

EFFECT OF PRE AND POSTNATAL NEUROSTEROID THERAPY ON NEURODEVELOPMENT AND BEHAVIOUR

Julia Catherine Shaw

Bachelor of Biomedical Science (Honours Class I)

School of Biomedical Sciences and Pharmacy

Faculty of Health and Medicine

University of Newcastle

A thesis submitted in fulfillment of the requirements for the degree of
Doctor of Philosophy (Experimental Pharmacology)

September 2017

This research was supported by an Australian Government Research
Training Program (RTP) Scholarship

DECLARATION

I hereby certify that the work embodied in the thesis is my own work, conducted under normal supervision.

I hereby certify that this thesis is in the form of a series of *papers. I have included as part of the thesis a written statement from each co-author, endorsed in writing by the Faculty Assistant Dean (Research Training), attesting to my contribution to any jointly authored papers. (*Refer to clause 39.2 of the Rules Governing Research Higher Degrees for acceptable papers).

The thesis contains no material which has been accepted, or is being examined, 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 to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository, subject to the provisions of the Copyright Act 1968 and any approved embargo.

Signed: _____

Date: 15/09/17

Julia Shaw, Bachelor of Biomedical Science (Honours Class I)

University of Newcastle

This thesis is dedicated to mama bear and papa bear,

Jacqueline and Steven Shaw.

Thank you for doing all that you do for me.

This would not have been possible without you.

ACKNOWLEDGEMENTS

Completing this PhD has been the biggest milestone of my life thus far, if I knew 8 years ago that I would go on to hold a doctorate I would not have believed it. There is no way that this would have been possible without the ongoing support of so many people in my family, social, and workplace circles. Firstly, to my supervisors Jon Hirst and Hannah Palliser – you obviously can't do a PhD without supervisors and I believe that I hit the jackpot with mine. Thank you for always trusting in my ability (maybe a little too much!) and for providing such a welcoming environment for my introduction to research. Please take the fact that I wish to stay and work with you both as an enormous compliment, I look forward to what the future holds for us all. Jon, your wisdom never ceases to amaze me and your quirky sense of humour is something I will always remember fondly. An extra thank you to Hannah for allowing me to pursue the New Zealand collaboration with you and for accompanying me on those first intense trips (what an eye opener!). I think we formed a bond then that we would not have formed otherwise and I feel privileged to have performed this work with you. Whilst on the topic of New Zealand, much of my PhD would not have been possible without Max Berry and Rebecca Dyson. You are both such intelligent women and I am in awe of your dedication to your work. Thank you for entrusting me with samples to process and allowing me to spend time in your facility and learn from you both. Thank you to my mentor, Kirsty Pringle, who is another female scientist that I look up to and feel privileged to have worked with. Thank you for always welcoming me into your office for a chat when I needed, providing direction when I was lost, and for loving a drink as much as me – I can always count on you for a beverage in good and bad times.

Over the past 4 years I have had many ups and downs, and without Bridie Goggins and Sarah Tew by my side I don't know how I would have kept going. I have so much love in my heart for you both!! From holidaying in Europe, to life at the infamous Heartbreak Hotel and all the "fun times" it involved (it was the best of times, and it was the worst of times hehe), I would have been lost without you both and can never thank you enough for sticking with me through thick and thin. Bridie, quit following me already (actually please don't). To Jess Willard, you were off gallivanting and getting the most out of life for most of my PhD but we always picked up straight where we left off once you got back. You got me through my TAFE days and perhaps I may not have made it into Biomedical Science without you – I'm so glad I came up to introduce myself to you on our first day and still laugh at that memory. Sam Wilson, my arch nemesis turned loving friend, thank you for telling me I'm smart when I feel dumb, drinking cider with me every chance we get, and missing me when I'm working too hard and reminding me to take a break to see you.

Sarah Delforce, my work wifey, I love you. I can't imagine spending late nights at work with anyone else. Your constant presence (go home once in a while!) and offers of cups of tea got me through each working week. I hope we work together forever and ever and ever. I also thoroughly enjoyed scrolling for memes with Gabrielle Crombie (little Bigfoot), as our new late nights buddy, and love that I can share my dark sense of humour with you both. Samantha, we were destined to be close from the day you started at MBRC and I asked you to live with me – I could tell you were a good egg and my kind of person. Thank you for early morning chats when I needed, for being my occasional housemate, and for our weekends together in Mussie – I'm always up for visit that involves wine and cheese in our PJs. I'm so glad that you

are my desk buddy and I look forward to coming in to see your smiling face every morning – even when you're a grump, it makes stirring you up more fun hehe. To everyone else in the Hirst/Pringle groups – Saijey, Celine, Minoo (our token Smith member), Anya, Eugenie (the smartest person I know), Poonam, Yu Qi, Riaz, and Rohan, as well as past members Angie, Greer, Kirsten, and Eric – you are all what makes this such a wonderful work environment and I know that wherever you all end up in life that you will invoke joy in those around you.

Finally, thank you to my family and Kyle James. Kyle, you have had such a positive influence on me – I have so much more confidence in myself as well as happiness in my life since being with you. I love you, finding you felt like coming home, and there is nowhere else I would rather be. Thank you for letting me work when I needed to and for always being there with open arms at the end of the day. Caroline Ruppe, the best auntie anyone could ever have – your support, especially in the early days, has always meant so much to me and helped me more than you would know. Coming to have an afternoon cup of tea with mum I smile seeing your car parked out the front and feel lucky to have such a wonderful relationship with you. Geoffrey, Peffrey, Pfeffer, the Peff – Hello (you know the voice) my big little brother, thank you for deigning to speak to me sometimes. I cherish every word that comes out of your mouth as they are so few and far between. Never change, I love you the stubborn way that you are. Finally, a massive thank you to my parentals – Jacqueline and Steven, you are both my inspirations and are my rocks. The love and unwavering support that I receive from you both makes me feel so special. I am the luckiest to call you my parents. My love for you is endless.

CONTENTS

DECLARATION	2
ACKNOWLEDGEMENTS	4
CONTENTS	7
ABSTRACT	10
LIST OF TABLES	13
LIST OF FIGURES	14
LIST OF ABBREVIATIONS	16
PUBLICATIONS FOR INCLUSION	20
ADDITIONAL PUBLICATIONS	21
1.0 INTRODUCTION	22
1.1 PRETERM BIRTH	23
1.1.1 OCCURRENCE AND CAUSES	23
1.1.2 PREVENTION OF PRETERM LABOUR	25
1.1.3 OUTCOMES FOLLOWING PRETERM BIRTH	27
1.2 DEVELOPMENT OF THE BRAIN	30
1.2.2 CELL TYPES IN THE BRAIN	36
1.2.3 IMPORTANCE OF THE PLACENTA FOR NEURODEVELOPMENT <i>IN UTERO</i>	38
1.2.4 <i>EX UTERO</i> NEURODEVELOPMENTAL CONSEQUENCES FOLLOWING PRETERM BIRTH	39
1.3 NEUROSTEROIDS	44
1.3.1 THE <i>IN UTERO</i> NEUROSTEROID ENVIRONMENT AND FETAL NEURODEVELOPMENT	44
1.3.2 ALLOPREGNANOLONE ACTION ON GABA _A RECEPTORS	50
1.3.2.1 NEUROSTEROID MODULATION OF GABA _A RECEPTORS	54
1.3.2.2 GABA _A RECEPTORS AND GLUCOCORTICOIDS	55
1.3.2.3 THE GABAERGIC SYSTEM	56
1.3.3 ALLOPREGNANOLONE FOLLOWING PRETERM BIRTH	57
1.3.4 NEUROSTEROIDS AS A THERAPEUTIC INTERVENTION	58
1.4 GANAXOLONE	60
1.4.1 GANAXOLONE CLINICAL TRIALS	62
1.5 PRETERM MODEL, HYPOTHESIS, AND AIMS	62
1.5.1 GUINEA PIG PRETERM MODEL	62
1.5.2 HYPOTHESES	64
1.5.3 AIMS	65

2.0	METHODS	66
2.1	ANIMAL ETHICS	66
2.2	ANIMAL MODELS	66
2.2.1	PRETERM FETUS AND NEONATE	66
2.2.2	PRETERM JUVENILE	67
2.2.3	PRENATAL PROGESTERONE THERAPY	68
2.2.4	PRETERM NEUROSTEROID-REPLACEMENT THERAPY	69
2.3	ANIMAL CARE	70
2.3.1	HOUSING AND FEEDING	70
2.3.2	CAESAREAN SECTION DELIVERY	71
2.3.3	PRETERM CARE FOLLOWING CAESAREAN SECTION	72
2.3.4	PRETERM INDUCTION OF LABOUR	74
2.3.5	PRETERM CARE FOLLOWING INDUCTION OF LABOUR	75
2.4	BEHAVIOURAL TESTING	77
2.4.1	OPEN FIELD	77
2.4.2	ENVIRONMENT EXPLORATION	77
2.4.3	SOCIAL INTERACTION	78
2.4.4	ANALYSIS	78
2.5	TISSUE AND FLUID COLLECTION	79
2.5.1	EUTHANASIA	79
2.5.2	PLASMA	79
2.5.3	BRAIN	80
2.1.1	ORGANS	81
2.2	PLASMA STEROID ANALYSES	82
2.2.1	ALLOPREGNANOLONE PLASMA RADIOIMMUNOASSAY	82
2.2.2	CORTISOL PLASMA ENZYME IMMUNOASSAY	84
2.2.3	PROGESTERONE PLASMA CHEMILUMINESCENT MICROPARTICLE IMMUNOASSAY	85
2.3	SALIVA STEROID ANALYSES	86
2.3.1	CORTISOL SALIVA ENZYME IMMUNOASSAY	86
2.3.2	PROGESTERONE SALIVA ENZYME IMMUNOASSAY	87
2.4	IMMUNOHISTOCHEMISTRY	88
2.4.1	TISSUE PREPARATION	88
2.4.2	STAINING PROCEDURES	88
2.4.3	ANALYSIS	90
2.5	REAL-TIME POLYMERASE CHAIN REACTION	93
2.5.1	TISSUE PREPARATION	93
2.5.2	RNA EXTRACTION AND GEL	93
2.5.3	REVERSE TRANSCRIPTION	96
2.5.4	PRIMER DESIGN	97
2.5.5	REAL TIME POLYMERASE CHAIN REACTION	99
2.5.6	COMPARATIVE CT METHOD OF ANALYSIS	101
2.6	WESTERN BLOT	101
2.6.1	RIPA EXTRACTION	101
2.6.2	BCA ASSAY	102

2.6.3	WESTERN BLOT	103
2.6.4	STAINING PROCEDURES	104
2.6.5	ANALYSIS	105
2.7	STATISTICAL ANALYSIS	106
2.7.1	CHAPTER THREE	106
2.7.2	CHAPTER FOUR	106
2.7.3	CHAPTER FIVE	106
2.7.4	CHAPTER SIX	107
2.7.5	CHAPTER SEVEN	108
3.0	“PRETERM BIRTH AFFECTS GABA_A RECEPTOR SUBUNIT MRNA LEVELS DURING THE FOETAL-TO-NEONATAL TRANSITION IN GUINEA PIGS”	109
4.0	“LONG-TERM EFFECTS OF PRETERM BIRTH ON BEHAVIOUR AND NEUROSTEROID SENSITIVITY IN THE GUINEA PIG”	122
5.0	“DISRUPTION OF THE CEREBELLAR GABAERGIC SYSTEM IN JUVENILE GUINEA PIGS FOLLOWING PRETERM BIRTH”	133
6.0	“ADMINISTRATION OF PROGESTERONE THROUGHOUT PREGNANCY INCREASES MATERNAL STEROIDS WITHOUT ADVERSE EFFECT ON MATURE OLIGODENDROCYTE IMMUNOSTAINING IN THE GUINEA PIG”	162
7.0	“NEUROSTEROID REPLACEMENT THERAPY USING THE ALLOPREGNANOLONE-ANALOGUE GANAXOLONE FOLLOWING PRETERM BIRTH IN THE GUINEA PIG”	175
8.0	DISCUSSION	209
8.1	EFFECTS OF PREMATURE EXPOSURE TO THE <i>EX UTERO</i> ENVIRONMENT ON NEURODEVELOPMENT AND BEHAVIOUR	210
8.2	EFFECTS OF PRENATAL PROGESTERONE AND NEUROSTEROID-REPLACEMENT THERAPIES ON NEURODEVELOPMENT AND BEHAVIOUR	218
8.3	CONCLUSION	224
8.4	FUTURE DIRECTIONS	225
9.0	REFERENCES	227
10.0	APPENDIX	250
10.1	IMMUNOBLOTTING OF PLACENTAL 11BHSD2	250
10.2	SUPPLEMENTARY DATA TABLE FOR CHAPTER FIVE	251
10.3	SUPPLEMENTARY DATA TABLES FOR CHAPTER SIX	252

ABSTRACT

Children that are born preterm are at an increased risk of developing late onset cognitive problems and behavioural disorders, such as attention deficit hyperactivity disorder (ADHD) and anxiety, with males being particularly vulnerable. The mechanisms by which this happens are poorly understood; however, actions and modulation of GABA_A receptor signalling by the neurosteroid allopregnanolone has a major role in late gestation neurodevelopment, and we believe that the early loss of placentally-derived allopregnanolone following preterm birth is pivotal to the development of these disorders. There is increasing interest in the development of the hippocampus and cerebellum following preterm birth and the potential involvement of GABAergic pathways in neurodevelopmental disorders. In these studies, we propose that the early loss of the intrauterine trophic environment as a result of preterm birth alters the development of the hippocampus and cerebellum, contributing to ongoing neurobehavioral disorders. We anticipate that neurosteroid-replacement therapy with ganaxolone (GNX) following preterm birth may prevent the deficits in neonatal development that contribute to these disorders. We further propose that maternal administration of progesterone, which is commonly administered prophylactically to women at risk of preterm labour, may have unforeseen effects on fetal neurodevelopment due to the ability of progesterone to be metabolized to a number of steroids with varying effects on development including allopregnanolone and cortisol.

We found that there is an adaptive increase in the mRNA levels of GABA_A receptors involved in neurosteroid action after term birth in the guinea pig neonate,

but not after preterm birth. The increased levels in the term neonate may compensate for the dramatic decline in allopregnanolone levels following separation from the placenta, and this lack of an adaptive increase in the preterm neonate may heighten the adverse effect of the premature decline in neurosteroid exposure. Preterm neonates also had deficits in myelination of the hippocampus, subcortical white matter, and cerebellum. At juvenile age these deficits remained in the hippocampus, subcortical white matter, and female cerebellum. Interestingly, increased myelination of the male cerebellum at juvenility, suggesting a deficit in axonal pruning, was observed in conjunction with a dysregulation of the cerebellar GABAergic system. In addition to the white matter alterations at juvenility, male guinea pigs that were born preterm exhibited a hyperactive, ADHD-type phenotype, whilst females had anxious behaviour. Unexpectedly, maternal progesterone therapy did not affect fetal allopregnanolone or cortisol steroid levels, nor did it have an effect on myelination of the hippocampus. Circulating maternal cortisol was increased, but fortunately the placental enzymatic barrier protected the fetus from this potentially damaging rise in cortisol. Novel neurosteroid-therapy replacement therapy using ganaxolone during the immediate neonatal period following preterm birth improved the neurobehavioural outcomes of the male juvenile offspring by ameliorating myelination deficits and returning behavioural phenotypes to term-born levels.

Therefore, through utilizing our guinea pig model of preterm birth, we were able to conclude that the GABAergic system and its effects on myelination are disrupted following preterm birth and that this occurs in parallel with a hyperactive phenotype in males, and conversely an anxious phenotype in females. Furthermore, whilst prenatal progesterone therapy does not influence fetal allopregnanolone levels,

restoring the *in utero* neurosteroid environment for preterm neonates following preterm birth may be a viable therapy to prevent the onset of behavioural and learning disorders.