>
</tr>
<tr>
<td>APP96-110</td>
<td>Ac-NWCKRGRKQCKTHPH-NH2</td>
<td>2</td>
<td>+5</td>
</tr>
<tr>
<td>COG1410</td>
<td>Ac-AS-Aib-LRKL-Aib-KRLL-NH2</td>
<td>2</td>
<td>+3</td>
</tr>
</tbody>
</table>

*R = L-arginine, r = D-arginine, N = asparagine, W = tryptophan, C = cysteine, K = lysine, G = glycine, Q = glutamine, T = threonine, H = histidine, P = proline, A = alanine, Aib = 2-Aminoisobutyric acid or 2-methylalanine, S = serine, L = leucine.

4.2 Primary cortical neuronal cultures

Primary cortical neurons were isolated and cultured as described in Meloni et al. (2014). Briefly, cortical tissue extracted from E18-19 Sprague-Dawley rats were dissociated in Dulbecco’s modified Eagle medium (DMEM; Life Technologies, Australia) supplemented with 1.3 mM L-cysteine, 0.9 mM NaHCO₃, 10 U/mL papain (Sigma
MATERIALS AND METHODS

Aldrich, USA), 50 U/mL DNase (Sigma Aldrich, USA), and washed in cold DMEM/10% horse serum. Neurons were resuspended in Neurobasal media (NB; Life Technologies, Australia) containing 2% B27 supplement (B27; Life Technologies, Australia). Neurons were seeded into 96-well plastic plates (Nunc, Australia) and maintained in a CO₂ incubator (5% CO₂, 95% air balance, 98% humidity) at 37°C. On day in vitro (DIV) 4, one third of the culture medium was removed and replaced with fresh NB/2% B27 containing the mitotic inhibitor cytosine arabinofuranoside (final concentration 0.5 µM; Sigma Aldrich, USA), and on DIV 8, one half of the culture medium was replaced with NB/2% B27. Neuronal cultures were used for in vitro studies on DIV 11 – 13. Under these conditions, cultures routinely consist of > 95% neurons and 1 – 5% astrocytes.

4.3 Glutamic acid excitotoxicity model and peptide treatments

The in vitro glutamic acid excitotoxicity model is well established in A/Prof Meloni’s laboratory. This model is representative of a major secondary neurodamaging event that occurs following TBI. The glutamic acid model is routinely performed on cortical neurons maintained in 96-well plastic culture plates (Meloni et al., 2014; Meloni et al., 2015a). Peptides were added to culture wells 10 minutes prior to glutamic acid (L-glutamic acid; Sigma Aldrich, USA) exposure by removing media and adding 50 µL of MEM (Minimal Essential Medium; Life Technologies, Australia) /2% B27 containing the specific peptide. To induce excitotoxicity, 50 µL of MEM/2% B27 containing glutamate (200 µM; final concentration 100 µM) was added to the culture wells and incubated at 37°C in a CO₂ incubator for 5 minutes (note: peptide concentration is reduced by half during this step). Media in wells was then replaced with 100 µL of MEM/2% B27 and cultures incubated for a further 20 – 24 hours at 37°C in the CO₂ incubator. For all experiments, untreated controls with or without glutamic acid treatment underwent the
same incubation steps and media additions. In this model, neuronal survival following glutamic acid treatment typically ranges from 2 – 10% (i.e. 90 – 98% cell death).

4.3.1 Neuronal viability assessment

Cell viability was examined with light microscopy to qualitatively assess morphological cell death at 30 – 40 minutes and 24 hours after glutamic acid exposure. The MTS colorimetric viability assay (Promega, Australia) was used to determine cell viability at 24 hours after glutamate exposure. The MTS assay quantifies the ability of cytosolic enzymes in viable cells to reduce the tetrazolium salt (MTS; 4,5-dimethyliazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) to the water-soluble brown formazan salt, which is detected spectrophotometrically at 490 nm.

4.4 Animals and husbandry

All experimental protocols and the use of animals in this project was approved by the Animal Ethics Committee of the University of Western Australia and follows the guidelines outlined in the “Australian Code for the Care and Use of Animals for Scientific Purposes 8th edition”, as established by the Australian Health and Medical Research Council. In the design of these studies, every effort was made to minimise the amount of animal discomfort.

Two different strains of male rats (Rattus norvegicus) were used for the project, namely Sprague-Dawley and Long-Evans (Animal Resource Centre, Murdoch, Australia), and habituated at the University of Western Australia’s Biomedical Research Facility (Shenton Park, Australia) for a week prior to surgery. The animals weighed 350 – 450 g
and were housed in cages with two to four animals under controlled conditions before and after surgery; a 12-hour light-dark cycle, and free access to food and water ad libitum.

4.5 Traumatic brain injury model and peptide administration

4.5.1 Anaesthesia and surgical preparation

Halothane (Pharmachem, Australia) was used as the anaesthetic during surgery, as it is considered less neuroprotective than isoflurane (Statler et al., 2000, 2006; Zheng & Zuo, 2004). General anaesthesia was induced using a face-mask and 5% halothane (mix 30% O₂/70% N₂O). The rat was then intubated and maintained with 1 – 2% halothane.

A sagittal incision was made on the dorsal surface of the head, connective tissue beneath the scalp was cleared to expose the coronal, sagittal, and occipital suture lines. Once thoroughly cleaned, a stainless steel disc (“helmet”; diameter = 10 mm, depth = 3 mm) was fixed onto the exposed skull between bregma and lambda points of the suture lines, with cyanoacrylate adhesive. Throughout the surgical procedure, foot-pinch and eye-reflex assessments were carried out to confirm depth of anaesthesia.

4.5.2 Weight-drop impact-acceleration injury model

The Marmarou weight-drop impact-acceleration injury model, extensively described elsewhere (Foda & Marmarou, 1994; Marmarou et al., 1994), was adapted for this project. Once the stainless steel helmet was fixed, rats were transferred onto a foam bed (Foam to Size, USA; 43 x 12 x 12 cm) and secured with masking tape across the upper back and pelvic region on the dorsal surface. The rat was then temporarily disconnected from the ventilator (< 1 min) to induce the injury (Figure 4.1). After the contact surface of the
weight was aligned with the top of the helmet, a 435 g brass weight was dropped from a height of 180 cm through a hollow aluminium tube, directly onto the helmet attached to the rat.

Figure 4.1 Marmarou's weight drop model of TBI, as adapted for this project. Weight (435 g) was dropped from a height (180 cm) onto the anaesthetised rat placed prone on a foam bed. The falling weight directly strikes the helmet (inset), which acts to evenly distribute the energy across the skull surface, and thus the brain.

Following impact, the foam bed was pulled away from the weight-drop apparatus to prevent a second impact of the weight upon rebound, and the rat was immediately reconnected to the ventilator. Seizures lasting 5 – 10 seconds were often observed immediately following injury, which is common following moderate to severe TBI. When seizures occurred, rats remained secured to the foam bed to reduce any chance of further injury. Once seizure had ceased, the rat was removed from the foam bed and returned to the surgical table. The steel helmet was subsequently removed, along with
residual cyanoacrylate from the exposed skull surface. The head wound was then sutured before the rat was placed in a supine position to be prepared for intravenous treatment delivery. Sham animals underwent the same surgical and TBI procedures, but were placed adjacent to the apparatus as the weight was released.

4.5.3 Treatment administration

All treatments were administered intravenously (600 μL over 6 min) at 30 minutes post-impact, following procedures routinely carried out for our rat stroke models. A small incision was made superficial to the right internal jugular vein to expose the vessel. Once the infusion site of the jugular vein was located, the rostral end was tied off with a suture, before the infusion cannula (SteriHealth, Australia; PE 50; diameter 1 mm (outer) x 0.5 mm (inner); 35 cm length) was prepared, primed, inserted into the vein, and secured in place with a suture. Peptide and vehicle (0.9% saline) treatments were randomised, and all personnel carrying out animal procedures were blinded to treatments.

4.6 Post-surgical animal care and monitoring

At the conclusion of surgery, pethidine (IM: 1 mg in 0.2 mL saline) and bupivacaine were administered (SC: 0.1 mg in 0.2 mL saline per site) to the head surgical wound. A 2 mL volume of injectable saline was also subcutaneously administered to aid hydration. To avoid hypothermia, rat cages were placed on a heating mat during post-surgical monitoring. Animals were monitored at least twice a day (AM and PM) and administered pethidine (SC: 1 mg in 0.2 mL saline) if their behaviour was indicative of discomfort. Saline (SC: 2 mL) was administered if animal weight continued to decline and to aid
MATERIALS AND METHODS

hydration. Rats were also provided with sweetened cereal and raspberry-flavoured water gel to encourage food intake and weight gain.

4.7 Functional assessments

TBI can result in memory and motor functional deficits in the rat. Therefore, to examine the effect of peptide treatment on memory and motor function outcomes, the project utilised the Barnes maze, adhesive tape removal, and rotarod tests. Functional assessments were performed early morning (commencing at 0730 hours) in the days after TBI, beginning with the Barnes maze, followed by the adhesive tape removal, and rotarod test. Specific details of the assessment regimen are provided in the relevant chapters.

4.7.1 Barnes maze test

Figure 4.2 Barnes maze configuration. Circular table with 20 equally-spaced holes around the periphery. The middle of the table served as the start point for each animal as indicated by the orange dot.

The Barnes maze (Barnes, 1979) is a spatial learning and memory test where animals are required to find the location of an escape tunnel from 20 other holes set in a round table top (Figure 4.2). During the test, a bright light is placed above the table to serve as a mild aversion stimulus. The table is surrounded by a heavy black curtain, with no other visual
MATERIALS AND METHODS

stimuli other than a cue card with horizontal black and white lines (line thickness: 1 Hz at 1 m). The escape tunnel location remained constant between each assessment day.

Each trial began with the rat at the centre of the table, covered by a plastic container. Latency was measured from the time when the plastic container was removed and the light switched on, to the time the rat located and entered the escape tunnel. Each animal was given 180 seconds to locate the escape tunnel. In the event that the rat could not locate the escape within the given time, the light was kept on, and the rat was guided into the escape tunnel. The light was then switched off, and the rat returned to his cage.

4.7.2 Adhesive tape removal test

![Image of adhesive tape removal test](image)

**Figure 4.3** Male Sprague-Dawley rat demonstrating paw placement of the 10 x 10 mm adhesive tape.

The adhesive tape removal test assesses sensorimotor function (Bouet et al., 2009). Alternating between right and left forepaws for each trial, a 10 x 10 mm piece of adhesive tape (Diversified Biotech, USA) is placed on the palmar surface of the forepaw (Figure 4.3). Before commencing the assessment, each rat is left to habituate in the transparent plexiglass cage (29 x 19 x 13 cm) test environment for a minimum of 30 seconds. For each trial, latency was measured from the time the experimenter released the rat with the applied adhesive tape, until complete removal of the adhesive tape from the paw. Rats
were allowed 180 seconds per trial to remove the tape. In the event that the rat was unable to completely remove the adhesive tape, the experimenter intervened to remove the tape.

4.7.3 Rotarod test

The rotarod assesses vestibulomotor function (Hamm, Pike, O’Dell, Lyeth, & Jenkins, 1994). The assessment involved placing the animal facing away from the experimenter on a rotating rod (Model number: MK-630B; Muromachi, Japan). Rotations started at a speed of four revolutions per minute (rpm), and incrementally increased to 40 rpm. Each rat was required to remain on the rod for as long as possible. Latency was recorded from the time animal was placed on rod until falling from the rod onto a metal platform, which stopped the timer and the rotation of the rod. In the event that the rat turned around to face the assessor or stood up on its hind-limbs, the assessor gently guided the rat back onto the rod in the correct position.

4.8 Tissue processing

At the conclusion of functional assessment (usually 3 – 4 days post-TBI), animals were euthanised via an initial isoflurane anaesthesia (induction chamber; 4% isoflurane), followed by a 1 mL intraperitoneal injection of pentobarbital (325 mg/mL; Virbac, Australia). Transcardial perfusion consisted of introducing a large bore needle (PrecisionGlide™ 16G 1 ½ in.; BD, Australia) through the left ventricle, piercing the right atrium, followed by perfusion with 4% formalin (approximately 100 mL), and rinsed with heparinised saline (approximately 200 mL). The brain was subsequently removed and stored in 10% neutral buffered formalin (Amber Scientific, Australia) for one week before dehydration and embedding in paraffin wax (PathWest, Australia). For the
collection of fresh tissue, rats were perfused with ice-cold heparinised saline only. Brains were sagittally dissected, with one half post-fixed as described above and one half frozen in isopentane with liquid nitrogen, and stored at -80°C.

4.9 Histological assessment for brain injury

Formalin-fixed, paraffin-embedded coronal sections (10 µm) were mounted onto Superfrost® glass slides (76 x 26 mm; Menzel-Gläser, Germany) and treated with two changes of xylene, before rehydration by incubating in decreasing concentrations of ethanol (two changes each of 100 and 95%, one change of 70%) and finally, water. Sections were stained with either Nissl or Bielschowsky’s silver reagents to assess for cornu ammonis (CA) hippocampal neuronal and axonal damage, respectively. Light microscopic images were captured using an Olympus DP-70 digital camera fitted to an Olympus IX70 inverted microscope.

4.9.1 Nissl staining

Coronal sections from approximately bregma -3.8 were placed in 10% cresyl violet (Nissl) solution for 6 minutes at 40°C before dehydrating in increasing concentrations of ethanol (one change each of 70, 95, and 100%) and two changes of xylene. Slides were mounted with DePeX mounting medium.

The Nissl stained sections were imaged using light microscopy at 400X magnification to assess the number of viable appearing CA1, CA2, and CA3 pyramidal hippocampal neurons. Pyramidal neurons with an intact cell body and the presence of an obvious nucleus and/or nucleolus were counted along the entire length of the CA1, CA2, and CA3
hippocampal regions using the Image J counter (v1.51j8; National Institutes of Health, USA).

4.9.2 Bielschowsky’s silver staining

Coronal sections from approximately bregma -4.5 were used for Bielschowsky’s silver staining. Following rehydration, slides were treated with a 10% AgNO₃ solution for 8 – 10 minutes at 40°C, followed by ammoniacal silver (1 drop NH₄OH in 10% AgNO₃) for 18 minutes, before reducing (50 mL developer stock solution: 0.25 g sodium citrate, 2 drops concentrated HNO₃, 10 mL 37 – 40% formaldehyde; 50 mL developer working solution: 8 drops each of concentrated NH₄OH and developer stock solution) to a visible metallic silver. Slides were dehydrated in one change each of 95 and 100% ethanol, two changes of xylene, and mounted with DePeX mounting medium.

Silver stained sections were imaged using light microscopy at 200X to determine the extent of axonal injury in the corpus callosum (Figure 4.4a). The severity of axonal injury was semi-quantitatively assessed in three consecutive sections to obtain an overall grade, ranging from 0 (indicating absence of injury) to 4, where axons displayed disorganised architecture, undulation, varicosities, and disordered orientation of oligodendrocyte nuclei (Figure 4.4b). A more detailed description of the grading criteria can be found in Chapters 5 and 7.
Figure 4.4 Bielschowsky’s silver staining and axonal injury grading of rat brain. (a) Area of the corpus callosum used to semi-quantitatively determine the severity of axonal injury in rat brains. (b) The grading scale used to assess axonal injury, with representative images of silver-stained corpus callosum arranged in increasing severity (L–R) from grade 0–4.

4.10 Statistical analysis

All statistical analyses were carried out using R 3.4.2 and presented as mean ± standard error of the mean (SEM) using the ggplot2 package (Wickham, 2009), unless otherwise specified within chapters. Group comparisons were analysed by an analysis of variance (ANOVA), followed by a pairwise-t-test without adjustments for p-value. Animal mortality was analysed using Chi-square test. Axonal injury grading was analysed by a Kruskal-Wallis non-parametric test. For statistical purposes, peptide treatment groups were compared to the vehicle treatment group. A value of p < 0.05 was considered statistically significant for all datasets.
4.11 References


Chapter 5

Assessment of R18, COG1410, and APP96-110 in excitotoxicity and traumatic brain injury.

Published: Translational Neuroscience
ASSESSMENT OF R18, COG1410, AND APP96-110 IN EXCITOTOXICITY AND TRAUMATIC BRAIN INJURY

Abstract
Cationic arginine-rich and poly-arginine peptides (referred to as CARPs) have potent neuroprotective properties in an in vitro glutamic acid excitotoxicity neuronal injury model [3–6], as well as in vivo after both permanent and transient middle cerebral artery occlusion (MCAO) stroke in rats [4,7–10]. Furthermore, increasing arginine content and positive charge are critical for peptide neuroprotective potency (Meloni et al., 2015a), with poly-arginine peptide R18 identified as our lead peptide (18-mer of arginine; Table 1). The COG1410 and APP96-110 peptides are derived from the apolipoprotein E and amyloid precursor proteins, respectively, and have been demonstrated to improve outcomes in several acute brain injury models, including TBI [11–18]. Interestingly, both COG1410 and APP96-110 are also cationic and arginine-rich (Table 1).

In view of the involvement of excitotoxicity in secondary central nervous system injury cascades, and the pathophysiological parallels between stroke and TBI, it is likely that CARPs could also have beneficial effects following TBI [19,20]. Therefore, we assessed our lead peptide, R18, along with the COG1410 and APP96-110 peptides in an in vitro glutamic acid neuronal excitotoxicity injury model and an in vivo impact-acceleration TBI model.

Introduction
Neuroprotective pharmacological agents aimed at minimising harm to the brain and improving patient outcomes after a traumatic brain injury (TBI) are currently lacking. As such, TBI places a massive burden on society and the economy, a situation further compounded by its rising incidence [1]. Current surgical and rehabilitative interventions are limited in their ability to improve outcomes caused by TBI, and a large proportion of survivors endure debilitating neurological deficits as a consequence [2]. With limited efficacious treatment options, the development of an effective neuroprotective agent would be of great clinical significance in terms of reducing the impact of TBI on patients and the wider community.

Our laboratory has demonstrated that cationic arginine-rich peptides including poly-arginine peptides (hereafter referred to as CARPs) have potent neuroprotective properties in vivo after both permanent and transient middle cerebral artery occlusion (MCAO) stroke in rats [4,7–10]. Furthermore, increasing arginine content and positive charge are critical for peptide neuroprotective potency (Meloni et al., 2015a), with poly-arginine peptide R18 identified as our lead peptide (18-mer of arginine; Table 1). The COG1410 and APP96-110 peptides are derived from the apolipoprotein E and amyloid precursor proteins, respectively, and have been demonstrated to improve outcomes in several acute brain injury models, including TBI [11–18]. Interestingly, both COG1410 and APP96-110 are also cationic and arginine-rich (Table 1).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence*</th>
<th>Net charge at pH 7</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>R18</td>
<td>H-RRRRRRRRRRRRRRRR-OH</td>
<td>+18</td>
<td>98%</td>
</tr>
<tr>
<td>APP96-110</td>
<td>Ac-NWCKRGKQCKTPH-NH2</td>
<td>+5</td>
<td>98%</td>
</tr>
<tr>
<td>COG1410</td>
<td>Ac-AS-Aib-LRKL-Aib-KRLL-NH2</td>
<td>+3</td>
<td>98%</td>
</tr>
</tbody>
</table>

* = Aib = 2-Aminoisobutyric acid or 2-methylalanine

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**Methods**

**Peptides used in this study**
Details of the peptides used in this study are provided in Table 1. Peptides for this study were synthesised by Mimotopes (Australia) and purified by high performance liquid chromatography. For both *in vitro* and *in vivo* studies, peptides were respectively prepared as a 500 μM stock solution in water for irrigation (Baxter, Australia), and in 0.9% sodium chloride for injection (Pfizer, Australia). Reconstituted peptides were stored at -20°C until use.

**Primary cortical neuronal cultures**
The isolation and culturing of primary cortical neurons was undertaken as previously described [3]. Briefly, cortical tissue extracted from E18-19 Sprague-Dawley rats were dissociated in Dulbecco's modified Eagle medium (DMEM; Life Technologies, Australia) supplemented with 1.3 mM L-cysteine, 0.9 mM NaHCO₃, 10 U/mL papain (Sigma Aldrich, USA), 50 U/mL DNase (Sigma Aldrich), and washed in cold DMEM/10% horse serum. Neurons were resuspended in Neurobasal media (Life Technologies, Australia) containing 2% B27 supplement (B27; Life Technologies) and glutamine (0.5 mM), penicillin G (0.1 mg/mL) and streptomycin (0.06 mg/mL). Neurons were seeded into 96-well plastic plates (Nunc, Australia) or 96-well glass wells (7 mm diameter, ProTech, Australia) pre-coated with poly-D-lysine (70 – 150 kDa; Sigma-Aldrich) and maintained in a CO₂ incubator (5% CO₂, 95% air balance, 98% humidity) at 37°C until use on days in vitro 10 to 14. On day in vitro four the mitotic inhibitor cytosine β-D-arabinofuranoside (1 μM; Sigma-Aldrich) was added to the cultures to inhibit the proliferation of non-neuronal cells. Under these conditions, cultures routinely consist of > 95% neurons and 1 - 3% astrocytes.

**Glutamic acid excitotoxicity model and peptide treatments**
The glutamic acid model is routinely performed on cortical neurons maintained in 96-well plastic culture plates [3,4]. Peptides were added to culture wells 10 minutes prior to glutamic acid (50% glutamic acid; Sigma Aldrich) exposure by removing media and adding 50 μL of MEM (Minimal Essential Medium; Life Technologies; Cat. No.: 11090. Supplemented with glutamine, penicillin G and streptomycin as above)/2% B27 containing the specific peptide To induce excitotoxicity, 50 μL of MEM/2% B27 containing glutamate (200 μM; final concentration 100 μM) was added to the culture wells and incubated at 37°C in the CO₂ incubator for 5 minutes (note: peptide concentration reduced by half during this step). Media in wells was then replaced with 100 μL of MEM/2% B27 and cultures incubated for a further 20 - 24 hours at 37°C in the CO₂ incubator. For all experiments, untreated controls with or without glutamic acid treatment underwent the same incubation steps and media additions. In this model, neuronal survival following glutamic acid treatment typically ranges from 2 - 10% (i.e. 90 - 98% cell death).

**Neuronal viability assessment**
Cell viability was examined with light microscopy to qualitatively assess morphological cell death at 30 - 40 minutes and 24 hours after glutamic acid exposure. The MTS colorimetric viability assay (Promega, Australia) was used to quantitatively assess cell viability 24 hours after glutamic acid exposure. The MTS assay quantifies the ability of cytosolic enzymes in viable cells to reduce the tetrazolium salt (MTS; 4,5-dimethylimidazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) to the water-soluble brown formazan salt, which is detected spectrophotometrically at 490 nm.

**Intracellular calcium kinetics**
Intracellular calcium influx was monitored in neuronal culture wells (glass wells) using Fura-2 AM (5 μM; Sigma Aldrich, Australia) in real time using a fluorescent plate reader, as previously described [5]. The aim of these experiments was to determine the relative change in intracellular calcium before and after glutamic acid exposure. Briefly, cells were loaded with the fluorescent calcium ion indicator Fura-2 AM in 50 μL MEM/2% B27, 0.1% pluronic F-127 (Sigma-Aldrich), for 20 minutes at 37°C in the CO₂ incubator. Fura-2 AM solution was removed from wells, replaced with 50 μL MEM/2% B27 containing peptide (1 or 5 μM) or NMDA/AMPA receptor blockers (MK801/CNQX; 5 μM/5 μM; Tocris Bioscience, UK) and incubated for 10 minutes at 37°C in the CO₂ incubator. Control cultures received 50 μL of MEM/2% B27 only. After the 10-minute incubation period, media in wells was replaced with 50 μL of balanced salt solution (mM: 116 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.8 MgSO₄, 1 NaH₂PO₄; pH 7.2) and wells were transferred to a spectrophotometer (CLARIOstar, BMG Labtec, Australia) while maintaining temperature at 37°C. Starting at 30 seconds prior to glutamic acid addition (50 μL of 200 μM in MEM/2% B27; final concentration 100 μM) to wells, spectrophotometer measurements (excitation 355 nm/emission 495 nm) were recorded every 5 seconds until 2 minutes after glutamic acid addition. Control wells did not receive glutamic acid treatment. Experiments were performed in triplicate. For fluorescent kinetic tracers, data was converted to reflect proportional neuronal calcium influx relative to both control (no glutamic acid) and glutamic acid treated control, with no glutamic acid control taken as 0% calcium influx (baseline) and glutamic acid control treated as 100% calcium influx.

**Traumatic brain injury model and peptide administration**
This study was approved by the Animal Ethics Committee of the University of Western Australia and follows the guidelines outlined in the “Australian Code for the Care and Use of Animals for Scientific Purposes”. Male Sprague-Dawley rats weighing 360 – 400 g were housed under controlled conditions with a 12-hour light-dark cycle and free access to food and water *ad libitum* before and after surgery. A total of 36 animals underwent the procedure, but only 27 survived to the four-day end-point (25% mortality). Nine peptide-treated animals either died unexpectedly, or were euthanased for welfare reasons as per the animal ethics guidelines such as excessive weight-loss (>15%), or persistent respiratory distress (see Table 2 for details). Peptide-treatment groups consisted of 4 – 5 animals, while sham and vehicle treatment groups consisted of 5 and 8 animals, respectively.

The Marmarou weight-drop impact-acceleration injury model [21] was adapted for
To avoid hypothermia, rat cages were placed on a heating mat during post-surgical monitoring.

Post-surgical animal care and monitoring

At the conclusion of surgery, pethidine (IM: 1 mg in 0.2 mL saline) and bupivacaine were administered (SC: 0.1 mg in 0.2 mL saline per site) to the head surgical wound. A 2 mL volume of injectable saline was also subcutaneously administered to aid hydration. To avoid hypothermia, rat cages were placed on a heating mat during post-surgical monitoring and housed in a holding room maintained at 26 - 28°C. Post-surgery, animals were monitored twice a day. Pethidine (SC: 1 mg in 0.2 mL saline) was administered if the animal’s behaviour was indicative of discomfort, and saline (SC: 2 mL) if animal weight continued to decline. Rats were also provided with sweetened nourishments to encourage food intake.

Functional assessments

Due to the exploratory nature of the study, three behavioural tests (Barnes maze, adhesive tape removal, and rotarod) were assessed to measure potential injury deficits. These assessments were carried out daily, starting at 24 hours after surgery and continued until study end-point (4 days post-TBI). Each animal was given three attempts at each test, and the first attempt was used for statistical analyses.

The Barnes maze is a well-characterised spatial learning and memory test [22] that utilises a rat’s innate need to escape brightly-lit areas. Animals are required to find the location of an escape hole from 20 other holes set in a round table top within 180 seconds. During the test, a bright light is placed above the table to serve as a mild aversion stimulus. The adhesive tape test assesses sensorimotor function [23], and has been previously used to detect deficits in a similar model of TBI [24]. Alternating between right and left forepaws, a 10 mm x 10 mm piece of adhesive tape (Diversified Biotech, USA) is placed on the palmer surface of the forepaw, and the time taken to remove the tape recorded; animals were allowed 180 seconds to remove the tape. The rotarod test is commonly used in experimental TBI [25], and assesses vestibulomotor deficits. The test involves placing the animal on a rotating rod (Model number: MK-630B; Muromachi, Japan) at a speed of four revolutions per minute (rpm), increasing incrementally to 40 rpm; the time that the animal remained on the rod is recorded (maximum time allowable was 180 s).

Histological assessment for axonal injury and hippocampal neuronal loss

Rats were euthanased 4 days post-TBI with pentobarbital (IP: 100 mg/kg) and transcardially perfused with 200 mL of saline followed by 200 mL of 10% neutral buffered formalin. Brains were removed and post-fixed in 4% formalin for 1 week before embedding in paraffin. Brains were sectioned in 10 µm coronal sections at approximately bregma -4.5 for Bielschowsky’s silver staining, and bregma -3.8 for Nissl staining. Stained sections were imaged using light microscopy to assess axonal injury within the corpus callosum and neuronal loss in the cornu ammonis (CA) of the hippocampus. The severity of axonal injury was semi-quantitatively assessed in three consecutive sections to obtain an overall grade, ranging from 0 (indicating absence of injury) to 4, with increasing grade indicating increasing levels of axons displaying disorganised architecture, undulation and varicosities, and disordered orientation of oligodendrocyte nuclei. To determine the number of surviving hippocampal neurons, CA1/CA2 and CA3 pyramidal neurons with an intact cell body and the presence of an obvious nucleus and/or nucleolus at 400X magnification were counted in the entire length of both hippocampi using Image J counter (v1.51j8; National Institutes of Health, USA). Light microscopic images were captured using an Olympus DP-70 digital camera fitted to an Olympus IX70 inverted microscope.

Statistical analysis

All statistical analyses were carried out using SPSS (v.24; IBM, USA) and presented as mean ± standard error of the mean (SEM). Cell viability, calcium kinetics measurements, and CA counts were analysed by an analysis of variance (ANOVA), followed by a Fisher
Results

Effect of peptides on cultured neurons exposed to glutamic acid excitotoxicity
The neuroprotective dose response efficacy of R18, COG1410, and APP96-110 peptides was compared in cortical neuronal cultures exposed to glutamic acid excitotoxicity (Figure 1). As previously reported [5], R18 was highly neuroprotective following glutamic acid excitotoxicity, achieving a 98% protective effect at the 1 μM dose. In contrast, COG1410 and APP96-110 provided only modest neuroprotection following glutamic acid excitotoxicity, achieving approximately 10 - 15% protection at the 1 and 10 μM dose, respectively.

Effect of peptides on neuronal intracellular calcium kinetics following glutamic acid excitotoxicity
The ability of R18, COG1410, and APP96-110 peptides at concentrations of 1 and 5 μM to reduce neuronal intracellular calcium influx following exposure to glutamic acid was assessed using a Fura-2 AM calcium kinetics assay (Figure 2a - d). At 1 and 5 μM, R18 significantly reduced neuronal intracellular calcium influx (Figure 2a, d). Comparatively, APP96-110 significantly reduced neuronal calcium influx at 5 μM, but not at 1 μM, while COG1410 displayed a non-significant reduction in calcium influx at 5 μM.

Animal survival rate and weight loss following TBI
All sham and vehicle-treated animals survived to end of experiment day 4. In contrast, 2 of 7, 3 of 9, and 4 of 9 animals in the R18 (P = 0.83), COG1410 (P = 0.28) and APP96-110 (P = 0.18) treatment groups respectively, did not survive to day 4. Details regarding animal deaths are summarised in Table 2. No significant differences in weight loss were observed between peptide and vehicle treatment groups (data not shown).

Effect of peptides on functional outcomes following TBI
When administered 30 minutes after injury, R18, COG1410, and APP96-110 peptide treatments did not result in any significant improvements in functional outcomes when compared to vehicle (Figures 3 - 5). Although not statistically significant, R18 treatment did improve functional measurements in the adhesive tape (days 1 – 4; Figure 3a - d) and rotarod (days 3 and 4; Figure 4c, d) tests. Similarly, the COG1410 treatment group displayed an improvement in the rotarod test (day 4), but not to a statistically significant level. In contrast, APP96-110 treatment appeared to worsen Barnes maze performance (days 2 - 4; Figure 5b - d).

Effect of peptides on histological outcomes following TBI
Severity of axonal injury was significantly reduced in the R18 (P = 0.02) and COG1410 (P = 0.05) treatment groups, while APP96-110 had no effect on axonal injury (Figure 6). In the absence of injury, axons within the corpus callosum appeared smooth and well-organised, with oligodendrocyte nuclei arranged parallel to axons (Figure 7a). Following TBI, the architecture of callosal axons showed varying degrees of disorganisation and undulation and at times displayed varicosities, with loss of the normal longitudinal orientation of oligodendrocyte nuclei (Figure 7b - e). Hippocampal CA neuronal loss was increased in vehicle-treated animals compared to sham animals, but not to a statistically significant level (Figure 8). Similarly, no significant differences were observed in CA1/CA2 and CA3 hippocampal counts between peptide and vehicle treatment groups (Figure 8a, b).

Discussion
Following previous studies in our laboratory demonstrating the neuroprotective efficacy of CARPs, including our lead peptide R18 in in vivo models of stroke [3–5,8–10], the present study set out to determine if R18 could exert beneficial effects in an in vivo model of TBI. Two other peptides, COG1410 and APP96-110, which have previously demonstrated positive effects in in vivo TBI models were also included for comparative purposes.

An initial in vitro glutamic acid neuronal excitotoxicity study demonstrated R18 to be...
more neuroprotective and reduced neuronal intracellular calcium influx to a greater degree than both COG1410 and APP96-110. While modest, the ability of COG1410 and APP96-110 to reduce excitotoxic neuronal calcium influx is of interest, as these two peptides were not designed based on this specific mechanism of action. The modest inhibitory action of COG1410 and APP96-110 on calcium influx is likely due to their low cationic charge and the presence of only two arginine residues. We have previously demonstrated that CARPs can reduce excitotoxic calcium influx [4,7,26], and that calcium influx inhibition is influenced by peptide cationic charge and arginine content [5,7].

In the *in vivo* study, administration of R18, COG1410 and APP96-110 after TBI did not appear to have any significant effects in reducing CA neuronal loss, however CA injury was modest, with vehicle-treated and sham animals not recording significantly different CA

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**Figure 2.** Intracellular calcium assessment using Fura-2 AM after glutamic acid exposure in cortical neuronal cultures. a – c: Fluorescent tracers; fluorescence intensity (FI) of neuronal cultures 30 s before and after the addition (arrow) of glutamic acid (100 μM final concentration) expressed as a percentage of intracellular neuronal calcium influx. Peptides (1 and 5 μM) and NMDA/AMPA receptor blockers (MK801/CNQX; 5 μM/5 μM) were added to neuronal cultures for 10 min and removed (time = 0) before glutamic acid addition. Glutamic acid control received glutamic acid exposure only. Control did not receive peptide or glutamic acid treatment. d: Neuronal calcium influx expressed as trapezoidal area under the curve (AUC). Data are presented as mean ± SEM; N = 3. * P < 0.05 when compared to glutamic acid control.
Figure 3. Adhesive tape test measurements at days 1-4 (a-d) post-TBI. Peptide dose 300 nmol/kg. Sham (N = 5), vehicle (N = 8), APP96-110 (N = 5), COG1410 (N = 4), and R18 (N = 5). Data are presented as mean ± SEM. There were no significant differences between vehicle and peptide treatment groups.

Figure 4. Rotarod measurements at days 1-4 (a-d) post-TBI. Peptide dose 300 nmol/kg. Sham (N = 5), vehicle (N = 8), APP96-110 (N = 5), COG1410 (N = 4), and R18 (N = 5). Data are presented as mean ± SEM. There were no significant differences between vehicle and peptide treatment groups.
neuronal cell counts. In contrast to the results obtained for hippocampal CA injury, R18 and COG1410 significantly reduced axonal injury following TBI, while APP96-110 was ineffective. Diffuse axonal injury is a feature common across all severities of traumatic brain injury [27] and is caused by both mechanical and chemical disturbances [28–31]. The mechanical forces produce axonal stretching, while chemical disruptions can induce glutamate excitotoxicity, both of which lead to an increase in neuronal intracellular calcium influx. Furthermore, the toxic increase in neuronal intracellular calcium can subsequently increase mitochondrial calcium levels with detrimental effects on the organelle, which ultimately lead to cell death [31,32]. Consequently, given the capacity of R18 and COG1410 to reduce excitotoxicity-induced neuronal intracellular calcium, in addition to the known ability of CARPs to target and exert positive effects on mitochondria [20,33,34], this could explain the observed positive effects on axonal injury. Furthermore, the reduced axonal injury in R18 and COG1410 treatment groups could explain the trends in improved functional outcomes for these treatments.

The peptides did not have any significant effect on functional outcomes following TBI, although some positive trends were observed for R18 and COG1410 in the adhesive tape and/or rotarod tests. The lack of significant functional improvements most likely reflects the low number of animals used and/or the high variability associated with functional outcomes after TBI. While the small sample size is a limitation of the study, additional

**Figure 6.** Barnes maze measurements at days 1 - 4 (a - d) post-TBI. Peptide dose 300 nmol/kg. Sham (N = 5), vehicle (N = 8), APP96-110 (N = 5), COG1410 (N = 4), and R18 (N = 5). Data are presented as mean ± SEM. There were no significant differences between vehicle and peptide treatment groups.

**Figure 7.** Axonal injury at day 4 post-TBI. Axonal injury grade of sham (N = 5), vehicle (N = 8), APP96-110 (N = 5), COG1410 (N = 4), and R18 (N = 5) treatment groups. Peptide dose 300 nmol/kg. Data are presented as box plots showing median and grade distribution; *P < .05 when compared to vehicle treatment group.
animals were not recruited to the study for several reasons: i) the study was considered largely exploratory to establish experimental procedures and uncover any initial evidence of functional and/or histological treatment effects; and ii) on animal welfare grounds due to the unexpectedly high mortality rate experienced in the peptide treatment groups, particularly COG1410 and APP96-110.

Both COG1410 and APP96-110 peptides have previously demonstrated the capacity to reduce axonal injury and functional outcomes following TBI [12,14]. However, APP96-110 was given intracerebroventricularly rather than intravenously, which may account for its lack of efficacy in the present study, despite reducing in vitro excitotoxicity-induced neuronal intracellular calcium levels. Intracerebroventricular administration of APP96-110 represents a more direct route of delivery into the brain and overcomes many of the systemic pharmacokinetic interactions that would reduce uptake into the brain if administered intravenously such as the blood brain barrier, elimination by the kidney and liver, and degradation by vascular proteases. Similarly, successful outcomes with COG1410 have been achieved with higher doses [11,14,15,35] than used in the present study. The 300 nmol/kg dose was chosen in the present study because R18 is neuroprotective at this dose following MCAO [10], and to increase the possibility of uncovering a differential treatment effect with

\[
\text{Where, a = 0, b = 1, c = 2, d = 3, e = 4}
\]

**Figure 7.** Representative images of axonal injury grading criteria from 0 – 4 (a – e) following Bielschowsky’s silver stain, as indicated in the key. Examples of axons exhibiting undulated appearance and varicosities are designated ‘V’. Areas showing disordered arrangement of oligodendrocyte nuclei are designated ‘N’. Scale bar represents 100 μm.

**Figure 8.** Number of normal appearing CA neurons per millimetre of hippocampus at day 4 post-TBI. a: CA1/CA2; and b: CA3 regions at approximately bregma -3.8. Peptide dose 300 nmol/kg. Data are presented as mean ± SEM. Sham (N = 5), vehicle (N = 8), APP96-110 (N = 5), COG1410 (N = 4), and R18 (N = 5). There were no significant differences between sham, vehicle, and peptide treatment groups.
R18, COG1410, and APP96-110.

Neuroprotection by the APP96-110 peptide is purported to act mainly via its heparin-binding activity [12], however its exact mechanism has not been elucidated. A similar peptide also derived from the amyloid precursor protein suggests that APP96-110 could modulate calcium influx and activate NFκB transcription factors [36]. Similarly, COG1410 may also affect calcium influx [37] through interactions with cell-surface heparan sulfate proteoglycans during internalisation into the cell [38], but it is generally argued that its neuroprotective action is mainly through interactions with the lipoprotein receptor-associated protein [39]. In light of these reports and the findings of the present study, we propose that the neuroprotective and/or calcium influx inhibitory activity of COG1410 and APP96-110 are mediated at least in part by the positive charge and arginine content of the peptides. In support of this, a more recent CARP derived from selected non-sequential amino acids from the same apolipoprotein E region as COG1410 (CN-105; Ac-VSRRR-NH2, charge +3) has demonstrated histological and functional benefits in a closed-head TBI model [40]. In addition, the CN-105 peptide has also demonstrated efficacy in in vivo models of stroke [41] and intracerebral haemorrhage [42]. While the in vivo study uncovered potential therapeutic effects with R18 and COG1410, several difficulties were encountered with the animal functional assessment procedures. With regards to the rotarod assessment, it was observed that with repeated exposure, rats had a tendency to leap off the rotating rod during the test, leading to a shorter recorded latency. This behaviour was observed in both sham and injured rats. It was also observed that in the Barnes maze test, the bright light became a less effective aversion stimulus with rats willing to explore the table rather than enter the escape hole. Considering this, we suggest additional functional tests and/or minimising the exposure of animals to these tests in future studies. For example, as an alternative learning and memory test the Morris water maze may be employed.

In conclusion, this study has demonstrated that the CARP R18 is more effective than either COG1410 or APP96-110 at reducing neuronal death and calcium influx following in vitro excitotoxicity. In addition, R18 showed greater efficacy than APP96-110 in reducing axonal injury and improving some functional outcomes after TBI. Additional dose response and therapeutic time window studies are required to further evaluate the potential of R18 as a neuroprotective therapy for TBI.

Acknowledgements

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Conflicts of interest

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

References


Chapter 6

Poly-arginine peptide R18D reduces neuroinflammation and functional deficits following traumatic brain injury in the Long-Evans rat.

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Poly-arginine Peptide R18D Reduces Neuroinflammation and Functional Deficits Following Traumatic Brain Injury in the Long-Evans Rat

Li Shan Chiu1,2 · Ryan S. Anderton1,2,4,5 · Jane L. Cross1,2,3 · Vince W. Clark1,2,3 · Neville W. Knuckey1,2,3 · Bruno P. Meloni1,2,3

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Abstract
We have previously demonstrated that the poly-arginine peptide R18 can improve histological and functional outcomes following traumatic brain injury (TBI) in the Sprague–Dawley rat. Since D-enantiomer peptides are often exploited in pharmacology for their increased stability and potency, the present study compared the effects of R18 and its D-enantiomer, R18D, following TBI in the Long-Evans rat. Following a closed-head impact delivered via a weight-drop apparatus, peptide was administered at a dose of 1000 nmol/kg at 30 min after TBI. Treatment with R18D, but not R18 resulted in significant reductions in sensorimotor (p = 0.026) and vestibulomotor (p = 0.049) deficits as measured by the adhesive tape removal and rotarod tests. Furthermore, treatment with R18 and R18D resulted in a significant reduction in brain protein levels of the astrocytic marker, glial fibrillary acidic protein (p = 0.019 and 0.048, respectively). These results further highlight the beneficial effects of poly-arginine peptides in TBI, however additional studies are required to confirm these positive effects.

Keywords Neuroprotection · Arginine-rich · Peptide · TBI · Inflammation

Introduction
In the absence of an efficacious neuroprotective therapeutic, traumatic brain injury (TBI) persists as a global burden. Survivors are often plagued with somatic complaints, cognitive dysfunction, and physical impairment for the remainder of their life. TBI also has long-term effects on society and the economy such as low return-to-work rates (Gabbe et al. 2016), considerable financial burden on the health care system, and proclivity for criminal behaviour in both males (Timonen et al. 2002; Schofield et al. 2006; Williams et al. 2010) and females (O’Sullivan et al. 2015). Therefore, a neuroprotective treatment that can minimise injury and improve outcomes after a TBI is urgently needed. The multifaceted pathophysiology of TBI presents significant challenges in developing an effective neuroprotective therapeutic. Thus, developing a multifunctional agent that targets several neuroprotective and/or neurodamaging pathways will likely provide the best translational opportunity for clinical success.

We have recently demonstrated that cationic arginine-rich peptides (CARP), including poly-arginine peptides, are highly neuroprotective in excitotoxic and oxygen glucose deprivation neuronal cell culture models (Meloni et al. 2015a, b, 2017; MacDougall et al. 2016; Chiu et al. 2017a), and in middle cerebral artery occlusion (Milani et al. 2016b, a, 2017, 2018; Meloni et al. 2017), hypoxic-ischemic encephalopathy (HIE) (Edwards et al. 2018), and TBI (Chiu et al. 2017a) animal models. In addition, our laboratory has also demonstrated that CARPs reduce excitotoxic neuronal calcium influx and reduce neuronal surface expression of the NMDA receptor subunit protein.

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Li Shan Chiu
lishan.chiu@research.uwa.edu.au

1 Perron Institute for Neurological and Translational Science, QEII Medical Centre, 8 Verdun St, RR Block, Nedlands, WA 6009, Australia
2 Centre for Neuromuscular and Neurological Disorders, The University of Western Australia, Nedlands, Australia
3 Department of Neurosurgery, Sir Charles Gairdner Hospital, QEII Medical Centre, Nedlands, WA, Australia
4 School of Heath Sciences, The University Notre Dame Australia, Fremantle, WA 6160, Australia
5 Institute for Health Research, The University Notre Dame Australia, Fremantle, WA 6160, Australia
NR2B9c (MacDougall et al. 2016; Chiu et al. 2017a). Other studies have demonstrated that CARPs reduce the activity and/or surface expression of other ion channels and receptors (e.g. NCX, CaV2.2, TNFR) (Fotin-Mleczek et al. 2005; Brittain et al. 2011; Brustovetsky et al. 2014; Meloni et al. 2015a) that exacerbate ionic disturbances or cell death pathways that may be associated with a TBI. Additionally, CARPs have other properties that may be beneficial in terms of neuroprotection such as the ability to target and stabilise mitochondria (Chen et al. 2015), mitigate ROS production (Szeto 2006; Dai et al. 2011; Szeto et al. 2011), activate pro-cell survival signalling (Gu et al. 2013; Yang et al. 2016), inhibit proprotein convertases that activate matrix metallocproteinases (Cameron et al. 2000; Kacprzak et al. 2004; Fugere et al. 2007; Ramos-Molina et al. 2015), and reduce inflammation (Laskowitz et al. 2006; Tu et al. 2017). Furthermore, CARPs have cell penetrating properties which allow them to cross the blood brain barrier and enter the brain.

The use of chiral compounds in pharmacology has long been known to enhance stability and potency of the compound (Roques et al. 1993; Abadji et al. 1994). In the case of peptides, this can result in increased uptake into the brain (Wei et al. 2014). For example, peptides synthesised with D-enantiomer amino acids are more resistant to degradation by peptidases after intravenous administration (Wei et al. 2014). The poly-arginine peptide R18 (18-mer) and its D-enantiomer R18D have been assessed in both neuronal cell culture excitotoxic and in vivo stroke and HIE models in our laboratory (Milani et al. 2018; Edwards et al. 2018). The studies revealed a similar neuroprotective efficacy in the in vitro excitotoxic and animal HIE models, while a single-dose study in a permanent middle cerebral artery occlusion stroke model revealed that R18D reduced infarct volume to a greater extent than R18. However, both peptides reduced hemisphere swelling to a similar degree.

Considering our previous studies assessing R18 and/or R18D in TBI and in stroke-related models this study had several objectives. The first was to examine R18 and R18D efficacy in reducing functional deficits associated with TBI, as functional improvement is the ultimate goal of any neuroprotective therapy. Secondly, we also examined global changes in the inflammatory response following TBI and treatment with R18 and R18D by measuring GFAP, IL-6, TNFα, and IL-1β expression in total protein brain lysates. As only modest effects were observed with 300 nmol/kg R18 in our previous study, we increased the dose to 1000 nmol/kg. Furthermore, neuroprotective effects and injury outcomes can differ in different strains of rats. Given that our previous TBI study employed Sprague–Dawley rats, the present study used Long-Evans rats.

**Methods**

**Peptides Used in this Study**

R18 (H-RRRRRRRRRRRRRRRRRRRRR-OH; net charge +18; R = L-arginine) and R18D peptide (H-rrrrrrrrrrrrrrrrrrrrrr-OH; net charge +18; r = D-arginine) peptides were purified by high performance liquid chromatography to at least 98% and subjected to peptide hydrolysis and liquid chromatography-mass spectrometry to obtain a precise measure of peptide content (Mimotopes). Lyophilised peptide stocks were resuspended in 0.9% sodium chloride for injection (Pfizer) and 650 µL was aliquoted into 3 mL syringes. Reconstituted peptides were stored at −20 °C until use.

**Traumatic Brain Injury Model and Peptide Administration**

This study was approved by the Animal Ethics Committee of the University of Western Australia and follows the guidelines outlined in the “Australian Code for the Care and Use of Animals for Scientific Purposes”. Male Long-Evans rats weighing 375–435 g were housed in pairs under controlled conditions with a 12-h light–dark cycle and free access to food and water ad libitum before and after surgery. A total of 40 animals underwent the TBI procedure. Two animals, one R18- and one R18D- treated animal, experienced breathing difficulties during surgical recovery within an hour of removal from ventilation and thus were euthanased. Two separate studies examining either R18 or R18D efficacy were performed. The R18 study consisted of 6 peptide, 8 vehicle, and 5 sham treated animals. The R18D study consisted of 7 peptide, 9 vehicle, and 5 sham treated animals.

A moderate-severe, diffuse TBI was induced as previously described (Chiu et al. 2017a) using a well-characterised weight-drop impact-acceleration model (Foda and Marmarou 1994; Marmarou et al. 1994). Briefly, animals underwent anaesthetic induction with 5% halothane (mix 30% O₂/70% N₂O gas), intubation, and were maintained using 1–2% halothane. TBI was induced by releasing a 435 g brass weight from a 180 cm height, onto a steel disc (1 cm diameter and 2 mm thick) adhered to the skull of the animal with cyanoacrylate. At 30 min post-impact, treatments were administered intravenously (600 µL over 6 min) through the right internal jugular vein using an infusion pump. Treatments consisted of the vehicle control (0.9% NaCl for injection), and R18 or R18D at 1000 nmol/kg. Sham animals underwent the same surgical procedure and received vehicle treatment but were not subjected to TBI. All personnel carrying-out treatment administration and animal procedures were blinded to treatment status.
Post-surgical Animal Care and Monitoring

At the end of surgery, pethidine (IM: 1 mg in 0.2 mL saline) and bupivacaine were administered (SC: 0.1 mg in 0.2 mL saline per site) to the head surgical wound. A 2 mL volume of injectable saline was also administered subcutaneously to aid hydration. Cages were placed on a heating mat during a 1 to 2 h post-surgical monitoring period, and subsequently housed in a holding room maintained at 26–28 °C. Animals were monitored at least twice a day and provided with sweetened cereal and gel packs to encourage food intake.

Functional Assessments

The functional assessments were used to identify deficits in spatial learning and memory (Barnes maze), sensorimotor (adhesive tape removal), and vestibulomotor (rotarod) function as previously described (Chiu et al. 2017a). All three assessments were carried out in the order described above on the day prior to surgery (day 1; baseline), and days 2 and 4 post-TBI. Each animal had three attempts and a maximum of 180 s to complete the Barnes maze and adhesive tape removal tests, while no time limit was set for the rotarod test. Functional recovery of each animal was determined by comparing the post-surgery latency for days 2 and 4 post-TBI for each test to its own pre-surgery (day 1; baseline) latency and expressed as percentage change from baseline. An animal recording a positive or a negative value on days 2 or 4 post-TBI indicated an improvement or decline in functional recovery, respectively.

Protein Extraction and Western Blotting

After the completion of functional measurements on day 4 post-TBI, animals were administered a lethal injection of pentobarbitone (325 mg/mL; Virbac), and transcardially perfused with ice-cold 0.9% saline. The brain was extracted, and total protein isolated from fresh frozen brain tissue using RIPA lysis buffer (10 mM Tris–HCl, pH 8.0; 1 mM EDTA; 1% Triton X-100; 0.1% sodium deoxycholate; 0.1% SDS; 140 mM NaCl; 1 mM PMSF) containing protease inhibitor (one tablet in 10 mL; Roche). Protein lysates were centrifuged to collect supernatant which was subsequently stored at −80 °C. Representative protein samples from each treatment group were then probed via Western blot. After the completion of functional measurements on day 4 post-TBI, animals were administered a lethal injection of pentobarbitone (325 mg/mL; Virbac), and transcardially perfused with ice-cold 0.9% saline. The brain was extracted, and total protein isolated from fresh frozen brain tissue using RIPA lysis buffer (10 mM Tris–HCl, pH 8.0; 1 mM EDTA; 1% Triton X-100; 0.1% sodium deoxycholate; 0.1% SDS; 140 mM NaCl; 1 mM PMSF) containing protease inhibitor (one tablet in 10 mL; Roche). Protein lysates were centrifuged to collect supernatant which was subsequently stored at −80 °C. Representative protein samples from each treatment group were then probed via Western blot.

Western blotting was performed as previously described (MacDougall et al. 2016). Briefly, proteins were separated by SDS-PAGE, transferred to PVDF membranes, and blocked in PBS-Tween 20 (0.1%) containing 1 mg/mL ovalbumin for 1 h. Membranes were then incubated overnight at 4 °C in GFAP (1:1000; Sigma-Aldrich), IL-6 (1:1000; Invitrogen), TNFα (1:1000; Invitrogen), IL-1β (1:1000; Bio-Rad), or β-tubulin (1:3000; Invitrogen) primary antibodies prepared in PBS-Tween 20 (0.1%) with 1 mg/mL ovalbumin. Protein was detected by incubating for at least an hour at room temperature in goat anti-mouse DyLight 800 (1:10,000; Bio-Rad) or goat anti-rabbit StarBright 700 (1:3000; Bio-Rad) with added hFab-Rhodamine (β-tubulin 1:10,000; Bio-Rad) as the loading control. For β-tubulin, a HRP-conjugated goat anti-mouse secondary antibody (1:20,000; Bio-Rad) was used. Blots were visualised using the ChemiDoc system (Bio-Rad). Quantification and band densitometry of Western blots was performed using ImageJ software (v1.51j8; National Institutes of Health, USA).

Statistical Analysis

All statistical analyses of functional assessments and densitometry were conducted in R 3.5.1 and presented as mean ± standard error of the mean (SEM). An analysis of variance (ANOVA) was performed, followed by a Fisher’s LSD post-hoc test. For statistical analysis of functional outcome and western blots, peptide treatment groups were compared to the vehicle treatment group. A value of p<0.05 was considered statistically significant for all data sets.

Results

Functional Assessments

The adhesive tape and rotarod tests demonstrated significant sensorimotor and vestibulomotor deficits in the TBI injured animal groups (Figs. 1, 2). In contrast, sham animals not subjected to TBI did not show deficits across any of the functional assessments (Figs. 1, 2, 3).

For the adhesive tape test, at day 2 post-TBI, R18D-treated animals performed significantly better (p=0.026) than vehicle-treated animals (Fig. 1a), while there was no difference in performance between R18 and vehicle treated animals (Fig. 1b). At day 4 post-TBI, R18D-treated animals demonstrated an improvement in performance from baseline, however this was not significantly different in performance from vehicle-treated animals, which remained below baseline (Fig. 1c). Additionally, R18-treated animals demonstrated an improvement in performance approaching baseline, however this was not significantly different from vehicle treated animals, which remained below baseline (Fig. 1d).

For the rotarod test, at day 2 post-TBI, R18D-treated animals performed significantly better (p=0.049) than vehicle-treated animals (Fig. 2a), while there was no difference in performance between R18 and vehicle treated animals (Fig. 2b). At day 4 post-TBI, R18D-treated animals demonstrated an improvement in performance compared to vehicle treated animals, albeit not to a statistically significantly
level (Fig. 2c), while there was no difference in performance between R18 and vehicle treated animals (Fig. 2d).

At days 2 and 4 post-TBI, Barnes maze performance remained above baseline and did not differ significantly between peptide and vehicle treated and sham animals (Fig. 3a–d).

Western Blot Analysis for Neuroinflammatory Response

Western blot analysis of brain lysates collected on day 4 post-TBI, demonstrated that treatment with R18D (p = 0.048) and R18 (p = 0.019) resulted in significantly reduced GFAP expression by 52% and 67% respectively, compared to treatment with vehicle (Fig. 4a). However, neither peptide was significantly better than the other (p = 0.50) at reducing GFAP expression. Treatment with R18D (p = 0.11) and R18 (p = 0.37) also reduced cytokine IL-6 protein levels by 44% and 23% respectively in the brain, albeit not to a statistically significant level (Fig. 4b). Contrastingly, R18 (p = 0.062) treatment increased TNFα (Fig. 4c), and IL-1β increased in both R18 (p = 0.59) and R18D (p = 0.19) treatment groups (Fig. 4d), however none were significant when compared to vehicle.
Discussion

The present study has demonstrated that treatment with R18D reduced TBI-associated sensory and vestibular motor deficits in the Long-Evans rat. Moreover, both R18 and R18D reduced TBI-associated reactive astrocytic cellular (GFAP) responses. These findings are consistent with our proposed application of CARPs as a therapeutic in TBI (Chiu et al. 2017b), and previous data from our laboratory which has demonstrated that treatment of Sprague–Dawley rats with R18 following a TBI reduced the extent of axonal injury and provided positive trends for functional recovery (Chiu et al. 2017a). While the present study has confirmed that both the L- and D-isooform R18 peptides can provide beneficial outcomes following TBI, the more favourable effects observed following R18D treatment could be related to the superior stability of the D-enantiomer peptides. Interestingly, the Barnes maze test did not demonstrate any obvious deficits in performance following TBI, which suggests that it may not be sufficiently sensitive for use following this TBI model in the Long-Evans rat.

Increased levels of GFAP within the brain is associated with the proliferation of astrocytes and gliosis, which play a key role regulating neuroinflammation, and can have both
detrimental and regenerative functions following brain tissue injury. Elevated GFAP expression following TBI is a commonly used biomarker of astrocytic activation and neuronal degeneration (Huang et al. 2015), and correlates with TBI severity (Hsieh et al. 2017). Therefore, measurement of GFAP is a good pre-clinical biomarker for the assessment of potential neuroprotective treatments in TBI. In this regard, the present study has for the first time demonstrated that R18 and R18D can reduce GFAP levels in the brain following TBI. In this regard, the present study has for the first time demonstrated that R18 and R18D can reduce GFAP levels in the brain following TBI, which is in line with previous studies demonstrating that other CARPs such as COG1410 (AS[Aib]LRKL[Aib] KRLL; net charge +4) (Hoane et al. 2007) and APP96-110 (NWCKRGRQCKTHPH; net charge +5) (Plummer et al. 2018) also reduced GFAP expression following brain injury. The exact mechanism whereby CARPs such as R18 and R18D reduce GFAP expression is not known but may be due to the neuroprotective actions of the peptide limiting neural injury directly, or by inhibiting pathways responsible for astrocyte activation and inflammation.

The suggestion that R18 and R18D could reduce levels of the pro-inflammatory cytokine IL-6 in the brain of rats following TBI is of potential interest. IL-6 expression increases in rodent brain (Taupin et al. 1993; Shohami et al. 1994) and in human serum soon after TBI (Yousefzadeh-Chabok

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et al. 2015; Gill et al. 2017), with increased levels associated with worse outcomes. Clinically, serum IL-6 has been implicated in elevated intracranial pressure following TBI (Hergenroeder et al. 2010), however the exact role of elevated brain levels of the cytokine with respect to brain injury is poorly understood (Chakraborty et al. 2016). Other CARPs administered following neurotrauma such as COG133 (Ac-LRVRASLRLRKLRL-NH₂, net charge +7.1), CN-105 (Ac-LSRRNH₂, net charge +3), and PACAP (Ac-HSDGIFTJEIYKQRKQRKQKRNKH₂, net charge +10.1) have demonstrated downregulation of IL-6 mRNA (Laskowitz et al. 2017) and/or expression of other inflammatory cytokines (Lynch et al. 2003; Mao et al. 2012). In addition, CARPs can reduce the release of IL-6 from C2C12 myotubes exposed to TNF-α (Lightfoot et al. 2015), synovial fluid mononuclear cells of rheumatoid arthritis patients exposed to VEGF (Yoo et al. 2005), dendritic cells stimulated with toll-like receptor ligands (Kandler et al. 2006), and reduce serum levels in collagen-induced arthritic mice (Yoo et al. 2005).

Both TNFα and IL-1β were elevated in the brain following treatment with R18 and/or R18D, but not to statistically significant levels. Although there is still uncertainty surrounding the exact role of the neuroinflammatory response after injury, there are suggestions that cytokines may have both deleterious and beneficial effects at different phases of secondary injury (Shohami et al. 1999; Ziebell and Morganti-Kossmann 2010). Given that sensorimotor and vestibulomotor deficits were significantly reduced, but profound changes were not observed in IL-6, TNFα, or IL-1β may be attributable to their dual role in the inflammatory response. Similarly, CN-105 demonstrated neuroprotective properties in TBI, despite not reducing mRNA levels of IL-6 and TNFα (Laskowitz et al. 2017).

There are several mechanisms whereby CARPs may suppress the acute inflammatory response following a
TBI. For example, CARPs can inhibit the TLR4/MyD88/NF-κβ signalling pathway (Mao et al. 2012) through specific inhibition of NF-κβ. In support of this, the CARP AIP6 (RLRWR, net charge +3) can block binding of the NF-κβ p65 sub-unit to DNA and inhibit the transcription of pro-inflammatory genes (Wang et al. 2011). Furthermore, proteasomal activity increases following TBI, and CARPs such as R8 (RRRRRRRR, net charge +8) (Kloß et al. 2009), PR39 (RRRRPPYLPYPRPFFPFPRPLP- PRLPPFPFRPPFRP, net charge +10) (Gao et al. 2000), and PR11 (RRRRPPYLPYPR, net charge +5) (Gaczynska et al. 2003; Anbanandam et al. 2008) are potent inhibitors of the proteasome, an effect that would inhibit proteasomal degradation of the NF-κβ inhibitory sub-unit I-κβ and maintain NF-κβ in an inactive state. In addition, CARPs may affect the cell-surface levels of receptors and/or interfere with binding of ligands to cell-surface receptors such as tumour necrosis factor receptor (Fotin-Mleczek et al. 2005), vascular endothelial growth factor receptor (Yoo et al. 2005), and toll-like receptor (TLR) (Mao et al. 2012) that mediate inflammatory responses. In this regard, the TLR4/MyD88/NF-κβ signalling pathway is also known to activate the JAK-STAT pathway, which is known to regulate GFAP and astrogliosis (Woodcock and Morganti-Kossmann 2013).

**Limitations**

Two separate studies examining R18 and R18D efficacy at only one dose were performed, thereby precluding a direct side-by-side dose and statistical comparison between the two peptides. In addition, relatively low animal numbers were used, and the study had a short 4-day end point. Future studies examining peptide mechanisms of action, dosing regimens, extended end-points, and additional functional tests (e.g. ladder test, Morris water maze, wire hang) are needed to confirm peptide efficacy after TBI. It would be important in future studies to undertake histological analysis of brain tissue following TBI to confirm that improvements in functional parameters correlate with reductions in brain injury, and to confirm efficacy in other models of TBI. Furthermore, a more segmented analysis of the inflammatory markers may have uncovered more differential effects in different brain regions.

Additionally, the current study did not include sham control animals treated with R18 and R18D as we were not anticipating any confounding effects from the peptides given we have never observed side effects from peptide treatment. Regardless, we acknowledge that the inclusion of these groups would have been beneficial in understanding the safety and tolerability of intravenously administered R18 and R18D in healthy, male Long-Evans rats.

**Conclusions**

In line with CARPs having multiple potential mechanisms of neuroprotection following TBI (Chiu et al. 2016, 2017b), we have demonstrated in a weight-drop impact-acceleration model of TBI that both R18 and R18D poly-arginine peptides can reduce GFAP and, to a lesser extent, IL-6 expression in brain tissue, and that R18D reduced the severity of functional deficits after TBI. These findings provide further evidence that CARPs represent a novel class of neuroprotective agent with promising application as an acute treatment for TBI.

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**Compliance with Ethical Standards**

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

**Ethical Approval** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

**References**


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Chapter 7


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Li Shan Chiu a,b*, Ryan S. Anderton a–d, Vince W. Clark a,b,e, Jane L. Cross a,b,e, Neville W. Knuckey a,b,e, Karine Mardon f, Rajiv Bhalla f, Bruno P. Meloni a,b,e

a Perron Institute for Neurological and Translational Science, Nedlands, Australia
b Centre for Neuromuscular and Neurological Disorders, The University of Western Australia, Nedlands, Australia
c School of Heath Sciences, The University Notre Dame Australia, Fremantle, Western Australia, 6160, Australia
d Institute for Health Research, The University Notre Dame Australia, Fremantle, Western Australia, 6160, Australia
e Department of Neurosurgery, Sir Charles Gairdner Hospital, QEII Medical Centre, Nedlands, Western Australia, Australia
f Centre for Advanced Imaging, University of Queensland, St Lucia, Queensland, Australia

*Correspondence author.
Perron Institute for Neurological and Translational Sciences,
QEII Medical Centre,
8 Verdun St, RR Block, Nedlands, Western Australia, 6009, Australia.
E-mail: lishan.chiu@research.uwa.edu.au
Ph: +61 8 6457 0317
Fax: +61 8 6457 0333
Abstract

Despite extensive studies, there are still no clinically available neuroprotective treatments for traumatic brain injury (TBI). Cationic arginine-rich peptides (CARPs), including polymers of arginine have demonstrated neuroprotective efficacy in excitotoxic and oxygen glucose deprivation in vitro models and in vivo models of stroke, hypoxic-ischaemic encephalopathy, and TBI. In previous studies we have demonstrated beneficial treatment effects of poly-arginine peptides R18 (18-mer of arginine; 300 nmol/kg) and R18D (18-mer of D-arginine; 1000 nmol/kg) in a rat model of impact-acceleration closed-head injury. In the current study, we examined the efficacy of R18D when intravenously administered at a low (100 nmol/kg) and high (1000 nmol/kg) dose 30 minutes after a closed-head injury in male Sprague-Dawley rats. Furthermore, following administration of R18D to healthy rats, serum levels and distribution in major organs were determined using mass spectrometry and positron-emission tomography (PET) imaging, respectively. At post-injury day 3, R18D at the high dose significantly reduced axonal injury, while the low dose showed a trend for reduced axonal injury. In the Barnes maze, both doses of R18D treatment appeared to improve learning and memory recovery compared to vehicle treatment at post-injury days 1 and 3, albeit not to a statistically significant level. Rotarod assessment of vestibulomotor recovery did not differ between R18D and the vehicle treatment groups. Free R18D peptide in serum was detected only at the 5-minute post-administration time-point. PET imaging of radio-labelled R18D for 60-minutes after administration revealed most of the peptide was rapidly eliminated by the kidneys, with only a small proportion taken up by the heart, liver, and spleen, and less by the brain. Together, the findings confirm that R18D has neuroprotective potential following TBI and a limited capacity to cross an intact blood brain barrier.
Introduction

Due its massive burden on affected patients, society and the economy, there is a dire need to develop a neuroprotective therapeutic for traumatic brain injury (TBI). This issue is further compounded by the complex and multifactorial pathophysiology associated with a TBI. Since many previous approaches to developing a neuroprotective therapeutic have focused on targeting specific aspects of the TBI injury cascade, one alternative approach is to develop a therapeutic with a multi-targeted neuroprotective mode of action.

Recent studies have demonstrated that cationic arginine-rich peptides (CARPs) are beneficial in both in vitro and in vivo stroke-related injury models (Meloni et al., 2017; Milani et al., 2017a, 2017b, 2018; Milani, Clark, et al., 2016; Milani, Knuckey, Anderton, Cross, & Meloni, 2016), and there is increasing evidence that these peptides exert their neuroprotective actions through multiple mechanisms (Chiu, Anderton, Knuckey, & Meloni, 2017; MacDougall, Anderton, Mastaglia, Knuckey, & Meloni, 2019; Meloni et al., 2015). For example, we have previously demonstrated that CARPs can protect neurons from excitotoxicity by inhibiting intracellular calcium influx and reducing neuronal surface expression of the N-methyl-D-aspartate (NMDA) receptor subunit protein NR2B9c (Chiu, Anderton, Cross, et al., 2017; MacDougall, Anderton, Edwards, Knuckey, & Meloni, 2016). Moreover, CARPs have the capacity to reduce the activity and/or surface expression of other ion channels and receptors (e.g. AMPAR, NCX, TRPV1, CaV2.2, CaV3.3, TNFR) that may exacerbate brain injury associated with excitotoxicity or neurotrauma (Brittain et al., 2011; Brustovetsky, Pellman, Yang, Khanna, & Brustovetsky, 2014; Ferrer-Montiel et al., 1998; García-Caballero et al., 2014; Planells-Cases et al., 2000; Xie et al., 2016). CARPs can also target and stabilise mitochondria, and reduce mitochondrial reactive oxygen species production (Birk, Chao, Liu, Soong, & Szeto, 2015; Horton, Stewart, Fonseca, Guo, & Kelley, 2008; Kown et al.,...
2003; Marshall et al., 2015; Narasimhulu, Selvarajan, Brown, & Parthasarathy, 2014; Rigobello, Barzon, Marin, & Bindoli, 1995; Szeto et al., 2011; Zhao et al., 2004), inhibit proprotein convertases that activate matrix metalloproteinases (Cameron, Appel, Houghten, & Lindberg, 2000; Fugere, Appel, Houghten, Lindberg, & Day, 2007), modulate inflammatory responses (Datta et al., 2010; Hilchie, Wuerth, & Hancock, 2013; Laskowitz et al., 2001; Li et al., 2017; Narasimhulu et al., 2014), and activate pro-cell survival signalling (Gu et al., 2013; Yang et al., 2016; Zhou, Fang, Acheampong, Mukhtar, & Pomerantz, 2003).

The neuroprotective actions of CARPs are further supported by several studies that reveal this class of peptide can also exert beneficial effects in animal models of TBI (Cao et al., 2016; Laskowitz et al., 2017; Miyamoto et al., 2014; Plummer et al., 2018). Studies in our laboratory have demonstrated that our current lead neuroprotective CARP, polyarginine R18, was able to significantly reduce the extent of injury in the corpus callosum in a closed head injury model in the rat (Chiu, Anderton, Cross, et al., 2017). Furthermore, the R18 D-enantiomer (R18D) significantly improved sensorimotor and vestibulomotor recovery, and reduced brain glial fibrillary acidic protein levels after closed head injury in rats (Chiu et al., 2019).

Following our positive results with R18D, the aim of the current study was to further investigate the effectiveness of the peptide to improve functional (Barnes maze and rotarod) and histological (axonal injury) outcomes when administered at a low (100 nmol/kg) and high (1000 nmol/kg) dose following an impact-acceleration closed-head injury in male Sprague-Dawley rats. An additional component of the study was to examine R18D uptake in serum and tissue distribution when administered to healthy, uninjured Sprague-Dawley rats.
Methods

*Peptides used in this study*

The R18D (H-rrrrrrrrrrrrrrrr-OH; r = D-arginine, net charge +18) peptide was synthesised by Mimotopes (Australia) and purified by high performance liquid chromatography to 98%. Peptide used for dose response and serum uptake studies were prepared in 0.9% sodium chloride for injection (Pfizer, Australia) before approximately 650 μL was aliquoted into 3 mL syringes. Reconstituted peptides were stored at -20 °C until use. For distribution studies, the peptide was first synthesised as a boc-protected precursor (Mimotopes) before being labelled with the fluorine-18 radioisotope (¹⁸F) by the University of Queensland’s Centre for Advanced Imaging (Glaser et al., 2013). Quality control and purification was performed via HPLC on an Eclipse Plus C18 column (4.6 x 150 mm, 5 μm) with a 0.1% trifluoroacetic acid (TFA) in water/acetonitrile mobile phase at 10 – 90% acetonitrile gradient over 30 minutes.

*Traumatic brain injury model*

This study was approved by the Animal Ethics Committee of the University of Western Australia and follows the guidelines outlined in the “Australian Code for the Care and Use of Animals for Scientific Purposes”. Male Sprague-Dawley rats weighing 370 – 410 g were housed in pairs under controlled conditions with a 12-hour light-dark cycle and free access to food and water *ad libitum* before and after surgery.

A weight-drop impact-acceleration model (Foda & Marmarou, 1994; Marmarou et al., 1994) of TBI was used to induce the injury as previously described (Chiu et al., 2019; Chiu, Anderton, Cross, et al., 2017). Briefly, rats underwent anaesthesia induction with 5% halothane (mix 30% O₂/70% N₂O gas), were intubated, and maintained under 1 – 2% halothane during attachment of the metal disc to the skull, jugular vein cannulation, and
the intravenous infusion of treatments. Animals were temporarily disconnected from the anaesthetic (< 1-min) to induce TBI. Treatments were randomised and consisted of the vehicle control (0.9% NaCl for injection) and R18D at a low (100 nmol/kg) or high (1000 nmol/kg) dose administered in a blinded fashion at 30 minutes post-impact (600 μL over 6-min) through the right internal jugular vein using an infusion pump.

A total of 26 animals underwent the procedure and 24 survived to the three-day post-TBI end-point (7.69% mortality). The two animal deaths consisted of one vehicle and one R18D-treated (100 nmol/kg) animal. Both animals experienced shallow, laboured breathing during the surgical recovery phase and died within one hour post-TBI. Sham and vehicle treatment groups consisted of six animals each, and the low and high dose R18D treatment groups consisted of five and seven animals respectively.

Post-surgical animal care and monitoring
At the conclusion of surgery, pethidine (1 mg in 0.2 mL saline, intramuscular) and bupivacaine were administered (0.1 mg in 0.2 mL saline per site, subcutaneous) to the head surgical wound. A 2 mL volume of injectable saline was also subcutaneously administered to aid hydration. Rat cages were placed on a heating mat during post-surgical monitoring, subsequently housed in a holding room maintained at 26 – 28°C. Rats were monitored at least twice a day, and if animal behaviour suggested they were in pain, pethidine (1 mg in 0.2 mL saline) was administered. If weight did not steadily increase, saline (2 mL) was administered once daily. Rats were also provided with sweetened nourishments (e.g. cereal, gel packs) to encourage food intake.
**Functional assessments**

The Barnes maze and rotarod tests were used to identify deficits in learning and memory, and vestibulomotor function, respectively, as previously described (Chiu, Anderton, Cross, et al., 2017). Latency for each assessment was measured the day prior to surgery (baseline), then at days 1 and 3 post-injury (PID1, PID3). Each animal was given three attempts at each test, with a time limit of 180 seconds for the Barnes maze and no time limit for the rotarod. Mean latencies for each treatment group on PIDs 1 and 3 were compared to baseline mean latency and presented as a percentage-change from baseline. A treatment group recording a positive or a negative value indicated an improvement or decline in functional recovery, respectively.

**Histological assessment for axonal injury**

Three days after TBI, animals were euthanased with pentobarbital (100 mg/kg, intraperitoneal) and transcardially perfused with normal saline, followed by 10% neutral buffered formalin. Brains were removed and post-fixed in 4% formalin for one week before embedding in paraffin. Sectioned 10 µm coronal slices corresponding to bregma - 4.5 were stained using Bielschowsky’s silver stain as previously described (Chiu, Anderton, Cross, et al., 2017). Stained sections were then imaged using light microscopy to qualitatively grade axonal injury (0 – 4) within the corpus callosum from three consecutive sections. Axonal injury grading ranged from 0 (indicating absence of injury) to 4. Increasing grade was indicative of increasing degree of axons displaying an increasing degree of disorganised architecture and orientation of oligodendrocyte nuclei, undulation, and varicosities. Images were captured using an Olympus DP-70 digital camera fitted to an Olympus IX70 inverted microscope.
Detection of R18D free peptide in serum following intravenous administration in rats

While anaesthetised (1 – 2% halothane, 30% O₂/70% N₂O), adult male Sprague-Dawley rats (290 – 310 g) were intravenously administered R18D (1000 nmol/kg) via the jugular vein. At 5, 10, 20, 30, 60, 120, and 180 minutes post-peptide administration, whole blood samples (400 µL) were collected into 1.5 mL microfuge tubes and immediately centrifuged at 15,000 x g for 5 minutes at 4°C. Following centrifugation, serum was aliquoted into 0.5 mL microfuge tubes before storing at -80°C. Thawed serum samples were used for multiple reaction monitoring mass spectrometric analysis for the R18D peptide (Proteomics International, Australia). Briefly, 90 µL serum samples were spiked with an internal standard (final concentration of 2.3 nmol/mL) and combined with a 50 mM acetic acid solution, loaded onto a Strata-X C18 desalting column, washed, and eluted with 40% acetonitrile/50 mM acetic acid. The extracted solution was dried down under vacuum and resuspended in 50 µL 0.1% formic acid. 20 µL was injected onto the column to be analysed via electrospray ionisation mass spectrometry (LC/MS) using an Agilent 1100 HPLC system (Agilent; USA) coupled to a 4000 Q-TRAP mass spectrometer (Sciex, USA). Separation was achieved by a linear gradient of water/acetonitrile/0.1% formic acid (v/v) over 10 minutes.

In vivo imaging of radio-labelled R18D tissue distribution following intravenous administration in rats

In vivo imaging of radio-labelled R18D (¹⁸F-R18D) distribution was carried out by the University of Queensland’s Centre for Advanced Imaging (Australia). Briefly, six healthy male Sprague-Dawley rats (290 – 310 g) were anaesthetised in 3% isoflurane in oxygen (2 L/min flow rate) and maintained at 1 – 2% throughout the procedure. A catheter was inserted into the lateral tail vein, and the animal placed in the Inveon PET/CT (Positron Emission Tomography/Computed Tomography) scanner (Siemens, Germany), all while
physiological monitoring was maintained. A single intravenous injection of \(^{18}\)F-R18D (≤ 500 µL, 50 MBq; 2 nmol/kg) in a solution of 10% ethanol/90% physiological saline was infused over a one- to two-minute period. A 60-minute dynamic PET scan was started simultaneously with the radiotracer injection, followed by a 15-minute CT attenuation scan, with the brain, heart, liver, spleen, and kidneys being organs of interest.

CT images were reconstructed using a Feldkamp conebeam back-projection algorithm provided by an Inveon Acquisition Workstation (IAW 2.1, Siemens). For the dynamic PET data acquisition, the emission data were normalized and corrected for decay and dead volume. The list-mode data were sorted into 41 frames (10 x 30 s, 25 x 60 s, and 6 x 300 s time frames). The resulting sinograms were reconstructed with filtered back-projection and an ordered-subset expectation maximization (OSEM2D) algorithm, and analysed using the Inveon Research Workplace software (IRW 4.1, Siemens) which allows fusion of CT and PET images and definition of region of interest (ROIs).

**Statistical analysis**

All statistical analyses were conducted in R 3.5.1 and presented as mean ± standard error of the mean (SEM). An analysis of variance (ANOVA) was performed, followed by a Fisher’s LSD post-hoc test. Data from the axonal injury assessment measurements were analysed using the Mann-Whitney U test. For statistical analysis of functional and histological outcomes, both R18D groups were compared to the vehicle treatment group. A value of \( p < 0.05 \) was considered statistically significant for all data sets.
Results

Functional outcomes

On PID1, Barnes maze assessment for learning and memory for the low and high dose R18D treatment groups resulted in a respective 8% and 30% decrease in performance from baseline, while the vehicle treatment group exhibited a 148% decrease (Figure 1a). On PID3, the low and high dose R18D groups displayed a respective 66% and 68% improvement from baseline, while the vehicle group displayed a 36% improvement from baseline (Figure 1b). While treatment with R18D appeared to provide positive effects on learning and memory recovery compared to vehicle, the results were not statistically significant. Sham animals did not appear to display any obvious functional deficits.

Figure 1 Learning and memory functional recovery on the Barnes maze at post-injury days (a) 1 and (b) 3. Data are presented as mean ± SEM; N = 5 – 7.

On PID1, rotarod assessment for vestibulomotor function for the low and high dose R18D treatment groups resulted in a respective 90% (p = 0.85) and 72% (p = 0.36) decrease in performance from baseline, while the vehicle treatment group exhibited an 87% decrease (Figure 2a). On PID3, the low and high dose R18D groups resulted in a respective 65% (p = 0.80) and 29% (p = 0.24) decrease in performance, while the vehicle group exhibited
a 58% decrease (Figure 2b). Although a positive trend for improvement on the rotarod was shown, this was not statistically significant. Sham animals did not appear to display any obvious functional deficits.

Figure 2 Vestibulomotor functional recovery on the rotarod at post-injury days (a) 1 and (b) 3. Data are presented as mean ± SEM; N = 5 – 7.

Histological outcomes

Based on Bielschowsky’s silver stain, axonal injury in the corpus callosum ranged from grade 0 to 3.5 across all treatment groups (Figure 3). Vehicle-treated animals recorded the highest average axonal injury score (2.25), despite one animal displaying no apparent axonal injury (Figure 3). Animals treated with the low and high doses of R18D reduced average axonal injury grades to 1.40 and 1.21 respectively, with the high dose significantly reducing injury compared to vehicle-treated animals (p = 0.044). As expected, sham animals displayed no axonal injury (grade 0).
**Figure 3** Grading of the extent of axonal injury in the corpus callosum as determined by Bielschowsky’s silver stain. Horizontal bar represents mean grade; N = 5 – 7. *p < 0.05 when compared to vehicle treatment group.

**Serum pharmacokinetics of R18D**

Following administration of R18D to animals and mass spectrometric analysis of serum samples, free R18D peptide was only detected at the five-minute post-administration time point (Figure 4).
Figure 4 Detection of R18D in serum collected five minutes after intravenous administration (1000 nmol/kg dose) in healthy, male Sprague-Dawley rats. Free R18D in serum as measured by HPLC with a retention time of around 0.4 minutes.

Tissue distribution of R18D

Radio-labelled R18D ($^{18}$F-R18D) administered to rats revealed that less than 10% of the injected dose had distributed to major organs after five minutes, and with levels in the organs remaining relatively constant over the 60-minute acquisition period. The majority of the peptide (6 – 7%) appeared to localise to the kidney (Table 1, Figure 5). Levels in heart, liver, and spleen ranged from 0.61 to 0.934% of the injected dose over the 60-minute acquisition period, while levels in brain were between 0.115 to 0.123% (Table 1). Limited distribution in the brain was also reflected in the PET/CT images obtained at the 5-, 10-, and 60-minute time-points post-peptide administration (Figure 6a – c).
**Figure 5** The time-activity curve obtained from animals injected with 18F-R18D; N = 6, SUV = standard uptake volume. Data are presented as mean ± SEM.

**Table 1.** Biodistribution data after intravenous injection of 18F-R18D to healthy male Sprague Dawley rats. Results are expressed as percentage of the injected dose per gram of tissue (%ID/g) (Mean ± SD, n=6)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>5-min</th>
<th>Mean %ID/g ± SD</th>
<th>10-min</th>
<th>Mean %ID/g ± SD</th>
<th>60-min</th>
<th>Mean %ID/g ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>0.123 ± 0.0151</td>
<td>0.113 ± 0.0152</td>
<td>0.116 ± 0.0189</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>0.912 ± 0.121</td>
<td>0.803 ± 0.107</td>
<td>0.934 ± 0.166</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0.681 ± 0.0954</td>
<td>0.610 ± 0.0869</td>
<td>0.711 ± 0.134</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>0.809 ± 0.221</td>
<td>0.717 ± 0.212</td>
<td>0.820 ± 0.189</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidneys</td>
<td>7.07 ± 1.54</td>
<td>6.85 ± 1.05</td>
<td>6.56 ± 2.04</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Values have been decay-corrected to the time of the injection of the radiotracer.*
**Figure 6** Biodistribution of $^{18}$F-R18D in a representative animal at (a) 5-min, (b) 10-min, and (c) 60-min (cumulative) after intravenous injection. Left image represents the axial view, middle image the coronal view, and right image the sagittal view. Red ‘B’ indicates the brain, and ‘K’ indicates kidneys.
Discussion

In line with our previous studies examining the effectiveness of R18D in the rat closed-head injury model, the current findings indicate that R18D intravenously administered at 1000 nmol/kg has therapeutic potential in TBI. Histological examination demonstrated that R18D could reduce the extent of axonal injury in the corpus callosum, while the peptide displayed a tendency to improve functional recovery in the Barnes maze and on the rotarod.

Diffuse axonal injury (DAI) is an important feature of TBI that is caused by both biomechanical and biochemical disturbances. Although the biomechanical primary phase of brain trauma is not amenable to pharmacological treatment, the secondary pathophysiological consequences associated with the biochemical aspect can be targeted. Secondary injury processes such as excitotoxicity, mitochondrial dysfunction, and inflammation contribute to disrupted axonal transport and degeneration (Büki, Okonkwo, & Povlishock, 1999; Maxwell, Domleo, McColl, Jafari, & Graham, 2003). Histologically, axonal injury is characterised by axons exhibiting a “beads-on-a-string” appearance and, in more severe cases, axonal retraction bulbs. The ability of R18D at the high dose, and low dose to a lesser degree, at reducing the extent of axonal damage is in line with an earlier study in our laboratory demonstrating that R18 at a 300 nmol/kg dose reduced axonal injury (Chiu, Anderton, Cross, et al., 2017).

With evidence that CARPs, including poly-arginine-18 peptides (e.g. R18, R18D), have beneficial immunomodulatory effects (Chiu et al., 2019; Laskowitz et al., 2017; Lynch et al., 2005) and can reduce the toxic accumulation of intracellular calcium (Chiu, Anderton, Cross, et al., 2017; MacDougall et al., 2016), it is becoming increasingly apparent that this class of peptide may provide a viable neuroprotective therapeutic for TBI.
Furthermore, as previous neuroprotective agents developed for TBI have generally targeted a single pathophysiological event and failed clinically, R18D represents a therapeutic with a multitude of neuroprotective actions, which greatly enhances its neuroprotective potential and translational clinical effectiveness (Chiu, Anderton, Knuckey, & Meloni, 2016; Chiu, Anderton, Knuckey, et al., 2017; Doppenberg & Bullock, 1997; Narayan et al., 2002; Van der Schyf & Youdim, 2009).

In a previous study, we demonstrated that treatment with R18D reduced TBI-associated sensorimotor and vestibulomotor deficits (Chiu et al., 2019), while in the present study only a positive trend for improved recovery was observed for learning, memory, and vestibulomotor function. There could be several reasons for different functional outcomes observed in the two studies. Aside from the inherent variability expected of functional assessments, different strains of rat were also used. Strain-specific differences in injury susceptibility and recovery outcome has been well-documented in TBI (Al Nimer et al., 2013; Marklund & Hillered, 2011; Reid et al., 2010; Tan, Quigley, Smith, & Hoane, 2009). However, the lack of significant findings suggest that the post-injury examination time-points and functional assessment regimen may not have been optimal. Additionally, higher animal numbers and a longer study end-point may have reduced variability and uncovered statistically significant differences with R18D treatment.

The current and previous studies suggest that R18D has potential to improve motor and cognitive outcomes after TBI. Furthermore, other CARPs have also demonstrated the ability to improve functional outcome in TBI models. For example, the peptides COG1410 (Ac-AS(Aib)LRKL(Aib)KRLL-NH₂, charge: +4) and CN-105 (Ac-VSRRRNH₂, charge: +3) provided significant improvements in spatial learning and memory in mouse TBI models (Laskowitz et al., 2007, 2017). Furthermore, pre-treatment with
PACAP38 (Ac-HSDGIFTDSYRKYQMAVKKYLAAVLGKRYKQRVKNK-NH₂, charge: +9.1) improved learning and memory function in rats subjected to a TBI (Mao et al., 2012). Interestingly, uninjured healthy rats treated with PACAP38 demonstrated an improvement in spatial memory and increased expression of anti-oxidative enzymes (Ladjimi et al., 2019).

To determine whether the significant findings in axonal injury and positive trends in functional recovery observed in this study were a result of R18D, serum pharmacokinetics and tissue distribution were examined. The finding that free R18D in serum was only detected at five minutes after administration was somewhat expected, as the high net positive charge of the peptide would cause it to rapidly bind to negatively charged serum proteins such as albumin and α1-acid glycoprotein (Nguyen et al., 2010; Sarko et al., 2010; Schartmann et al., 2018). However, while unbound R18D has a short serum half-life, protein-bound peptide has a half-life of several hours, as demonstrated for R9D (9-mer of arginine synthesised with D-arginine) (Doranz et al., 2001) when administered to human subjects. Importantly, despite its long serum half-life, repeated intravenous dosing with R9D (3 times per week for 4 weeks) at doses as high as 478 nmol/kg did not cause any adverse neurological side-effects or reactions (Doranz et al., 2001); indicating that single dose R18D will be safe for patient administration, though this requires confirmation in clinical studies.

With respect to tissue distribution, R18D uptake was predominantly by the kidney where it is likely to be eliminated. Low level uptake of R18D was observed in heart, spleen, and liver. Only a small fraction of the injected peptide appeared to locate to the brain. It should be noted that these studies were performed using a low peptide dose (2 nmol/kg) and used a relatively short data acquisition time post-administration (60-min). In addition,
the low level accumulation of R18D in the brain is also likely to reflect a lower capacity of the peptide to cross an intact versus compromised BBB. Given that a severe head injury can alter the integrity of the BBB, it is likely that R18D will have greater capacity to enter the brain and elicit its positive effects. For example, the poly-arginine-11 (R11) peptide was demonstrated to have a greater capacity to penetrate the brain after an ischaemic injury (Gotanda et al., 2014). Unfortunately, it was not possible to conduct the peptide distribution study on injured animals since the laboratory in which these studies were conducted was not equipped to perform the TBI procedure. This also precluded investigating other pharmacokinetic differences between injured and healthy animals.

In the event that peripheral administration of R18D is not proven optimal for targeted delivery to the brain and neuroprotection following TBI, several alternative brain delivery options could be explored (Nonaka et al., 2012; Sarko et al., 2010). For example, intranasal delivery is an alternate route of administration which may enhance R18D accumulation in the brain by overcoming the BBB (Hanson & Frey, 2008). However, whether an intranasal route is practical at the clinical levels after TBI would need to be determined. Alternatively, modifying the peptide by incorporating cyclic oligosaccharides, such as cyclodextrins, may improve peptide solubility and subsequent bioavailability, and in doing so, improve brain uptake (Nonaka et al., 2012). However, whether increased brain levels are required to improve neuroprotection and yet be safely administrated is currently unknown. Furthermore, the inclusion of multiple doses, both lower and higher than the ones used in this study, would have allowed a more thorough dose-response study and provided additional information on optimal dosing regimen.

This study has demonstrated that the poly-arginine-18 peptide R18D can reduce axonal injury and provide positive trends for functional recovery. In order to elucidate whether
R18D may be responsible for these benefits, further dose-response and long-term studies are required. Studies in healthy rats demonstrated that free R18D peptide has a short serum half-life and that only a limited amount of peptide localises in the brain.
Acknowledgements

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Conflicts of interest

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


R18D REDUCES AXONAL INJURY


Chapter 8

General discussion
8.1 Overview

TBI remains as one of the most socially and economically burdensome conditions in Australia and the world. The only interventions currently in practice are preventative measures and neurosurgery. Although these have come a long way in reducing TBI incidence and improving patient outcome after sustaining a TBI, neither address the core issue of secondary cellular damage that contributes to the severity of the neurological outcome and impairment of functional recovery.

Pharmacological neuroprotection strategies are urgently needed to further improve patient outcomes after TBI, however it is important to acknowledge that all previous pharmacological neuroprotective agents demonstrating pre-clinical success have failed when assessed at the clinical level. This translational failure may be explained by the multi-faceted aspects of TBI pathophysiology and its variable manifestation in humans. For these reasons, not only has TBI been difficult to model in animals, it represents a challenging disorder in terms of developing an effective neuroprotective treatment. Neuroprotective strategies have traditionally targeted single aspects of TBI pathophysiology, but there is growing awareness that either combined or multi-targeted therapies are more likely to be effectively translated into the clinic.

This thesis examined a novel class of peptides known as cationic arginine-rich peptides (CARPs) as a potential neuroprotective agent for TBI, particularly poly-arginine peptides R18 and R18D. Poly-arginine peptides have demonstrated beneficial effects in cultured cortical neurons exposed to excitotoxic injury and in animal models of acute neuronal injury such as stroke. In rat models of middle cerebral artery occlusion (MCAO), poly-arginine peptides have reduced infarct volumes and demonstrated functional
improvements (Meloni et al., 2017; Milani et al., 2017a, 2017b, 2018; Milani, Clark, et al., 2016; Milani, Knuckey, Anderton, Cross, & Meloni, 2016). With excitotoxicity being a shared pathophysiological aspect of stroke and TBI, it was hypothesised that CARPs such as R18 and R18D could also be neuroprotective in TBI.

A weight-drop impact-acceleration model of TBI was used in the rat as part of this thesis, and used to compare our lead peptide, R18, to several peptides being developed in other laboratories for neuroprotection in TBI. Following an initial study with R18 indicating beneficial effects following TBI (Chapter 5), this led to further studies using an 18-mer peptide comprised of the more stable D-enantiomer of arginine, R18D (Chapter 6). Here, it was hypothesised that R18D would be more effective than R18 in the TBI model. Positive results obtained with R18D in the TBI model led to the examination of high and low doses of R18D in order to identify a potential dose-dependent neuroprotective effect (Chapter 7). In conjunction with the University of Queensland, an additional study was designed to elucidate R18D peptide distribution in healthy, uninjured rats (Chapter 7). Together, these studies yielded several key findings.

8.2 Key findings

8.2.1 Poly-arginine peptide R18 is more effective than COG1410 and APP96-110 peptides at protecting cultured cortical neurons from glutamic acid excitotoxicity

Excitotoxic injury is a pathophysiological feature common to most, if not all, acute neurological disorders, including TBI. Excitotoxicity results when glutamate, an excitatory neurotransmitter, fails to rapidly clear from the synaptic cleft and accumulates. This triggers overstimulation of post-synaptic glutamate receptors, eventuating in the toxic influx of calcium ions into neurons. In TBI, excitotoxicity is a prime
pharmacological target for neuroprotection because the accumulation of intracellular calcium results in induced mitochondrial permeability transition, leading to a loss in membrane potential, architectural breakdown, the release of pro-apoptotic factors, decreased ATP synthesis, and ROS production (Ahmed, Rzigalinski, Willoughby, Sitterding, & Ellis, 2008; Cheng, Kong, Zhang, & Zhang, 2012; Singh, Sullivan, Deng, Mbye, & Hall, 2006; Xiong, Gu, Peterson, Muizelaar, & Lee, 1997). The resulting imbalance in energy homeostasis and oxidative stress contributes to cell death.

Using an in vitro rat cortical neuron excitotoxicity injury model, it was demonstrated that concentrations of R18 as low as 0.5 µM were able to significantly maintain cell viability compared to untreated neurons (Chapter 5). In contrast, treatment of cortical neurons with APP96-110 or COG1410 did not provide any obvious neuroprotection up to concentrations of 10 µM in the excitotoxicity injury model (Chapter 5). R18, APP96-110, and COG1410 at concentrations of 1 and 5 µM were further evaluated for their ability to reduce neuronal intracellular calcium influx following exposure to glutamic acid. R18 was able to significantly reduce calcium influx compared to the untreated control at 1 and 5 µM, whereas APP96-110 was only effective at 5 µM and COG1410 had no obvious effect at reducing neuronal calcium influx (Chapter 5).

COG1410 and APP96-110 are two peptides that have shown promise as neuroprotective agents in animal models of TBI, but contain fewer arginine residues and are less cationic than our lead poly-arginine peptide, R18. The superior efficacy of R18 compared to COG1410 and APP96-110 in excitotoxicity (Chapter 5) is in line with earlier studies in the Meloni laboratory. These studies demonstrated that the neuroprotective properties of CARPs, including poly-arginine peptides, is highly dependent on both arginine content and cationic state (Meloni, Brookes, et al., 2015). One mechanism whereby poly-arginine
peptides ameliorate excitotoxic injury, is by reducing the level of neuronal cell-surface glutamate receptors, which likely occurs during endocytic and/or non-endocytic uptake of the peptide by neurons (MacDougall, Anderton, Mastaglia, Knuckey, & Meloni, 2019; Meloni, Milani, et al., 2015).

8.2.2 Poly-arginine peptides can improve functional recovery in male rats subjected to TBI

In addition to the more visible functional deficits such as motor impairments that occur in TBI patients, changes in personality, behaviour, cognition, and learning and memory are also common (Ponsford et al., 2014). Even for injuries classified as “mild”, a considerable proportion of patients do not regain full functionality at one year after TBI (McMahon et al., 2013). In order to determine the effects of a candidate neuroprotective agent on functional outcome, three tests were utilised to assess motor and cognitive outcomes after TBI in rats. The Barnes maze test was used to assess spatial learning and memory (Barnes, 1979), while the adhesive tape test assessed sensorimotor function (Alwis, Yan, Morganti-Kossmann, & Rajan, 2012; Bouet et al., 2009), and the rotarod assessed vestibulomotor function (Hamm, Pike, O’Dell, Lyeth, & Jenkins, 1994). Chapters 5 – 7 provide a detailed description of each testing regimen.

Both COG1410 and APP96-110 peptides were utilised as potential positive controls due to demonstrated improvements in sensorimotor (Hoane et al., 2007), vestibulomotor (Cao et al., 2016; Corrigan et al., 2014; Laskowitz et al., 2007; Plummer et al., 2018), or learning and memory (Kaufman et al., 2010; Laskowitz et al., 2007) function when administered in rodents post-TBI. Surprisingly, neither COG1410, APP96-110, nor R18 peptides improved functional outcomes compared to vehicle-treated animals (Chapter 5).
when intravenously administered at a dose of 300 nmol/kg 30 minutes post-TBI. This was partly attributed to the large variation in functional recovery and small animal numbers within groups. However, other factors may have contributed to the negative results such as sub-optimal dosage and route of administration, or the peptide undergoing proteolytic degradation before eliciting a therapeutic effect.

To improve peptide stability, an 18-mer peptide of D-arginine (R18D) was synthesised. It was then assessed alongside R18 to determine if there were enantiomer-specific effects in terms of improving outcomes after TBI. Long-Evans rats subjected to a TBI and treated with R18D (1000 nmol/kg) demonstrated reduced sensorimotor and vestibulomotor deficits compared to vehicle treatment, while learning and memory deficits were similar in the peptide and vehicle treatments groups (Chapter 6). In contrast, R18-treated animals performed no better than the vehicle treatment group of the same cohort (Chapter 6). However in Sprague-Dawley rats subjected to a TBI, beneficial effects following treatment with R18D (100 and 1000 nmol/kg) were less pronounced, demonstrating only positive trends for functional recovery (Chapter 7). Overall, reductions in functional deficits as a result of R18 and R18D treatment appear to vary with rat strain, dose, and the peptide enantiomer. Regardless, the results of these experiments do suggest that improvements in functional outcomes can be achieved with poly-arginine R18 peptides when administered after TBI.

8.2.3 Poly-arginine peptide R18D has limited distribution in the brain of healthy, male Sprague-Dawley rats

Despite promising pre-clinical results, many peptide-based therapeutics do not progress past Phase II/III clinical trials. One explanation for this failure is the lack of studies
examining how the peptide behaves \textit{in vivo} prior to clinical trials (Tolias & Bullock, 2004). Thus, it is highly recommended that all therapeutic agents undergo pre-clinical and clinical absorption, distribution, metabolism, and excretion (ADME) studies. Although full pharmacological profiling of R18 and R18D peptides was beyond the scope of this thesis, initial peptide serum pharmacokinetics and tissue distribution was explored in healthy, uninjured rats.

R18D was demonstrated to have a serum half-life of less than five minutes, and was rapidly taken up by the major organs, particularly the kidney, with very little of the injected dose reaching the brain. Other studies using cationic arginine-rich cell-penetrating peptide carriers have also showed preferential accumulation in the kidney, liver, heart, spleen, and brain in similar proportions to R18D (Cai et al., 2006; Sharma et al., 2013; Shin et al., 2005). Despite the short serum half-life, protein-bound peptides have a tendency to persist in tissues for at least several hours post-administration (Butterworth et al., 2002; Lee & Pardridge, 2001; Otvos et al., 2014; Sarko et al., 2010), potentially allowing greater time for the agent to exert its therapeutic effect. However, the limited capacity of R18D to enter the brain was somewhat surprising given that CARPs can cross the BBB (Banks, Robinson, & Nath, 2005; Gotanda et al., 2014; Gou, Wang, Yang, Xu, & Xiong, 2011; Stalmans et al., 2015). It is also known that CARPs can continuously traverse the barrier between circulation and the brain (Stalmans et al., 2015). Ultimately, this determines the concentration that a peptide can achieve in this organ, which may be altered after a TBI. While additional R18 peptide brain uptake studies are needed, significant functional and histological improvements were demonstrated in injured rats treated with R18 peptides, indicating that therapeutic levels of the peptide are capable of entering the brain, and/or that very low brain concentrations of R18 is required for neuroprotection.
8.2.4 *R18 reduces the extent of axonal injury in the corpus callosum of male Sprague-Dawley rats subjected to TBI*

Diffuse axonal injury (DAI) is a common pathological phenomenon that can result from a TBI of any severity, and significantly contributes to poorer outcomes (Vieira et al., 2016). The mechanical and biochemical aspects of TBI are both thought to contribute to the breakdown of axonal structural integrity. Strain and tearing of the axonal fibres is caused by changes in pressure gradients of the intracranial cavity as a result of sudden movement of the head (Davceva, Basheska, & Balazic, 2015). This is then compounded by the events of secondary injury including toxic neuronal calcium ion influx, mitochondrial dysfunction, and subsequent ROS production (Büki, Okonkwo, Wang, & Povlishock, 2000; Wolf, Stys, Lusardi, Meaney, & Smith, 2001). Higher grades of DAI often leave patients at greater risk of unfavourable outcomes (van Eijck, Schoonman, van der Naalt, de Vries, & Roks, 2018), and may be exacerbated by complications such as hypoxia and hypotension (Vieira et al., 2016).

DAI in the corpus callosum was induced in rats using Marmarou’s weight-drop model of impact-acceleration TBI (Foda & Marmarou, 1994; Marmarou et al., 1994) (Chapters 5 & 7). The severity of axonal injury was histologically assessed in sections stained by Bielschowsky’s silver method and graded using a 5-point scale, where ‘0’ indicated absence of axonal pathology. A detailed description of the grading criteria is provided in Chapters 4, 5, and 7. In injured rats, intravenous administration of R18 at a dose of 300 nmol/kg (Chapter 5) and R18D at 1000 nmol/kg (Chapter 7) was demonstrated to significantly reduce the extent of axonal injury compared to vehicle-treated animals. In a similar study using the same model and rat strain published after the results described in Chapter 5, the APP96-110 peptide was found to significantly reduce the extent of axonal
injury at intravenous doses of 26 and 260 nmol/kg, as determined by counting the number of amyloid precursor protein (APP)-positive cells in the corpus callosum (Plummer et al., 2018). Another study assessing the COG1410 peptide with the controlled cortical impact (CCI) model of TBI in mice also demonstrated reduced numbers of APP-positive cells in the corpus callosum in peptide-treated mice although there were no significant differences in severity of axonal injury between peptide- and vehicle-treated mice based on silver staining (Jiang & Brody, 2012). Given that these studies seem to indicate that silver-staining is a less sensitive indicator of axonal injury severity, future studies may benefit from immunohistochemical methods that better discern the extent of injury within axons after treatment with R18 peptides.

Additionally, it should also be noted that histopathological analysis of Nissl-stained brain sections from all studies of this thesis did not detect any significant differences in the number of viable hippocampal neurons between peptide, vehicle, and sham treatment groups (Chapter 5). Although this finding differs from the literature, it does indicate that the TBI model used in this thesis does not result in hippocampal neuronal death, or overt changes in neuronal cell body morphology as assessed by Nissl staining at three to four days post-injury. However, significant differences were observed on the Barnes maze between sham and vehicle treatment groups (Chapters 7), which suggests learning and memory deficits were incurred as a result of the TBI. Given the major role of the hippocampus in spatial learning and memory (Jarrard, 1993), and that the Nissl method only stains nucleic acids within the neuronal cell body, it is possible that TBI-induced damage to the axons of hippocampal neurons or to synaptic terminals may have contributed to these cognitive deficits.
8.2.5 *R18D reduces the neuroinflammatory response in male Long-Evans subjected to TBI*

Inflammation is an integral feature of secondary injury events associated with a TBI and can have both beneficial and detrimental effects, thus representing a potential target for neuroprotection. In addition to the acute inflammatory response, chronic inflammation may also persist after injury (Gentleman et al., 2004; Johnson et al., 2013; Ramlackhansingh et al., 2011), potentially contributing to residual behavioural deficits, including chronic depression, and an increased risk of developing a neurodegenerative disease in the long-term (Fenn et al., 2014). The acute phase of the neuroinflammatory response after TBI is characterised by glial activation, leukocyte recruitment, and pro- and anti-inflammatory cytokine and chemokine production (Ziebell & Morganti-Kossmann, 2010). Therefore, immunomodulating therapies that can dampen the deleterious effects of neuroinflammation are potentially beneficial following TBI as demonstrated in preclinical studies (Bergold, 2016).

Evidence suggests that CARPs have immunomodulating properties when administered post-TBI. The pituitary adenylate cyclase-activating polypeptide (PACAP38: Ac-HSDGIFTDSYRKYKQMAVKKYLAAVLGKRYKQRVKNK-NH₂; net charge +10.1) has been shown to inhibit the TLR4/MyD88/NF-κβ signalling pathway (Mao et al., 2012), and AIP6 (RLRWR; net charge +3) can block binding of the NF-κβ p65 subunit to DNA, thus inhibiting the transcription of pro-inflammatory genes including TNFα, IL-1β, IL-6, inducible nitric oxide synthase, cyclooxygenase-2, and prostaglandin E₂ (Wang et al., 2011). In addition to PACAP38, other CARPs such as COG133 (Ac-LRVRLASHLRKLRKLL-NH₂; net charge +7.1) and CN-105 (Ac-VSRRR-NH2; charge +3) can downregulate gene expression of inflammatory markers such as TNFα,
IL-6, and Bax (Lynch et al., 2003; Mao et al., 2012) among several others (Laskowitz et al., 2017). Conversely, although R18D was able to significantly reduce GFAP expression in the brain in this thesis, it did not have a significant impact on IL-6, TNFα, or IL-1β brain levels after TBI (Chapter 6). While surprising, this may largely be due to the timing (Helmy, Carpenter, Menon, Pickard, & Hutchinson, 2011; Simon et al., 2017) and nature of sample collection. However, there have been recent suggestions that some cytokines traditionally thought to be pro-inflammatory, may actually have protective properties at different phases of the secondary injury cascade (Shohami, Ginis, & Hallenbeck, 1999; Ziebell & Morganti-Kossmann, 2010). Therefore, additional studies investigating the immunomodulatory effects of TBI may further benefit from utilising different experimental end-points and segmented analyses of the inflammatory response in different brain regions.

### 8.3 Future directions

The findings from this thesis raise a number of questions which can inform future studies examining poly-arginine R18 peptides as a neuroprotective therapeutic for TBI, and these are discussed below.

#### 8.3.1 Assessing peptide efficacy using different methods of injury induction

TBI is a complex and heterogeneous condition with differences in individual pathology and long-term recovery. It is important to acknowledge that neuroprotective therapeutic outcomes may differ between patients (Saatman et al., 2008), and therefore imperative that the therapeutic efficacy of R18 peptides be confirmed in different models of TBI. The TBI model used in this thesis was developed by Marmarou and colleagues (Foda & Marmarou, 1994; Marmarou et al., 1994), which adds a rotational acceleration component
to the injury mechanics of earlier weight-drop models such as the Feeney (Feeney, Boyeson, Linn, Murray, & Dail, 1981) and Shohami (Shapira et al., 1988) methods. However, while ease of injury induction and relative inexpensiveness are major advantages, the degree of variability in injury outcomes is a potential limitation of the procedure (Chapters 5 – 7).

The CCI model is perhaps one of the most widely used TBI models. The duration, velocity, and depth of impact to the exposed brain can be adjusted by careful calibration (Berkner, Mannix, & Qiu, 2016), allowing for high reproducibility and consistency in injury characteristics of ranging severity. The CCI model traditionally produces a more focal injury, however modifications which incorporate elements of the Marmarou model have more clinically relevant injury mechanics (Petraglia et al., 2014). Fluid percussion injury (FPI) is another widely-used model of TBI, where the force is delivered by a fluid pressure pulse generated by a pendulum striking a piston. FPI produces a localised injury similar to CCI, with additional diffuse sub-cortical neuronal and axonal injury that is comparable to clinical DAI when greater severities are induced (Berkner et al., 2016). Much like the Marmarou model, variability limits reliability of the midline-FPI model, particularly with regards to the extent of tissue damage and ranging physiological responses (Rowe, Griffiths, & Lifshitz, 2016). Penetrating ballistic brain (PBBI) and blast-like injuries are more complex and have a unique injury profile. These models have only recently been developed and mimic scenarios more likely found in the battlefield, which is of interest to military medicine.

A strategy employed by the “Operation Brain Trauma Therapy” consortium for screening neuroprotective agents was to use multiple study centres that specialised in either FPI, CCI, or PBBI models (Kochanek et al., 2011). Therefore, it would be highly advantageous
in future studies for poly-arginine R18 peptides to be examined in multiple TBI models across multiple centres. Additionally, the same model could also be used across multiple assessment centres (DeWitt et al., 2018). Furthermore, since clinical TBI is often part of polytrauma, introducing other co-morbidities of trauma, such as infection, hypoxia, and/or hypotension in TBI models when assessing neuroprotective agents (e.g. R18), would be highly advantageous as this may provide a better indication for translation efficacy.

8.3.2 Assessing peptide efficacy in phylogenetically higher animal TBI models

The majority of TBI drug-screening studies have been conducted in rodents, which are cost-effective organisms for in vivo assessment of pharmacological therapeutics. However, it is widely accepted that the lissencephalic cerebral cortex of rodents is a major limitation to faithfully recreating all histological and functional aspects of human TBI (DeWitt et al., 2018; Shultz et al., 2017). The cerebral cortices of higher order animals such as sheep, pigs, and non-human primates are gyrencephalic with considerably different white to grey matter ratios. Thus, it is imperative that the anatomical differences of the species used in TBI research should be considered in the interpretation of experimental results. Furthermore, it has been noted that demonstration of neuroprotective efficacy in large animal studies is critical for government support for clinical trials (DeWitt et al., 2018). Though crucial, such studies are unsurprisingly resource-intensive. Funding availability also often poses a significant challenge (Cook & Tymianski, 2012) in addition to the strong ethical concerns of such a study. The studies conducted in this thesis were largely exploratory, therefore use of a rodent TBI model was appropriate. Moreover, since the effects obtained with R18 and R18D were not consistent across different studies in terms of functional and histological efficacy,
additional studies are required in other rodent TBI models. At the present time, it would not be justifiable to progress to phylogenetically higher animals without further assessment of the peptide in rodent TBI models and different laboratories.

8.3.3 Alternative neurological and behavioural assessments after TBI

Individuals who have sustained a TBI can have wide-ranging functional deficits. Several functional domains may be affected and generally, a more severe TBI results in a patient having a worse Glasgow Outcome Scale–Extended (GOS-E) scores, and therefore reduced probability returning to normal life and successful re-integration into society (Hoofien, Vakil, Gilboa, & Donovick, 2001; Townshend & Norman, 2018). Since there is no uniform profile of disabilities following a TBI and there are no reliable methods for predicting patient functional outcomes, there is no consensus on the battery or schedule of assessments used to evaluate the efficacy of therapeutic interventions. Regardless, sensorimotor, vestibulomotor, and learning and memory deficits are often reported by patients (Centers for Disease Control and Prevention, 2015), thus these domains were used to assess functional outcome in this thesis.

Tests of reflexes and strength were not conducted in the current thesis. Although assessments for strength are rarely conducted in the context of TBI, both should be considered for future experiments, especially reflex tests. Assessing reflexes is valuable to determining therapeutic efficacy and has been found to correlate well with clinical measures of outcome (DeWitt et al., 2018) such as the GOS-E. Sensitivity of these assessments may be further increased by utilising composite scores from multiple functional assessments in order to provide insight into the potential mechanisms of injury on which a proposed therapeutic may act (Fujimoto, Longhi, Saatman, & McIntosh,
The Morris Water Maze (MWM) is more commonly used than the Barnes Maze to assess cognition. Coupled with automated video tracking system software (e.g. ANY-maze; Stoelting Co., Wood Dale, USA), multiple measures of maze performance (Tucker, Velosky, & McCabe, 2018; Vorhees & Williams, 2006) may be used to evaluate therapeutic efficacy, and therefore could be incorporated into future studies. Although both maze tasks assess learning and memory function, the MWM is considered more stressful (Harrison, Hosseini, & McDonald, 2009), which adds another behavioural domain that could be assessed. Given that the Barnes Maze did not consistently detect treatment effects, the MWM may be a more suitable alternative for assessing cognitive deficits in subsequent studies. Other tasks for consideration that assess vestibulomotor function include the beam-balance and beam-walking tests. Although the rotarod was determined to be more sensitive in comparison (Hamm, 2001), measurements taken in conjunction with gait analysis (e.g. DigiGait; Mouse Specifics Inc., Framingham, USA) could enhance sensitivity of the motor assessment protocol (Sashindranath, Daglas, & Medcalf, 2015).

8.3.4 Extending end-of-experiment time-point after TBI

Due to the high risk of animal welfare concerns with regards to injury severity, and the exploratory nature of the studies in this thesis, a 3- to 4-day experimental end-point was considered the most appropriate. Future studies may benefit from assessing animal recovery over weeks due to the complex and time-dependent nature of TBI pathophysiology (Kochanek et al., 2016), especially given the lack of consistent significant differences in histological and functional outcome (Chapters 5 – 7). In addition to the acute impact of TBI on patient outcomes, there is an increasing emphasis on its long-term impact due to the strong link between TBI and the later development of chronic
neurodegenerative disorders (Stocchetti & Zanier, 2016). Due to decreasing mortality associated with TBI, there is a greater proportion of survivors at risk of developing a chronic neurodegenerative disorder, therefore evaluating the efficacy of proposed interventions at longer time-points over months or even years, is critical.

8.3.5 Peptide mechanism of action

Broadly, this thesis aimed to determine whether R18 and R18D could provide positive effects when administered after TBI, thus their mechanism of action was not examined in depth. Based on the biological properties and previous neuroprotective and cytoprotective studies using CARPs, this class of peptide is known to have multiple mechanisms of action, and thus may be a more potent neuroprotective therapeutic than other previously developed compounds (Chapter 3). Given this thesis has demonstrated that treatment with R18 and R18D can provide positive effects after TBI, further investigations into their mechanisms of action are warranted. A better understanding of peptide mechanism of action may provide additional information to improving treatment regimen, mitigating risk of side-effects, therefore increasing its potential for clinical translation.

It is hypothesised that R18 and R18D enter cells through endocytic and non-endocytic mechanisms, which could be investigated in vitro using real-time imaging of fluorescent-labelled, peptide-treated neuronal cultures (Futaki et al., 2001; Kaplan, Wadia, & Dowdy, 2005). In addition, it will be important to confirm if the peptide targets intracellular events associated with TBI pathophysiology following internalisation, such as mitochondria, ROS production, and cell signalling. By characterising the interactions of poly-arginine peptides with intracellular targets, it will provide an insight into their potential efficacy in terms of dosage and its therapeutic window. This is especially critical to avoid
compromising safety, where high concentrations of peptide could become toxic (Swinney, 2004). Additionally, biochemical screening assays (e.g. free radical scavenging), genetic techniques (RNAi of signalling molecules), and computational methods for target inference (Schenone, Dančík, Wagner, & Clemons, 2013) may also be utilised to obtain information regarding intracellular mechanism of action.

8.3.6 Pharmacological profiling of poly-arginine peptides

There has recently been increased interest in peptide-based therapeutics, largely because of their structural diversity, low toxicity, potential for commercial success, and versatility in administration routes (Fosgerau & Hoffmann, 2015). Further to Chapter 8, section 8.3.5, more complete pharmacological profiling of R18 and R18D will increase their potential for clinical translation. Although parts of this thesis have established some of the pharmacodynamic properties of R18D, it is strongly recommended that future studies aim to determine drug ADME, and any potential toxicity in both healthy and TBI-induced rats as drug pharmacokinetics can differ between settings (Boucher & Hanes, 1998). Together, these findings will better inform effective dose, ideal administration route, and dosing regimen. Further steps should then be taken in additional pre-clinical studies to use routes of administration translatable to a clinical setting (DeWitt et al., 2018).

The majority of peptide-based therapeutics have been developed for intravenous delivery (Fosgerau & Hoffmann, 2015), and cell-penetrating peptides are a class of compounds often used as carriers for drug delivery. Like most naturally-derived peptides, the half-life of poly-arginine peptides is expected to be very short (Otvos & Wade, 2014), especially since only small amounts of unbound R18D were detected in serum at five minutes post-administration (Chapter 7). One caveat of a peptide-based therapeutic is
their propensity to undergo proteolytic degradation (Vlieghe, Lisowski, Martinez, & Khrestchatisky, 2010). Serum stability assays are a readily accessible method to determine peptide degradation in serum following intravenous administration (Ali et al., 2009; Amantana et al., 2007; Li et al., 2004). These methods can also be applied to urine samples to determine excreted metabolites (Ali et al., 2009; Li et al., 2004; Underberg, Hoitink, Reubsaet, & Waterval, 2000). It would also be valuable to determine if peptide stability differs between R18 and R18D since previous studies suggest that the D-peptide will have greater protease resistance (Adessi & Soto, 2002; Najjar, Erazo-Oliveras, Brock, Wang, & Pellois, 2017). Given it appears that little intact R18D enters the brain in healthy animals (Chapter 7), it would be useful to investigate if any degradation products (shorter poly-arginine peptides or free arginine) are generated after administration. If so, their respective activities should be profiled and measured to determine whether any therapeutic effect of the whole peptide is retained.

The cytochrome P450 family of enzymes play a major role in drug metabolism and clearance and are therefore important to consider for potential toxicity. Interactions between enzymes and peptide may be measured via biological assays, and studies with similar, cationic peptides suggest they may have a relatively limited ability to inhibit cytochrome P450 enzymes (Haug et al., 2016). Other techniques such as mass spectrometry imaging (Nilsson et al., 2015) and physiologically based computational modelling (De Buck et al., 2007; Kuepfer et al., 2016) can be widely applied throughout the suite of ADME investigations to complement findings from biological assays.

Potential toxicity of a candidate therapeutic is an important consideration in drug development, particularly given that 38% of drugs are abandoned in phase I clinical trials due to adverse side effects (Vlieghe et al., 2010). Animal studies from the Meloni
laboratory have not demonstrated any obvious deleterious toxic effects following peptide administration thus far (Milani et al., 2018). However, one study has found that treatment with a cationic anti-microbial peptide may be toxic to mammalian cells, such as in human erythrocytes (Nan, Lee, Kim, & Shin, 2010), and another where a cell-penetrating peptide may exhibit renal toxicity (Amantana et al., 2007). Interestingly, an arginine-rich pentapeptide that has demonstrated neuroprotective efficacy in preclinical neurotrauma studies (Laskowitz et al., 2017; Lei et al., 2016; Tu et al., 2017), including TBI, has been deemed safe in healthy humans when administered as a continuous intravenous infusion (Guptill et al., 2016). Moreover, the poly-D-arginine-9 peptide has also demonstrated intravenous administration safety in humans (Doranz et al., 2001). Finally, considering poly-arginine peptides are likely to undergo degradation, the amino acid by-products are unlikely to exert toxicity, thus making them preferable to other drug candidates (Vlieghe et al., 2010).

8.4 Concluding remarks

The current thesis has demonstrated the neuroprotective potential of R18 and R18D peptides as pharmacological therapeutics for TBI. Firstly, R18 was shown to be more effective than COG1410 and APP96-110 at reducing calcium influx and maintaining neuronal cell viability after a severe in vitro glutamic acid insult. A weight-drop impact-acceleration model of TBI was then used to assess peptide neuroprotective efficacy in male Sprague-Dawley rats. The initial study compared R18 with COG1410 and APP96-110, and demonstrated that treatment with R18 significantly reduced the extent of axonal injury and positive trends for functional recovery were also observed. When the efficacy of R18 and R18D peptides was compared in Long-Evans rats, only treatment with R18D significantly reduced sensorimotor and vestibulomotor deficits, while both peptides had
positive effects in reducing the neuroinflammatory response. Furthermore, it appears that the D-enantiomer of arginine may confer added stability to the peptide given that R18D was able to exhibit some neuroprotective effect at a lower dose than R18.

This thesis has highlighted the complex and varied nature of TBI, while simultaneously demonstrating neuroprotective effects of R18 and R18D in a TBI injury model. However, additional studies are required to examine the efficacy of poly-arginine R18 peptides in different TBI models and across different centres. Moreover, further studies investigating their mechanisms of action, pharmacokinetics, and pharmacodynamics are required.

Given the high prevalence and enormous medical and social impacts of TBI, the need to develop effective neuroprotective agents remains a high priority. Whether as a standalone intervention or coupled with other pharmacological agents, both R18 and R18D have potential for development as a therapeutic intervention to improve patient outcomes following TBI.
8.5 References


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