Potential Neurosteroid Replacement Therapy Following Premature Birth and Fetal Growth Restriction

by

Meredith Anne Kelleher
Bachelor of Biomedical Science (Hons)

A thesis submitted in fulfillment of
the requirements for the degree of
Doctor of Philosophy

July, 2012

School of Biomedical Sciences & Pharmacy
Faculty of Health
University of Newcastle
Australia

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent for this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying subject to the provisions of the *Copyright Act 1968*.

I hereby certify that the work embodied in this thesis has been done in collaboration with other researchers. I have included as part of the thesis a statement clearly outlining the extent of collaboration, with whom and under what auspices.

I hereby certify that the work embodied in this thesis contains a published paper and papers submitted or prepared for submission of which I am a joint author. I have included as part of the thesis a written statement, endorsed by my supervisor, attesting to my contribution to the joint publications.

Signed: _____ *Date:* _____

STATEMENT OF CONTRIBUTION TO JOINT PUBLICATIONS

I attest that I, Meredith Kelleher, have made a primary and original contribution to the publications, and manuscripts awaiting publication, included in this thesis, as detailed below and endorsed by my supervisors.

Chapter	Title	Status	Contribution
3	Sex-Dependent Effect of a Low Neurosteroid Environment and Intrauterine Growth Restriction on Fetal Guinea Pig Brain Development	Published	Experimental design and procedures Data analysis Manuscript preparation
4	Changes in neuroactive steroid concentrations after preterm delivery in the guinea pig	Submitted and under review	Experimental design and procedures Data analysis Manuscript preparation
5	Neuroactive Steroids in preterm guinea pigs following postnatal progesterone therapy	In preparation for submission	Experimental design and procedures Data analysis Manuscript preparation

Signed (Candidate): _____ *Date:* _____

Signed (Supervisor): _____ *Date:* _____

Signed (Supervisor): _____ *Date:* _____

This thesis is dedicated to
Elizabeth Jane Mullier (1918-2010)
a great woman and the most steadfast, loving
and proud grandma, who is truly missed.

ACKNOWLEDGEMENTS

I hope that over the course of this PhD I have learnt much about "Science" and at least a little about life. It is difficult not to descend into cliches and hyperbole when trying to express the gratitude that I feel to all those people that have supported me over the past four years. I have discovered that undertaking a PhD is truly an all-encompassing, challenging, humbling and foolhardy endeavour. I have also discovered the simple joy that can accompany a successful day in the lab, the elation at finally producing that single graph of results and the pleasure that comes with solving what seemed an impossible problem. In all these things, the people around me have truly been the most important, inspirational and encouraging part. Here, in this small way, I am trying to express my absolute and profound gratitude for all that those people have made it possible for me to complete this thesis and PhD.

First and foremost to my supervisors, Jon Hirst and Hannah Palliser, you have shaped the way I think and the scientist I have become. You have encouraged, challenged, questioned, supported and inspired me. Jon, your knowledge, helpful advice and good humour are always appreciated. It has truly been an honour to be able to work with you and I hope to have your advice and support in the years to come as I continue to learn and grow as a scientist. Hannah, I consider it a privilege to have been your first PhD student. I hope that (if my opinion counts for anything) I can express how great a job you have done and how significant you have been in my life and career so far. You encourage and inspire me daily. I am so fortunate to have you as a friend, and as a colleague. I would not have been able to reach this point without you. Thank you (and thanks to Dave too).

To all of the amazing friends and workmates who have witnessed "the process" and supported me through it, you have been more important to me than you could know and I will miss you greatly as I move on to new challenges. Bec (Beccy D, Rebecca Dyson), you kept me sane (??), entertained, fed, amused and

encouraged. I am especially grateful for your help and friendship and know that as you also near the end that you will do so stunningly. Kirsty, you are truly someone I respect, value and admire for your strength and brilliance at work and in life. Thank you for your perspective, warmth and above all friendship (and your delightfully distracting family). Della, you are a true friend that I would not have come across if not for choosing this course. Your friendship and help made this possible. To the people that have more recently become a part of our little work family: I wasn't always like this, it was the PhD (I'm sure this is true if only I could remember a time before it). Britt, you have been an amazing help over the past couple of years and I appreciate it greatly. Greer, remember how lucky you are to have Jon and Hannah, don't listen to anything I've said and good luck. Kate, I'm so glad to have you as a friend, my life is richer for it. Jo, your socks are always entertaining and so are you. Ian Wright, your knowledge and help was invaluable. To everyone at MBRC, you have all influenced and challenged me in different ways and I appreciate all of your warmth, help, advice and friendship.

To those good friends who have stuck with me, even when this PhD has kept me from giving much of my time, I appreciate all of your encouragement and often needed commiseration. Most particularly to Belinda, you have so much spirit and enthusiasm for life, thank you for sharing some of it with me over these many long years. To Jess and Megan, thanks for the fun, the friendship and the freedom.

To my family, although I may have bored you senseless with all this PhD talk, I truly appreciate your patience, encouragement and support. You are the people that set me on this path and made it possible for me to continue along it. Thank you Patrick, Naomi, Lauren, Dani, Phoebe and Heidi for providing me with the most fun, supportive and loving holiday destination. I am very lucky to have you as family. Dad, you have always encouraged me to ask questions, which is one of the greatest gifts. Mum, your love, strength, wisdom and kindness are truly special. Thank you for making this possible. I cannot express how grateful and proud I am to be your daughter.

Cheers.

TABLE OF CONTENTS

ABSTRACT

Events during gestation and changes in the intrauterine environment contribute to abnormal development and injury in the immature brain, influencing health and disease throughout life. Progesterone and its neuroactive steroid metabolite, allopregnanolone, are present in high concentrations during pregnancy. Allopregnanolone signalling at the $GABA_A$ receptor has important trophic and neuroprotective effects. The disruption of neuroactive steroid concentrations due to complications such as intrauterine growth restriction (IUGR) or preterm birth may therefore adversely affect brain development and increase perinatal brain injury.

Inhibition of allopregnanolone synthesis was assessed in fetal guinea pigs after surgery to induce IUGR. Both fetal brain and plasma allopregnanolone concentrations were reduced by finasteride treatment. Finasteride treatment and IUGR were associated with reduced myelination and IUGR with increased astrocyte activation in the brain.

A model of premature birth (0.87 gestation) was developed in the guinea pig to assess the effect of preterm postnatal changes in neuroactive steroid concentrations on the developing brain. Preterm guinea pigs exhibited less activity, higher mortality rates, reduced allopregnanolone concentrations and lower expression of steroid synthetic enzymes. Myelination in the hippocampus and cerebellum was also suppressed.

The potential of postnatal replacement of neuroactive steroids by progesterone treatment was examined in preterm neonates. Following progesterone therapy, cortisol levels were elevated, with implications for development. Sex differences were noted in plasma neuroactive steroid concentrations. Brain allopregnanolone concentrations in preterm neonates were increased at postnatal days 1 and 8 by progesterone administration. Exploratory behaviours were altered in progesterone treated preterm animals, demonstrating changes in brain function associated with treatment.

This thesis identifies changes in the perinatal guinea pig brain associated with altered neuroactive steroid concentrations and establishes the efficacy of progesterone replacement therapy in augmenting the endogenous synthesis of allopregnanolone in the preterm brain. Long-term studies to establish the developmental outcomes of postnatal progesterone/neuroactive steroid replacement after preterm birth and in combination with complications such as IUGR, hypoxic insults and infection are needed to identify new, safe and effective treatment options.

LIST OF FIGURES & TABLES

LIST OF PUBLICATIONS

Publications Arising from this Thesis:

Kelleher MA, Palliser HK, Hirst JJ

(Under preparation for submission to Pediatric Research)

Neuroactive steroids in preterm guinea pigs following postnatal progesterone therapy.

Kelleher MA, Hirst JJ, Palliser HK

(Submitted to Journal of Reproductive Science, 2012)

Changes in neuroactive steroid concentrations after preterm delivery in the guinea pig.

Kelleher MA, Palliser HK, Walker DW, Hirst JJ (2011)

Sex-dependent effect of a low neurosteroid environment and intrauterine growth restriction on fetal guinea pig brain development. *J Endocrinol.* 208(3): 301-309

Publication Arising from this Thesis, results not presented:

Dyson RM, Palliser HK, Kelleher MA, Hirst JJ, Wright IMR (2012)

The Guinea Pig as an animal model for studying perinatal changes in microvascular function. *Pediatr Res* 71(1): 20-24

LIST OF CONFERENCE ABSTRACTS

Bennett GA, Palliser HK, Kelleher MA, Saxby BM, Walker DW, Hirst JJ (2012) Prenatal maternal psychosomatic stress: Effects on fetal brain development following maternal neurosteroid treatment in guinea pigs *39th Annual Meeting of The Fetal and Neonatal Physiological Society, Utrecht, The Netherlands.* **Abstract O28**

Bennett GA, Palliser HK, Kelleher MA, Saxby BM, Walker DW, Hirst JJ (2012) Prenatal stress and effect of maternal neurosteroid treatment on fetal brain development in guinea pigs. *Perinatal Society of Australia and New Zealand Annual Scientific Meeting. Perinatal Society of Australia and New Zealand Annual Scientific Meeting.* **Abstract A098**

Kelleher MA, Palliser HK, Hirst JJ (2011) Sex and progesterone: Improving preterm survival? *38th Annual Meeting of The Fetal and Neonatal Physiological Society, Palm Cove, Queensland, Australia.* **Abstract 040**

Kelleher MA, Palliser HK, Hirst JJ (2011). Neurosteroid replacement therapy in the preterm neonate. *38th Annual Meeting of The Fetal and Neonatal Physiological Society, Palm Cove, Queensland, Australia.* **Abstract 133**

Kelleher MA, Palliser HK, Hirst JJ (2011) Progesterone replacement therapy & brain development in the preterm neonate. *Australian Society for Medical Research, 19th Annual NSW Scientific Meeting, Sydney, Australia.*
Abstract O3.5

Kelleher MA, Palliser HK, Hirst JJ (2011) A guinea pig model for the study of neuroactive steroid replacement in the preterm neonatal brain. *Annual Meeting of the Society for Gynecologic Investigation, Miami, USA.* **Abstract S-241**

Dyson RM, Palliser HK, Kelleher MA, Hirst JJ, Wright IMR (2010) The guinea pig as an animal model for studying microvascular function in the preterm neonate in early extrauterine life. *9th World Congress for Microcirculation, Paris, France.*

Kelleher MA, Palliser HK, Hirst JJ (2010) Premature birth results in ex utero brain development in a low neuroprotective steroid environment. *Annual Scientific Meeting of The Endocrine Society of Australia, Sydney, Australia.* **Abstract 477**

Dyson RM, Palliser HK, Kelleher MA, Hirst JJ, Wright IMR (2010) Preterm birth and intrauterine growth restriction: effect on microvascular function in the neonatal guinea pig. *Annual Scientific Meeting of The Endocrine Society of Australia, Sydney, Australia.* **Abstract 475**

Kelleher MA, Palliser HK, Walker DW, Hirst JJ (2010) Neuroprotective deficits in the preterm guinea pig brain. *37th Annual Fetal and Neonatal Physiological Society Meeting, University of Winchester, UK.* **Abstract O47**

Kelleher MA, Palliser HK, Hirst JJ (2010) Compromised neurosteroid biosynthesis in the preterm neonate. *Perinatal Society of Australia and New Zealand Annual Scientific Meeting, Wellington, New Zealand.* **Abstract A027**

Dyson RM, Kelleher MA, Palliser HK, Wright IM (2010) The guinea pig as an animal model for perinatal vascular changes? *Perinatal Society of Australia and New Zealand Annual Scientific Meeting, Wellington, New Zealand.* **Abstract A088**

Kelleher MA, Palliser HK, Walker DW, Hirst JJ (2009) Effect of intrauterine growth restriction and pharmacologic inhibition of 5α-reductase on enzyme expression in the fetal cerebellum. *Perinatal Society of Australia and New Zealand Annual Scientific Meeting, Darwin, Australia.* **Abstract P067**

Kelleher MA, Palliser HK, Yates DM, Sullivan RKP, Walker DW, Hirst JJ (2008) Effect of 5α-reductase inhibition on apoptotic brain cell death and the expression of neurosteroidogenic enzymes in the fetal and neonatal guinea pig. *Perinatal Society of Australia and New Zealand Annual Scientific Meeting, Gold Coast, Australia.* **Abstract A13**

Kelleher MA, Palliser HK, Yates DM, Sullivan RKP, Walker DW, Hirst JJ (2008) Effect of inhibition of 5α-reduced steroid synthesis on apoptotic brain cell death and neurosteroidogenic enzyme expression in the fetal and neonatal guinea pig. *Annual Meeting of the Society for Gynecologic Investigation, San Diego, USA.* **Abstract 227**

LIST OF ABBREVIATIONS

3α,5α-THP	3α,5α-tetrahydroprogesterone; allopregnanolone
3β-HSD	3β-hydroxysteroid dehydrogenase
5α-DHP	5α-dihydroprogesterone
5αR	5α-reductase enzyme
5αR1	5α-reductase enzyme type 1
5αR2	5α-reductase enzyme type 2
AC	adenylate cyclase
ADHD	attention deficit and hyperactivity disorder
AMPA	2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid
ANOVA	analysis of variance
ATP	adenosine triphosphate
B_o	tracer-antisera binding
Bax	Bcl-2-associated X protein
BBB	blood-brain barrier
Bcl-2	B-cell lymphoma 2 protein
BDNF	brain-derived neurotrophic factor
BLR	brain to liver weight ratio
BSA	bovine serum albumin
CA1	cornu ammonis area 1 of the hippocampus
Ca^{2+}	calcium ion
cAMP	cyclic adenosine monophosphate
Cl^-	chloride ion
CNS	central nervous system
CP	cerebral palsy
CPAP	continuous positive airway pressure
CSF	cerebrospinal fluid
CRH	corticotropin-releasing hormone

Cu^{2+}	copper ion
DAB	3,3'-diaminobenzidine
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGL	external granular cell layer
EIA	enzyme immunoassay
ERK	extracellular signal-regulated kinase
FGR	fetal growth restriction
Fin	finasteride
GA	gestational age
GABA	γ-amino-butyric acid
$GABA_A$	γ-amino-butyric acid type A receptor
GFAP	glial fibrillary acidic protein
H_2O	water
hCG	human chorionic gonadotropin
HCl	hydrogen chloride
HRP	horseradish peroxidase
IgG	immunoglobulin G
IGL	internal granular cell layer
IL	interleukin
i.p.	intraperitoneal
IQ	intelligence quotient
IUGR	intrauterine growth restriction
IVH	intraventricular haemorrhage
K^+	potassium ion
KCC2	potassium chloride co-transporter 2

$KMnO_4$	potassium permanganate
LPS	lipopolysaccharide; endotoxin
MAP-2	microtubule-associated protein 2
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
ML	molecular layer
MMP	matrix metalloproteinase
MOPS	3-(N-morpholino)propanesulfonic acid
mPR	membrane progesterone receptor
MRI	magnetic resonance imaging
Na^{2+}	sodium ion
NaCl	sodium chloride
NaN_3	sodium azide
NAPDH	nicotinamide adenine dinucleotide phosphate
NICU	neonatal intensive care unit
NKCC1	sodium potassium chloride co-transporter 1
NMDA	N-methyl-D-aspartate
NORT	novel object recognition test
NOS-2	nitric oxide synthase enzyme 2
NSB	non-specific binding
O_2	oxygen
OF	open field
OFR	oxygen free radical
P450scc	cholesterol side-chain cleavage enzyme
PAGE	polyacrylamide gel electrophoresis
PB	phosphate buffer
PBS	phosphate buffered saline
PEEP	positive end expiratory pressure
PFA	paraformaldehyde
PG	prostaglandin

PGRMC1	progesterone receptor membrane component 1
PI3K/Akt	phosphoinositide 3-kinase/protein kinase B
PIP	peak inspiratory pressure
PKA	protein kinase A
PKG	protein kinase G
PND	postnatal day
PPROM	preterm premature rupture of membranes
PR	progesterone receptor
PRE	progesterone response element
Pre-T	preterm postnatal day 1
Pre-T8	preterm postnatal day 8
+Prog	preterm postnatal day 1 with progesterone treatment
+Prog8	preterm postnatal day 8 with progesterone treatment
PVDF	polyvinylidene fluoride
PVL	periventricular leukomalacia
RDS	respiratory distress syndrome
RIA	radio-immunoassay
ROP	retinopathy of prematurity
RU486	mifepristone; progesterone receptor antagonist
σ1	sigma 1 receptor
s.c.	subcutaneous
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SGA	small for gestational age.
SIDS	sudden infant death syndrome
T1	novel object recognition test, trial 1 (familiarisation)
T2	novel object recognition test, trial 2 (recognition)
TBPS	t-butylbicyclophosphorothionate
TBS-T	tris-buffered saline with tween
TC	total counts

THDOC	tetrahydrodeoxycorticosterone
TNF-α	tumour necrosis factor-α
UCO	umbilical cord occlusion
WHO	World Health Organisation
<	less than
=	equal to
>	greater than
±	plus or minus
~	approximately
°C	degrees celsius
/	per
%	per cent
v/v	volume per volume
w/v	weight per volume
cm	centimetre
g	gram
hr	hour
kDa	kilodalton
kg	kilogram
L	litre
cpm	counts per minute
mA	milliamp
mg	milligram
mL	millilitre
mm	millimetre
mM	millimolar
mmol	millimole

ng	nanogram
nm	nanometre
nmol	nanomole
pH	scale of hydrogen ion activity
pmol	picomole
rpm	revolutions per minute
sec	second
V	volts
W	watts
µg	microgram
µL	microlitre
µm	micrometre
µmol	micromole