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Assessment of the neuroprotective efficacy of poly-arginine-18 (R18) peptides in a pre-clinical model of perinatal hypoxic-ischaemic encephalopathy (HIE)

by

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ABSTRACT

Hypoxic-ischaemic encephalopathy (HIE) is one of the leading causes of mortality and morbidity in infants, globally. This disorder eventuates following a reduction in oxygenated cerebral blood flow to the foetus *in utero*, leading to excitotoxic-mediated brain cell (e.g. neuron, glia and glial progenitor cell) death. Currently, there is no clinically appropriate neuroprotective treatment to reduce acute brain injury following HIE. Recent studies have demonstrated that poly-arginine and cationic arginine-rich peptides (CARPs; e.g. R18: R = arginine residues) exhibit potent neuroprotective properties in both *in vitro* and adult animal models of ischaemia, and therefore have the potential to be developed into a neuroprotective treatment to reduce brain injury following HIE. Therefore, the aim of this thesis was to assess the neuroprotective efficacy of CARPs in a model of perinatal HIE in the rat.

To elucidate the neuroprotective efficacy of CARPs, a novel surgical modification to the original *in vivo* Rice-Vannucci model of perinatal HIE was developed. Using 7-day old Sprague-Dawley rats, brain injury was induced following the permanent ligation of the common and external carotid arteries, followed by a period of transient hypoxia (8% O_2/92% N_2). Results from this experiment demonstrated that the occlusion of common and external carotid arteries reduced cerebral communicational and/or anastomotic blood flow, reducing variability and improving the reliability in the presence of a cerebral infarct. The demonstration and termination of cerebral communicational and/or anastomotic blood flow improved the pre-clinical assessment of neuroprotective therapies to treat HIE.

The CARPs, R18, R18D (D-enantiomer) and JNKI-1-TATD, were assessed in the modified Rice-Vannucci model of HIE when administered intraperitoneally, immediately after the cessation of hypoxia-ischaemia (HI; 8% O_2/92% N_2 for 2.5 h). Treatment with R18 and R18D significantly reduced infarct volume and improved behavioural assessments in this model. Surprisingly, the well-characterised neuroprotective peptide JNKI-1-1TATD, used as a positive control and benchmark, did not exhibit any significant neuroprotection. Succeeding positive results obtained following R18D administration immediately after HI, its therapeutic window was further assessed. R18D significantly decreased infarct volume and improved behavioural assessments when administered intraperitoneally up to 1 hour after the cessation of HI; correlating to 3.5 hours since HI onset. To confirm the neuroprotective mechanism of action of CARPs in HIE, an established *in vitro* primary cortical neuronal excitotoxic injury model was used. Results from this experiment demonstrate that CARPs reduce excitotoxic intracellular calcium influx in a dose-dependent fashion, providing evidence for a role in the reduction of several calcium-dependent pro-cell death cascades. The demonstration of significant neuroprotection following R18 peptide administration provides evidence for a novel therapeutic, which has the potential to reduce brain injury in infants who suffer HIE.

In summary, this thesis has identified a novel surgical modification to improve the reliability and reproducibility of the original Rice-Vannucci model of HIE. In addition, the administration of the R18 and R18D peptides following perinatal HIE, significantly reduces brain injury and improves behavioural assessments when administered up to 3.5 hours after the onset of HI. These findings demonstrate that CARPs provide an exciting and novel approach to reduce brain injury following HIE.

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DECLARATION

I hereby declare that:

- This thesis is submitted as part of the requirement for a Doctor of Philosophy degree as a result of my own work and research. All other sources have been indicated and acknowledged.
- Permission has been granted by co-authors for any work that has been co-published to be included in this thesis.
- This thesis has been substantially completed during the course of enrolment and its content has not previously been submitted or accepted for any other degree in this or any other institution.
- I understand that this work may be electronically scanned for detection of plagiarism.

Signed.....

Adam B. Edwards

Signed.....

Coordinating Supervisor: Bruno Meloni

Approval of final thesis

PUBLICATIONS ARISING FROM THIS THESIS

Edwards A., Anderton R., Knuckey N., Meloni B. 2018. Review: Perinatal hypoxic-ischaemic encephalopathy and neuroprotective peptide therapies: a case for cationic arginine-rich peptides (CARPs). *Brain Sciences*, 7; 8 (8). doi: 10.3390/brainsci8080147.

Edwards A., Anderton R., Knuckey N., Meloni B. 2018. Assessment of therapeutic window for poly-arginine-18D (R18D) in a P7 rat model of perinatal hypoxic ischaemic encephalopathy. *Journal of Neuroscience Research*, 96 (11): 1816-1826. doi: 10.1002/jnr.24315.

Edwards A., Cross J., Anderton R., Knuckey N., Meloni B. 2018. Poly-arginine R18 and R18D (D-enantiomer) peptides reduce infarct volume and improves behavioural outcomes following perinatal hypoxic-ischaemic encephalopathy in the P7 rat. *Molecular Brain*, 11 (1): 8. doi: 10.1186/s13041-018-0352-0.

Edwards A., Feindel K., Cross J., Anderton R., Clark V., Knuckey N., Meloni B. 2017. Modification to the Rive-Vannucci perinatal hypoxic-ischaemic encephalopathy model in the P7 rat improves the reliability of cerebral infarct development after 48 hours. *Journal of Neuroscience Methods*, 288. doi: 10.1016/j.jneumeth.2017.06.016.

CONFERENCE PROCEEDINGS ARISING FROM THIS THESIS

Edwards A., Anderton S., Knuckey N., Meloni B. 2017. *The poly-arginine-18 (R18) peptide is neuroprotective in a modified model of perinatal hypoxic-ischaemic encephalopathy; a dose-response and therapeutic window study*. Oral presentation at: Combined Biological Sciences Meeting; Perth, Australia.

Edwards A., Feindel K., Cross J., Anderton R., Clark V., Knuckey N., Meloni B. 2017. Neuroprotective efficacy of poly-arginine-18 (R18) peptides using an *in vivo* model of perinatal hypoxic-ishcaemic encephalopathy. *Journal of Cerebral Blood Flow and Metabolism*, 37 (IS) 1-97. doi: 10.1177/0271678X17695978.

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Edwards A., Anderton R., Knuckey N., Meloni B. 2016. *Babies' brains matter too*. Oral presentation: 3-minute thesis, 10th Federation of European Neurosciences Societies (FENS) Forum of Neuroscience; Copenhagen, Denmark.

Edwards A., Anderton R., Knuckey N., Meloni B. 2016. *Neuroprotective efficacy of poly-arginine peptides using an in vitro excitotoxicity model; implications for therapeutics*. Poster presentation at: 10th Federation of European Neurosciences Societies (FENS) Forum of Neuroscience; Copenhagen, Denmark.

Edwards A., Cross J., Anderton R., Knuckey N., Meloni B. 2016. *Neuroprotective efficacy of poly-arginine peptides using an in vitro model of neonatal hypoxic-ischaemic encephalopathy.* Poster presentation at: Combined Biological Sciences Meeting; Perth, Australia.

AWARDS ARISING FROM THIS THESIS

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Student Oral Presentation Prize

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Perth, Australia (2017).

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Berlin Brain and Brain Positron Emission Tomography Conference;

Berlin, Germany (2017)

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Student Oral Presentation Prize

Science on the Swan;

Fremantle, Australia (2018).

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LIST OF ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AUC	Area under the curve
BSS	Balanced salt solution
CARP	Cationic arginine-rich peptide
CBF	Cerebral blood flow
CCA	Common carotid artery
CCAO	Common carotid artery occlusion
CPP	Cell penetrating peptide
DNA	Deoxyribonucleic acid
ECA	External carotid artery
ECAO	External carotid artery occlusion
EPO	Erythropoietin
FAIR	Fluid attenuation inversion recovery
GABA	γ-aminobutyric acid
HI	Hypoxic-ischaemic or hypoxia-ischaemia
HIE	Hypoxic-ischaemic encephalopathy
ICA	Internal carotid artery

ICV	Intracerebroventricular
IL	Interleukin
IP	Intraperitoneal
IV	Intravenous
LPS	Lipopolysaccharide
MCAO	Middle cerebral artery occlusion
MEM	Minimum essential medium
$MgSO_4$	Magnesium sulfate
MMP	Matrix metalloproteinase
MPTP	Mitochondrial permeability transition pore
MRA	Magnetic resonance angiography
MRI	Magnetic resonance imaging
MTS	3-(4, 5, dimethyliazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H–tetrazolium salt
NMDA	N-methyl-$_D$-aspartic acid
P7	7-day-old
PASL	Pulsed arterial spin labelling
ROS	Reactive oxygen species
SD	Sprague-Dawley
SE-EPI	Spin-echo echo-planar imaging

TOF	Time of flight
TTC	Triphenyl tetrazolium chloride

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