



***In Utero* Androgen Administration Induces Changes in Gene Expression and Purkinje Cell Development in the Cerebellum**

Miss Lisa-Marie Wilson

BSc (Hons)

Doctorate of Philosophy

June 2015

A thesis submitted in partial fulfilment of the requirements of Edinburgh Napier University, for the award of Doctor of Philosophy

## **Declaration**

I hereby declare that the work presented in this thesis was carried out by the author, and where this is not the case, this is referred to in the text. This thesis is submitted for the fulfilment of the degree of Doctor of Philosophy, and not for any other degree or qualification.

Lisa-Marie Wilson

June 2015

## Abstract

Steroids play a major role in the development of the CNS with those brain areas involved in sexual behaviour having been the focus of most neuroendocrine studies to date *e.g.* the hypothalamus and pituitary gland (Feist and Schreck, 1996; Mong, *et al.*, 1999; Toran-Allerand, *et al.*, 1980). This occurs via intracellular and cell-surface receptors that regulate changes in protein synthesis (Mensah-Nyagan, *et al.*, 1999) to modify events related to neuronal survival and synapse formation (Breedlove, 1992).

Many steroidogenesis-associated enzymes have been described in the cerebellum and cortex, but the impact of steroids on their development has yet to be investigated. A steroidal fetal programming model was utilised to investigate how gene expression in these two brain regions is affected by steroid exposure during development, and examine how this may lead to a change in brain architecture and function.

The main aims of this study were to identify changes in the cerebellar expression of genetic markers of steroid metabolism, using quantitative real-time polymerase chain reaction (qRT-PCR), that result from exposure to testosterone propionate (TP) during development in male sheep. The cerebellum follows a clear developmental trajectory and contains an established cytoarchitecture that enabled us to readily identify the effects of TP treatment on the developing brain by utilising histological analyses. Further alterations of gene expression in the developing male ovine cortex were also identified using microarray analysis. The results provide novel findings in regards to androgen-sensitive gene expression in the developing ovine cerebellum and cortex but perhaps the most striking result was that androgen over-exposure delays cerebellar development, which may have consequences in later life on motor and/or cognitive function.

## **Acknowledgements**

There are a number of people I would like to take the time to thank, who have been there for me for the duration of my PhD. First and foremost, I would like to extend my gratitude to Dr. Claire Garden (Director of Studies) and Dr. Mick Rae (Second Supervisor) for their immense advice, guidance and support these past three and a half years. Claire's professionalism and brilliant mind has taught me a great deal in terms of designing and executing experiments and being able to clearly communicate my interpretation of results. Claire has also given me multiple opportunities such as taking tutorials, being a lab demonstrator, attending a varied selection of conferences, seminars and symposiums and dissecting brains, for which I am extremely grateful for. These experiences were worthwhile and will always be in my fond memories. Mick has been a pillar of support and always there when I needed advice. Our lengthy discussions about science and banging the world to rights always made me chuckle – his terrible jokes did not though. I would not have gotten this far without my two wonderful mentors, as they have motivated and inspired me to reach my full potential – not just as a scientist but as a person too. I am indebted to Claire and Mick and thank them for making my PhD such a worthwhile experience.

I would also like to thank Dr. Colin Duncan (University of Edinburgh) for allowing me to obtain the samples needed for my studies from his cohort subjects. Without his kindness, my project would have not been possible. Particular thanks is also extended Mr. Cathal Grace, brilliant technician and problem solver, known to the postgraduate students at Edinburgh Napier. His extensive knowledge and experience guided me through technical hitches – Cathal never gave up and always had an alternative technique/protocol when I felt nothing was ever going to work, which was often! Between 2010 and 2011, I had a sponsorship with PrimerDesign U.K. Ltd., which allowed for discount of consumables and training on good quantitative real-time polymerase chain reaction. I would like to thank them for their generosity and choosing me for their sponsorship scheme. Their dedicated staff have assisted me throughout my entire project.

The only people who knew exactly what I was feeling at any given time were the other postgraduate students within the Faculty of Health, Life and Social Sciences at Edinburgh Napier. I am very privileged to have met these talented and sometimes crazy people whom I know have become life long friends with. In some way, it feels like we are all one, big family and although we are heading off on different paths, I know these paths will cross again in the future. In particular, I would like to thank Seshadri Ramaswamy for his beautiful friendship, advice and for always being at the end of a phone if I needed help in an instant.

I would also like to take this opportunity to thank my family for putting up with me these past three and a half years – my Mum Anne-Marie, Dad Brian and brother Daniel. Their endless support, understanding, patience, strength and love are the basis for every success I have. I couldn't ask for a better family unit. I would also like to mention my grandparents, Giovanni, Anne and Jean, and my Aunt Angelina, who have always inspired me to work hard and reach my full potential.

Last and by no means least I need to extend my gratitude to my wonderful best friends, Nicola McDevitt, Kerry Allan, Mhairi-Clare Galletta and Lauren Clark. These ladies have always been there for me, through the fantastic times and the frustrating times. Without them, chocolate and a bottle of wine and/or gin, I probably would have gone insane during my project. I look forward to many more amazing times ahead. I love you all and appreciate you so much for being my shoulder to cry on during the lulls and someone to high five when things have gone my way.

I would like to dedicate this thesis to my cat Stella, who is my precious little princess. Stella was always around and happy to play or get her tummy rubbed, when Mummy needed a break from writing.

## Table of Contents

Declaration.....	ii
Abstract.....	iii
Acknowledgements.....	iv
Abbreviations.....	xvi
List of Figures.....	xix
List of Tables.....	xxii
CHAPTER 1.....	1
1.1 The cerebellum.....	2
1.1.1 Cerebellar structure.....	2
1.1.1.1 Cerebellar Purkinje Cells.....	3
1.1.1.2 Granule Cells.....	4
1.1.1.3 Golgi Cells.....	5
1.1.1.4 Stellate/Basket Cells.....	5
1.1.2 Gene products that facilitate the development of the cerebellum.....	5
1.1.2.1 BDNF.....	5
1.1.2.2 FOS.....	6
1.1.2.3 GAD1.....	6
1.2 The cortex.....	7
1.2.1 Structure and function.....	7
1.2.1.1 Cortex layers.....	8
1.2.1.2 Pyramidal cells.....	9
1.3 Fetal brain development.....	10
1.3.1 Prenatal period.....	10
1.3.1.1 Neuron production.....	10
1.3.1.2 Neuron migration.....	11
1.3.1.2.1 The cerebellum.....	11
1.3.1.2.1.1 Synaptogenesis.....	13
1.3.1.2.2 The cortex.....	14
1.3.1.2.2.1 Synaptogenesis.....	16
1.3.1.3 Neuron differentiation.....	16
1.3.1.4 Regressive events – fetal period.....	17

1.3.1.4.1 Apoptosis.....	18
1.3.1.5 Postnatal period.....	19
1.3.1.5.1 Postnatal proliferation and migration.....	19
1.3.1.5.2 Myelination.....	20
1.3.1.6 Regressive events occurring post-natally.....	22
1.3.1.6.1 Apoptosis – glial populations.....	22
1.3.1.6.2 Synaptic pruning.....	22
1.4 Steroids.....	24
1.4.1 Steroidogenesis and hormone receptor signalling.....	24
1.4.1.1 The aromatase pathway.....	25
1.4.1.2 The 5 $\alpha$ -reductase pathway.....	26
1.4.1.3 The progesterone pathway.....	26
1.4.1.3.1 Non-classical progesterone signalling – GABA.....	27
1.4.2 Peripheral steroids.....	30
1.4.3 Neurosteroids.....	30
1.4.3.1 The cerebellum.....	30
1.5 The impact of steroids on brain development.....	31
1.5.1 The role of androgen in brain development.....	31
1.5.1.1 How testosterone influences development of different brain regions.....	33
1.5.2 Clinical implications.....	33
1.5.2.1 Sexual differentiation.....	33
1.5.2.1.1 Gene expression and sexual dimorphism.....	35
1.5.2.2 Congenital adrenal hyperplasia.....	35
1.5.2.3 Neurodevelopmental disorders.....	35
1.5.2.3.1 Structural cerebellar abnormalities in neurodevelopmental disorders.....	36
1.5.2.3.2 Autism.....	37
1.5.2.3.3 Schizophrenia.....	38
1.6 Gene expression.....	40
1.6.1 Nuclear receptor signalling.....	40
1.7 Fetal programming.....	43
1.7.1 PCOS.....	43

1.7.2 Maternal stress .....	44
1.7.3 Alterations in the <i>in utero</i> endocrine environment.....	46
1.8 Why sheep are a good model for human pregnancy.....	47
1.9 Focus of study .....	50
1.9.1 The cerebellum .....	50
1.9.2 The cortex .....	52
 CHAPTER 2.....	 53
2.1 Methodology .....	54
2.1.1 Animal husbandry.....	54
2.1.1.1 Mating and pregnant ewe husbandry .....	54
2.1.1.2 Treatment regimes.....	56
2.1.1.3 Animal sacrifice and collection of specimens.....	58
2.1.1.3.1 Fetal, lamb and adult animal sacrifice .....	58
2.1.1.3.2 Tissue collection .....	58
2.1.2 RNA .....	59
2.1.2.1 RNA isolation .....	59
2.1.2.2 Determining RNA integrity .....	60
2.1.2.3 DNase treatment and determining RNA concentration .....	61
2.1.3 qRT-PCR optimisation .....	62
2.1.3.1 Genes of Interest identification.....	62
2.1.3.2 Primer design .....	64
2.1.3.3 Optimising Custom-Made Primers .....	65
2.1.3.4 Optimising Primer Concentration .....	66
2.1.3.5 Melt curve analysis .....	67
2.1.3.6 Optimising and validating custom-made primer sets .....	68
2.1.4 qRT-PCR.....	69
2.1.4.1 Pre-validated assays for cerebellar gene expression studies ...	69
2.1.4.2 Pre-validated assays for cortex gene expression studies .....	71
2.1.4.3 qRT-PCR Assay set up.....	72
2.1.4.4 Reference gene selection .....	74
2.1.4.5 Melt curve analysis .....	74
2.1.4.6 Calculating $\Delta\Delta C_T$ .....	74



2.1.4.7 Statistical analysis .....	75
2.1.5 Protein Preparation .....	76
2.1.5.1 Protein Dialysis .....	76
2.1.5.2 Protein extraction optimisation .....	77
2.1.5.3 Determining Protein Concentration.....	77
2.1.6 Western Blotting as a protein detection method.....	78
2.1.6.1 SDS-PAGE electrophoresis.....	79
2.1.6.2 Primary antibody selection.....	80
2.1.6.3 Western Blotting.....	82
2.1.7 Histology .....	83
2.1.7.1 Sample Processing.....	83
2.1.7.2 Haematoxylin and Eosin Staining .....	85
2.1.7.2.1 Visualisation .....	85
2.1.7.3 Nissl Staining .....	86
2.1.7.3.1 Visualisation .....	86
2.1.7.3.2 Statistical Analysis.....	86
2.1.7.4 Immunohistochemistry as a protein detection method.....	87
2.1.7.4.1 Immunohistochemistry .....	87
2.1.7.4.1.1 Visualisation for cerebellar tissue .....	88
2.1.7.4.1.2 Primary antibody selection .....	90
2.1.7.4.1.3 Localisation studies.....	91
2.1.7.4.1.4 Statistical Analysis.....	91
2.1.7.5 Simultaneous Immunofluorescence.....	92
2.1.7.5.1 Visualisation .....	92
2.1.7.6 Sequential Immunohistochemistry .....	93
2.1.7.1.6.1 Visualisation.....	94
2.1.8 Microarray experiments.....	95
CHAPTER 3.....	96
3.1 Aims and Objectives .....	97
3.1.1 Aim 1 and objectives .....	97
3.1.2 Aim 2 and objectives .....	97
3.2 Results .....	99

3.2.1 RNA integrity using denaturing paraformaldehyde gels .....	99
3.2.2 qRT-PCR optimisation .....	100
3.2.2.1 Determination of successful cloning of a PCR product for primer validation .....	100
3.2.2.2 Standard curve of cloned AR PCR product to show validation of custom-made primers.....	101
3.2.3 Gene expression analysis .....	102
3.2.3.1 The effect of excess fetal androgen on the mRNA expression of genes associated with steroidogenesis in the male cerebellum .....	105
3.2.3.1.1 Determination of whether the changes in gene expression observed are due to an androgenic or estrogenic effect .....	107
3.2.3.2 Protein analysis.....	108
3.2.3.2.1 Visualisation of protein bands .....	109
3.2.3.2.2 Western blotting.....	110
3.2.3.2.2.1 Anti-actin as a positive control .....	110
3.2.3.2.2.2 StAR protein expression in the developing cerebellum .....	111
3.2.3.2.2.3 5 $\alpha$ -reductase 2 protein expression in the developing cerebellum.....	112
3.2.3.2.2.4 Using lamb adrenal gland as a positive control tissue	113
3.2.3.3 Immunohistochemistry .....	114
3.2.3.3.1 Investigation of cell migration in the developing cerebellum .....	115
3.2.3.3.2 Exploring optimal tissue thicknesses for Nissl staining studies.....	116
3.2.3.3.3 Primary antibody titration studies.....	117
3.2.3.3.4 Localisation of AR in the developing cerebellum.....	118
3.2.3.3.5 Colocalisation of AR and calbindin in the cerebellum ....	119
3.2.3.3.5.1 Examining colocalisation by means of double immunofluorescence .....	119
3.2.3.3.5.2 Determining colocalisation of AR and calbindin by using sequential immunohistochemistry .....	120
3.2.3.3.6 Protein expression studies.....	121
3.2.3.3.6.1 StAR protein expression .....	121
3.2.3.3.6.2 PR protein expression.....	122
3.2.3.3.6.3 5 $\alpha$ -reductase 1 protein expression .....	123

3.2.3.3.6.4 5 $\alpha$ -reductase 2 protein expression .....	124
3.3 Discussion.....	125
3.3.1 Determining RNA integrity using A <sub>260</sub> /A <sub>280</sub> ratio .....	125
3.3.2 Fetal ovine cerebellar gene expression and alterations in response to excess androgen exposure in utero .....	126
3.3.2.1 Steroidogenic transporter, enzymes and steroid hormone signalling.....	126
3.3.2.2 <i>STAR</i> .....	127
3.3.2.3 <i>PR</i> .....	129
3.3.2.3.1 <i>PR</i> regulation by estrogen .....	130
3.3.2.4 Is the “TP effect” mediated by androgen or estrogen? .....	131
3.3.2.5 Trends in gene expression .....	132
3.3.2.6 No change in gene expression.....	132
3.3.2.6.1 Time point.....	133
3.3.2.6.2 Determining animal model species or anatomical region differences.....	134
3.3.3 Western blotting.....	135
3.3.3.1 Troubleshooting Western blot difficulties.....	135
3.3.4 Immunohistochemistry .....	137
3.3.4.1 Optimisation studies .....	137
3.3.4.1.1 Investigation of cell migration in the developing cerebellum .....	137
3.3.4.1.2 Exploring optimal tissue thicknesses for Nissl staining studies.....	139
3.3.4.1.3 Colocalisation of AR and calbindin in the cerebellum ....	140
3.3.4.1.3.1 Examining colocalisation by means of simultaneous immunofluorescence .....	140
3.3.4.1.3.2 Determining colocalisation of AR and calbindin by using sequential immunohistochemistry .....	142
3.3.4.2 Cerebellar cells that express the proteins of interest .....	143
3.4 Conclusions .....	145
CHAPTER 4.....	146
4.1 Aims and Objectives .....	147
4.1.1 Aim 1 and objectives .....	147

4.1.2 Aim 2 and objectives .....	147
4.1.3 Aim 3 and objectives .....	147
4.1.3.1 Purkinje cell number .....	148
4.1.3.2 Purkinje cell morphology .....	148
4.1.3.3 Apoptosis and proliferation .....	148
4.1.3.4 Procedure for measuring Purkinje cell morphology.....	148
4.2 Results .....	150
4.2.1 Gene analysis .....	150
4.2.1.1 The effect of excess fetal androgen on the mRNA expression of genes associated with cell and neuronal activity in the male cerebellum .....	152
4.2.2 Examination of the developing cerebellar layer widths after androgen exposure .....	154
4.2.3 Observation of Purkinje cells, using immunohistochemistry .....	155
4.2.3.1 The number of Purkinje cells increases after androgen over- exposure.....	155
4.2.3.2 Is the “TP effect” on Purkinje cell number mediated by androgen or estrogen? .....	156
4.2.3.3 Perturbed Purkinje cell development after prenatal excess androgen exposure.....	157
4.2.3.4 Purkinje cell observations .....	160
4.2.4 The apoptosis and proliferation cycle is modified in Purkinje cells due to excess androgen exposure.....	162
4.2.4.1 Apoptosis .....	162
4.2.4.2 Proliferation .....	164
4.3 Discussion.....	166
4.3.1 Fetal ovine cerebellar expression is unaltered in cell and neuronal activity markers.....	166
4.3.2 No change in developing cerebellar layer widths after androgen exposure .....	168
4.3.3 Purkinje cell morphology is altered due to androgen over-exposure	169
4.3.3.1 Is the “TP effect” on Purkinje cell number mediated by androgen or estrogen? .....	169
4.3.3.2 Purkinje cell number alterations in neurodevelopmental disorders.....	170
4.3.3.3 Purkinje soma diameter .....	170

4.3.3.4 PCL arrangement .....	170
4.3.3.5 Observations .....	171
4.3.3.6 Apoptosis and proliferation of altered Purkinje cells .....	173
4.4 Conclusions .....	175
CHAPTER 5.....	178
5.1 Aims and objectives .....	179
5.2 Results .....	180
5.2.1 Gene expression analysis .....	180
5.2.2 Neurodevelopmental disorder candidate genes mRNA expression data.....	182
5.3 Discussion.....	184
5.3.1 Fetal ovine cerebellar expression of neurodevelopmental disorder candidate genes.....	184
5.3.2 No change in gene expression of candidate genes as a response to prenatal androgen over-exposure .....	184
5.3.2.1 Schizophrenia .....	184
5.3.2.2 Autism .....	185
5.4 Conclusions .....	187
CHAPTER 6.....	189
6.1 Aims and objectives .....	190
6.1.1 Aim 1 and objectives .....	190
6.1.2 Aim 2 and objectives .....	190
6.1.3 Aim 3 and objectives .....	190
6.2 Results .....	191
6.2.1 Identifying pyramidal cells by Nissl staining.....	191
6.2.2 Examining the cortex using neuronal markers .....	192
6.2.3 Localisation of AR in the developing cortex.....	193
6.2.4 Cortex Gene Expression using Microarray Analysis.....	194
6.2.4.1 Differences in gene expression in the cortex, as a result of prenatal androgen over-exposure.....	194
6.2.4.2 Determining whether gene expression changes are due to an androgenic or estrogenic effect.....	196
6.2.5 qRT-PCR validation of the microarray data .....	198

6.2.6 Comparison of microarray and validation results .....	201
6.3 Discussion.....	202
6.3.1 No identification of pyramidal cells of the developing cortex .....	202
6.3.1.1 No protein expression of AR in the developing cortex .....	204
6.3.1.2 Testosterone may be indirectly mediated by the estrogen receptor.....	205
6.3.1.3 Summary .....	205
6.3.2 Microarray analysis of GD 90 male ovine cortex tissue.....	206
6.3.2.1 Microarray validation using qRT-PCR .....	206
6.3.2.1.1 Differences between microarray and qRT-PCR approaches.....	206
6.3.2.2 Microarray analysis - changes in gene expression .....	208
6.3.2.2.1 <i>ERBA BETA1</i> .....	208
6.3.2.2.2 <i>NPY3R</i> .....	208
6.3.2.2.3 <i>COL4A2</i> .....	209
6.3.2.2.4 <i>POMC</i> .....	209
6.3.2.2.5 <i>APP</i> .....	210
6.3.2.3 An evaluation of the SLIT/ROBO pathway .....	211
6.4 Conclusions .....	213
CHAPTER 7 .....	215
7.1 Summary.....	216
7.1.1 Methods.....	216
7.1.2 The cerebellum .....	216
7.1.3 The cortex .....	217
7.1.4 Summary of results .....	218
7.2 Limitations in methodology.....	219
7.2.1 Sample availability.....	219
7.2.2 Protein expression.....	219
7.3 Androgenic or estrogenic effect? .....	220
7.3.1 Examining androgen receptor activity to confirm whether TP effect is indeed mediated by androgen .....	221
7.3.2 Lack of androgen receptors in the cortex.....	222
7.4 The consequences of prenatal androgen over-exposure.....	223
7.4.1 The cerebellum.....	223

7.4.1.1 Structural cerebellar alterations.....	223
7.4.2 The cortex .....	225
7.4.2.1 Microarray validation .....	225
7.4.2.2 Gene expression alterations.....	225
7.4.2.3 Establishing whether cytoarchitectural alterations are observed in the cortex.....	226
7.5 Plasticity during development .....	227
7.6 Delayed cerebellar development in other models.....	228
7.6.1 Thyroid hormone.....	228
7.6.2 Biphenyls.....	229
7.6.3 SRC-1 .....	229
7.6.4 Vulnerability during cerebellar development.....	230
7.7 Are the observed changes carried on in later life – the effect of disruption during cerebellar development.....	231
References.....	233

## **Abbreviations**

Ab – antibody  
ABC complex – avidin-biotin complex  
ADHD – Attention deficit hyperactivity disorder  
AH-POA - anterior hypothalamic/preoptic area  
APS - ammonium persulphate  
AR - androgen receptor  
ARE – androgen response element  
AVPV - anteroventral periventricular nucleus  
BCA - bicinchoninic acid  
BLAST - Basic Local Alignment Search Tool  
Bp - base pair  
BSA - bovine serum albumin  
CAH - congenital adrenal hyperplasia  
CaMK - calcium/calmodulin-dependent protein kinase II  
cAMP – cyclic adenosine monophosphate  
cDNA - complementary deoxyribonucleic acid  
CNS – central nervous system  
CREB - CRE binding protein  
CRE - cAMP-responsive element  
CREM - CRE modulator  
Ct - cycle threshold  
CYP - cytochrome  
DAB - 3,3'-diaminobenzidine  
DAPI - 4',6-diamidino-2-phenylindole  
DES - diethylstilbestrol  
DEX - dexamethasone  
dH<sub>2</sub>O - distilled water  
DHT - dehydrotestosterone  
DNA - deoxyribonucleic acid  
ECL - Enhanced chemiluminescence  
ED – embryonic day  
EGC - external granule cell  
EGL – external granule layer



ELISA - enzyme-linked immunosorbent assay  
ER - estrogen receptor  
ERE- estrogen response element  
GABA - gamma-aminobutyric acid  
GAD - glutamate decarboxylase  
GAS7 - growth arrest specific gene  
GD – gestational day  
GFP – green fluorescent protein  
GOI – gene of interest  
GW – gestational week  
GWAS - genome wide association studies  
H&E - haematoxylin and eosin  
HRP - horseradish peroxidase  
HSD - hydroxysteroid dehydrogenase  
IGL – internal granule layer  
IU – international unit  
IZ – intermediate zone  
Kb – kilobase  
kDa - kilodalton  
M – molar  
MIQE - Minimum Information for Publication of Quantitative Real-Time PCR  
Experiments  
ML – molecular layer  
ml - millilitre  
 $\mu\text{g}$  – microgram  
 $\mu\text{l}$  – microlitre  
 $\mu\text{m}$  - micron  
mM - millimolar  
mRNA - messenger ribonucleic acid  
Mw - molecular weight  
MZ – marginal zone  
NCBI - National Center for Biotechnology Information  
NIH - National Institutes of Health  
ng – nanogram  
nm – nanomolar

OD – optical density  
PAGE - polyacrylamide gel electrophoresis  
PBS - phosphate buffered saline  
PBS-T - phosphate buffered saline - tween  
PCL – Purkinje cell layer  
PCR - polymerase chain reaction  
PCOS - polycystic ovary syndrome  
PND - post-natal day  
POMC - pro-opiomelanocortin  
PP- preplate  
PR – progesterone receptor  
PTM - post-translational modification  
qRT-PCR - quantitative real time - polymerase chain reaction  
RIA - radioimmunoassay  
RNA - ribonucleic acid  
ROBO – roundabout  
RT - reverse transcriptase  
SDS - sodium dodecyl sulphate  
SDN-POA - sexually dimorphic nucleus of the preoptic area  
SNPs - single nucleotide polymorphisms  
SP - subplate  
StAR - steroidogenic acute regulatory protein  
SVZ - subventricular zone  
T – testosterone  
TBS – tris buffered saline  
TBS-T - tris buffered saline - tween  
TEMED - tetramethylethylenediamine  
TP - testosterone propionate  
UTR - untranslated region  
VZ – ventricular zone  
WM – white matter  
21-OH - 21-hydroxylase

## List of Figures

Figure 1.1: Schematic of the major anatomical subdivisions of the cerebellum.....	2
Figure 1.2: Schematic representation of the developed cerebellar cortex.....	3
Figure 1.3: Diagrammatic representation of the cerebellar cortex.....	4
Figure 1.4: Schematic diagram of the brain.....	8
Figure 1.5: Schematic diagram of cytoarchitecture of a developed cortex.....	9
Figure 1.6: Mouse cerebellar neurogenesis.....	12
Figure 1.7: Neuronal migration – cortex.....	15
Figure 1.8: The steroid synthesis pathway.....	29
Figure 1.9: Steroid hormone receptor binding.....	40
Figure 1.10: Comparison of timing of cerebellar development for rat, human and sheep.....	48
Figure 2.1: Primer concentration matrix.....	66
Figure 2.2: Melt curve analysis .....	67
Figure 2.3: The process and conditions of protein preparation to find the optimal conditions for protein analysis.....	77
Figure 3.1: Denaturing agarose paraformaldehyde RNA gel.....	99
Figure 3.2: Colony PCR .....	100
Figure 3.3: AR standard curve .....	101
Figure 3.4: Summary of gene expression changes.....	106
Figure 3.5: The transferring of protein from gel to membrane.....	109
Figure 3.6: Positive control - anti-actin blot.....	110
Figure 3.7: Anti-StAR and $\beta$ -actin blots.....	111
Figure 3.8: Anti-5 $\alpha$ -reductase 2 blot.....	112
Figure 3.9: Positive tissue control - lamb adrenal gland.....	113
Figure 3.10: Haematoxylin and Eosin Staining of male ovine cerebellar tissue.....	115
Figure 3.11: Nissl Staining of cerebellar tissue of control male sheep.....	116

Figure 3.12: An example of dilution titers using anti-PR in male ovine cerebellar tissue.....	117
Figure 3.13: Localisation of AR in cerebellar tissue.....	118
Figure 3.14: Colocalisation of AR and Calbindin in Purkinje cells of the cerebellum – immunofluorescence.....	119
Figure 3.15: Colocalisation studies to show the location of AR and Calbindin in Purkinje cells of the cerebellum – sequential immunohistochemistry.....	120
Figure 3.16: Positive Purkinje cells/mm <sup>2</sup> expressing StAR protein.....	121
Figure 3.17: Positive Purkinje cells/mm <sup>2</sup> expressing PR protein.....	122
Figure 3.18: Positive Purkinje cells/mm <sup>2</sup> expressing 5 $\alpha$ -reductase 1 protein..	123
Figure 3.19: Positive Purkinje cells/mm <sup>2</sup> expressing 5 $\alpha$ -reductase 2 protein..	124
Figure 4.1: mRNA expression levels of cell and neuronal activity markers....	153
Figure 4.2: Measuring the widths of each cerebellar layer.....	154
Figure 4.3: Purkinje cell number – TP-treated samples.....	155
Figure 4.4: Purkinje cell number – DES-treated samples.....	156
Figure 4.5: Alterations in Purkinje cell soma diameter and PCL width.....	157
Figure 4.6: The percentage of Purkinje cells that cover the PCL.....	159
Figure 4.7: Purkinje cells and their dendritic network.....	160
Figure 4.8: Purkinje soma development.....	161
Figure 4.9: Apoptosis graphs.....	162
Figure 4.10: Apoptosis photographs.....	163
Figure 4.11: Proliferation graphs.....	164
Figure 4.12: Proliferation photographs.....	165
Figure 5.1: mRNA expression level of neurodevelopmental disorder candidate gene markers.....	185
Figure 6.1: Identifying the cortex layers.....	191
Figure 6.2: Identifying pyramidal cells using cell markers.....	192
Figure 6.3: Localisation of AR in the developing cortex.....	193
Figure 6.4: Microarray validation using qRT-PCR.....	199

Figure 6.5: mRNA expression of ROBO receptors.....200

## List of Tables

Table 2.1: Treatment cohorts and sample numbers.....	56
Table 2.2: Genes of interest list.....	63
Table 2.3: Custom-made primer sequences designed by PrimerDesign (U.K.).....	69
Table 2.4: Primer sequences that have been published in Hogg <i>et al.</i> , (2011).....	70
Table 2.5: Primer sequences that have been published for the gene of interests examined for microarray validation.....	71
Table 2.6: Mastermix compositions for PrimerDesign and Taqman assays.....	72
Table 2.7: Mastermix compositions for MWG Operons assays.....	73
Table 2.8: Worked example of $2^{\Delta\Delta CT^{*}(-1)}$ method and calculation for geometric reference mean.....	75
Table 2.9: Composition of 10% and 12% resolving gels for SDS-PAGE.....	79
Table 2.10: Composition of 5% stacking gel for SDS-PAGE.....	79
Table 2.11: Tissue processing protocol.....	81
Table 2.12: Antibody list for Western blot analysis.....	83
Table 2.13: Antibody list for immunohistochemistry.....	90
Table 2.14: Antibody list for localisation studies.....	91
Table 3.1: A list of genes of interest examined – steroid signalling and metabolism.....	103
Table 3.2: mRNA expression for each steroid signalling and metabolism GOI.....	104
Table 3.3: Summary of p values and fold shifts and differences for GOIs.....	105
Table 3.4: Summary of the genes altered as a result of steroid over-exposure.....	107
Table 4.1: A list of the genes of interest that were examined – cell and neuronal activity markers.....	150
Table 4.2: mRNA expression for each cell and neuronal activity GOI.....	151
Table 5.1: A list of the genes of interest examined – candidate genes for neurodevelopmental disorders.....	180

Table 5.2: mRNA expression for each neurodevelopmental disorder candidate genes.....	181
Table 6.1: Genes altered in the cortex using microarray analysis – TP vs Control samples.....	195
Table 6.2: Genes altered in the cortex using microarray analysis – TP vs DES samples.....	197
Table 6.3: Comparison of microarray and validation results.....	201

## **CHAPTER 1**

### **Introduction**



## 1.1 The cerebellum

### 1.1.1 Cerebellar structure

The cerebellum is part of the hind brain and is divided into three lobes – the anterior, posterior and flocculonodular lobes (Figure 1.1). Longitudinally, the cerebellum has a central vermis and two lateral hemispheres (Sotelo, 2004). The cerebellum is covered with a cortex and embedded in its core of white matter, the deep nuclei provide cerebellar output. Major roles of the cerebellum include the coordination of voluntary movements *e.g.* postural and limb movements; and motor skills, and evidence has suggested that it also has a part to play in cognitive functions *e.g.* language (Diamond, 2000) .

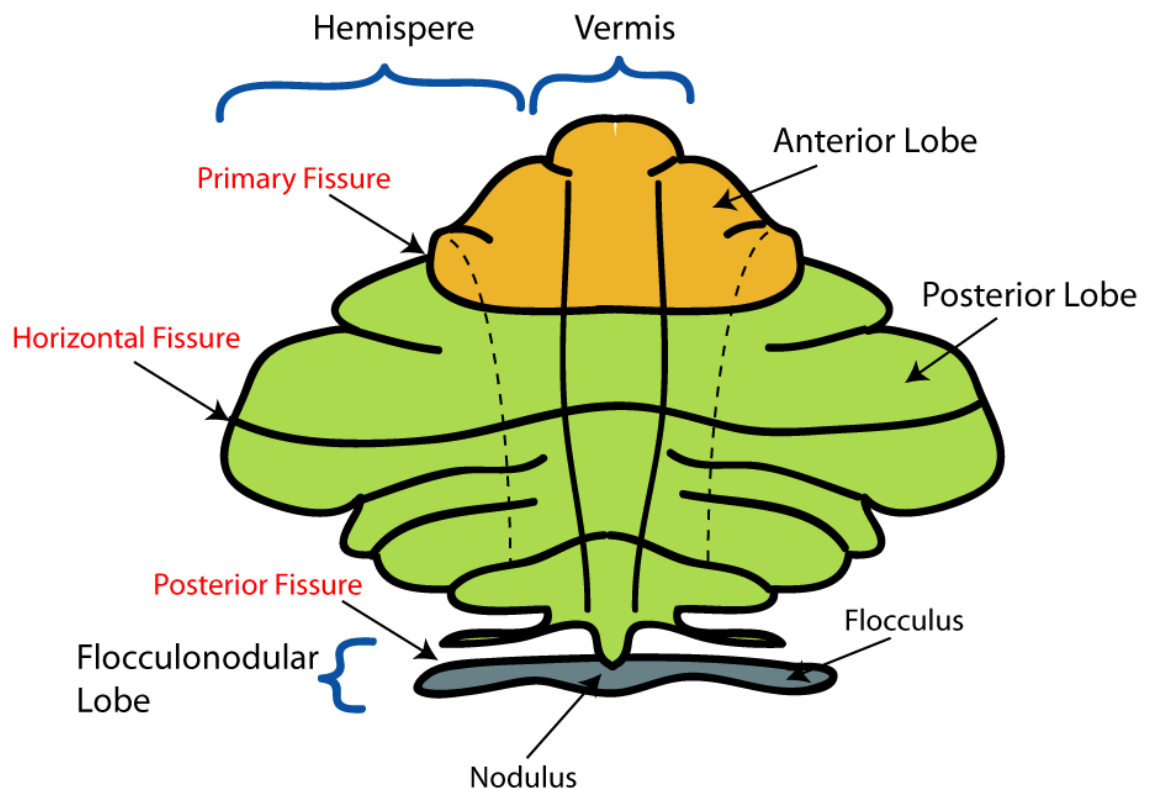


Figure 1.1: Schematic of the major anatomical subdivisions of the cerebellum. This is a superior view of an "unrolled" cerebellum, which places the vermis in one plane. Taken from <http://www.rudyard.org/cerebellum-diagram>

The cerebellar cortex contains four main types of neurons: granule cells, Purkinje cells and two types of inhibitory interneurons: Golgi cells and the stellate/basket cells (Sotelo, 2004). The cortex receives three kinds of input: from mossy fibres, climbing fibres and parallel fibres (Paxinos, 2004).

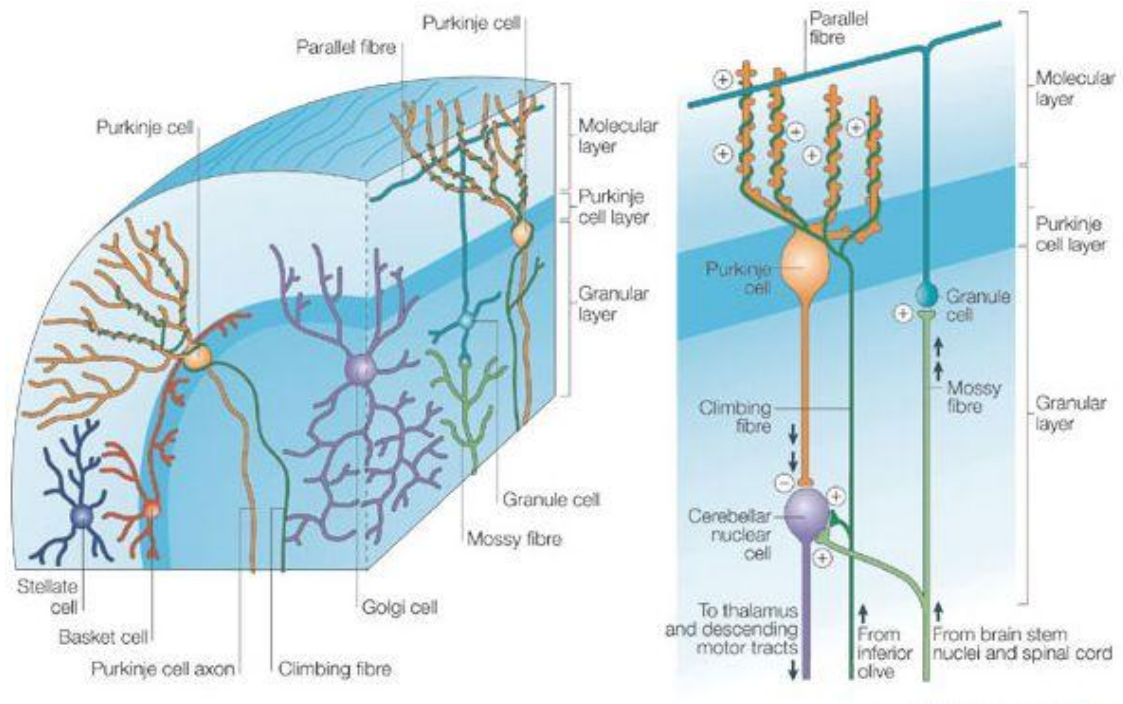


Figure 1.2: Schematic representation of the developed cerebellar cortex. The different cerebellar layers and the different types of fibres which provide the cerebellar input are illustrated. Taken and adapted from (Squire, *et al.*, 2003)

### 1.1.1.1 Cerebellar Purkinje Cells

Purkinje cell dendrites are aligned into a flat plane and are all orientated in the same direction – at right angles to the long axis of the folium in which they are located, and their soma diameter is approximately 80µm (Ohyu *et al.*, 1997). Altman (1972) demonstrated that rat Purkinje cells differentiate just after birth and the formation of the cerebellar cortex becomes complete during neonatal life. This is achieved through the process of migration of external granule cells to the IGL (discussed in further detail below), neuronal and glial growth and synaptogenesis. Purkinje cells can be identified through their large nuclei and cytoplasm in comparison with the other cells in the cerebellum (Salouci *et al.*,

2012). The Purkinje cell's main function is to control the sole output of motor coordination in the cerebellum (Salouci *et al.*, 2012) but it has also been identified as a major site for neurosteroid formation in the brain (Tsutsui, 2006). These neurons can produce several different neurosteroids and therefore Purkinje cells have served as an excellent cellular model for the study of neurosteroid actions in the cerebellum (Tsutsui, 2008).

### 1.1.1.2 Granule Cells

In the fetal human period, the internal granule cell layer is formed by further proliferation and migration of the external granule cells and is situated below the layer of Purkinje cells (see Figure 1.3). The lamina dissecans layer separates the internal granular layer from the Purkinje cells. During the inward migration of postmitotic granule cells, the Purkinje cells enlarge and develop dendritic trees (Milosevic and Zecevic, 1998). Granule cells are small in size (their soma diameter is approximately 10 $\mu$ m) but are the most numerous type of neuron found in the cerebellum (Mulac-Jericevic, *et al.*, 2000). Many granule cells synapse with a single Purkinje cell via parallel fibres (Sotelo, 2004).

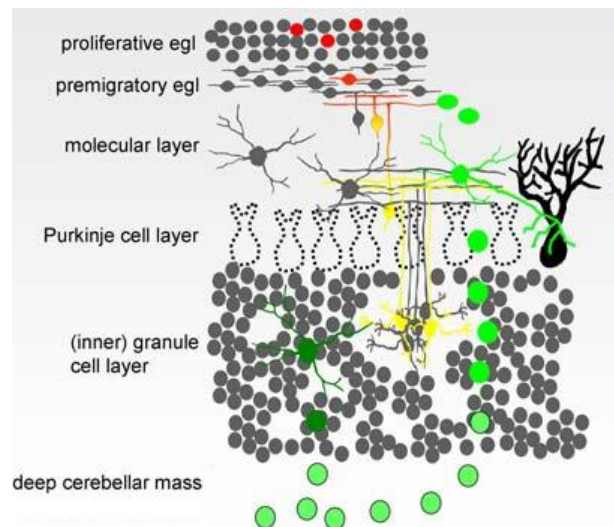


Figure 1.3: Diagrammatic representation of the cerebellar cortex. The external granule cell layer (egl) is where granule cells migrate towards the inner granule cell layer (igl). Granule cells form synapses with Golgi cells in the molecular layer. Stellate/basket cells in the molecular layers form inhibitory synapses with Pk cells. Diagram taken and adapted from (Schilling *et al.*, 2008).

### **1.1.1.3 Golgi Cells**

Golgi cells are inhibitory interneurons that are found within the granule cell layer of the cerebellum. These cells synapse onto the dendrites of granule cells and receive excitatory input from mossy fibres, and parallel fibres, that are long granule cell axons (Wall, 2003) . This circuitry allows for feed-forward and feed-back inhibition of granule cells. The Golgi cell acts by altering the mossy fibre - granule cell synapse using GABA as its transmitter and is therefore GABAergic.

### **1.1.1.4 Stellate/Basket Cells**

Basket cells project to the Purkinje cell bodies whereas stellate cells project to the more distant dendritic shafts (Schilling *et al.*, 2008). These cells are GABAergic inhibitory neurons. It is thought that basket and stellate cells differentiate as follows: the first cells secure highly adhesive Purkinje cell territories around the axon hillock and cell body whereas cells which reach Purkinje cells later on have to settle for less adhesive distal dendritic shafts (Voogd and Glickstein, 1998). Those molecular layer interneurons which arrive first in the molecular layer settle in the lower molecular layer (Altman and Bayer, 1997) and it has been suggested that this is where basket cells reside, cells that arrive later in the molecular layer and settle more superficially, attach to more distal dendrites *i.e.* stellate cells.

## **1.1.2 Gene products that facilitate the development of the cerebellum**

### **1.1.2.1 BDNF**

BDNF is a critical neurotrophin that regulates cell differentiation, cell survival, neurotransmission, and synaptic plasticity in the CNS (Numakawa, 2010). Estrogens stimulate the production of nerve growth factors, such as brain-derived neurotrophic factor (BDNF) and enhance neuronal survival (Beyer, 1999). Morrison and Mason (1998) demonstrated the complexities of the relationship between BDNF exposure and Purkinje cell development and survival. Some extra BDNF may boost survival, but too much BDNF triggers excess glutamate production that can become toxic to the Purkinje cells. In the current study, *BDNF* mRNA expression will be evaluated, in order to determine whether prenatal androgen over-exposure alters the expression of this gene in the fetal cerebellum which may ultimately have consequences on cerebellar development. There is also evidence to suggest that neurons that fail to grow or migrate to their appointed sites or are unable to make the right synaptic

connections can have drastic effects in later life. For example, schizophrenia is thought to be a neurodevelopmental disorder with its origin during the critical phases of gestational life (Lewis and Levitt, 2002).

#### **1.1.2.2 FOS**

*FOS* is involved in cell differentiation including synaptogenesis of granule cell dendrites with mossy fibers and parallel fiber contacts with Purkinje cell spines (Chen, *et al.*, 1998). Therefore, *FOS* is a marker of neuronal activation in the cerebellum (Chen, *et al.*, 2005). Neal and Wade (2007) demonstrated that testosterone exposure negatively regulates *FOS* expression in the amygdala of adult male green anole lizards, which implies that there could be a decline in neuronal activity. The current study has investigated whether *FOS* expression is regulated by testosterone in the developing ovine cerebellum.

#### **1.1.2.3 GAD1**

GABA synthesis in mammals is catalyzed by the two isoforms of glutamate decarboxylase (GAD) encoded by the *GAD1* and *GAD2* genes (Erlander *et al.*, 1991). Coordinated regulation of the *GAD1* and *GAD2* genes is essential for the proper differentiation of GABAergic neurons. *GAD1* is expressed abundantly in Purkinje cells of the cerebellum and is a common Purkinje cell marker (Chan-Palay *et al.*, 1981). In the present study, *GAD1* has been utilised as a Purkinje cell marker for gene expression studies.

#### **1.1.2.4 GFAP**

Glial cells are crucial in the development of the nervous system and are involved processes such as synaptic plasticity and synaptogenesis (Ullian *et al.*, 2004). Glial fibrillary acidic protein (*GFAP*) is a member of the intermediate filament family that provides support and strength to cells. Several molecules of *GFAP* protein bind together to form the main intermediate filament found in specialized brain cells called astrocytes. Astrocytes are star-shaped cells that support the functions of nerve cells in the central nervous system (Westmoreland *et al.*, 2001). Testosterone has been shown to down-regulate *GFAP* mRNA expression in facial nuclei of adult male hamsters (Jones *et al.*, 1997) which implies that this gene can be modulated by steroids. The current study will utilise *GFAP* as a glial marker, in order to determine if there are any alterations in gene expression caused by excess testosterone *in utero*.

## **1.2 The cortex**

### **1.2.1 Structure and function**

The general function of the cortex is the temporal organisation of actions toward biological or cognitive goals, such as the execution of all forms of action (somatic movement, eye movement, emotional behaviour, intellectual performance and speech (Fuster, 1997). The cortex, the lateral region in particular, specialises in the temporal structuring of new and complex goal-directed series of actions, whether in the form of behaviour, speech, or reasoning. Furthermore, the participation of the cortex in the choice between alternatives, in decision making, and in executing temporally structured actions are the reasons that the cortex has also been considered the “central executive” (Fuster, 2002).

Humans and other primates are characterized by a markedly prolonged period of postnatal brain development during which cultural traditions and practices, including language, are acquired. In humans, a relatively large proportion of brain size growth takes place postnatally, allowing for social and environmental factors to powerfully impact the establishment of neural connectivity (Teffer and Semendeferi, 2012). Macaque monkeys have been studied most extensively as a comparative model of neurodevelopment as they are born with brains that are already around 70% of adult mass. In sheep, brain mass has reached 50% adult size at birth (McIntosh *et al.*, 1979); whereas in humans, only approximately 25% of adult mass is achieved at birth (Robson and Wood, 2008). Figure 1.4 depicts a schematic diagram of a developed brain with annotations of certain areas, whereby the cortex comprises of the frontal, parietal and occipital lobes.

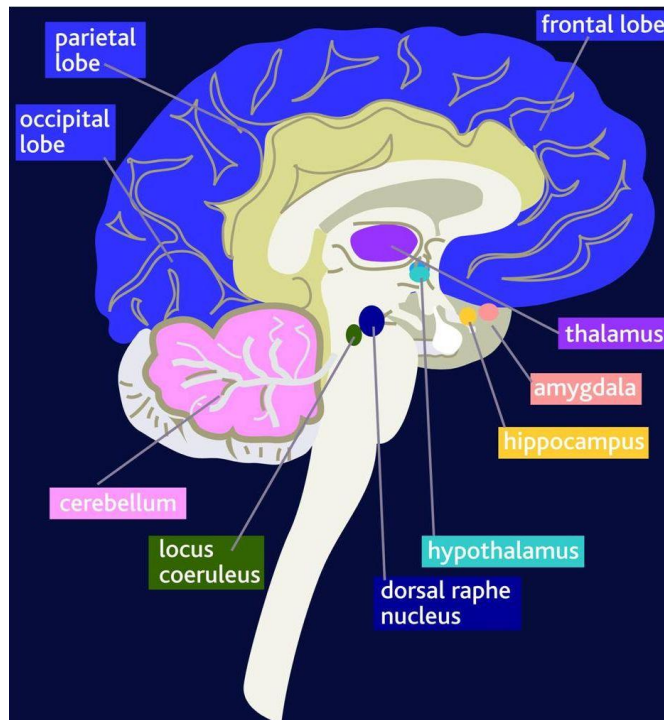


Figure 1.4: Schematic diagram of the brain. Here, it depicts the location of the cortex (frontal, parietal and occipital lobes). Taken and adapted from CNSForum.com

### 1.2.1.1 Cortex layers

The isocortex consists of up to six well-defined layers of cells (Figure 1.5). The outermost molecular or plexiform layer (I) contains nonspecific afferent fibres that come from within the cortex or from the thalamus. The external granular layer (II) is a dense layer composed of small pyramidal and stellate cells. The external pyramidal layer (III) contains pyramidal cells, frequently in row formation. The internal granular layer (IV) is usually a thin layer with closely packed stellate cells. The internal pyramidal or ganglionic layer (V) contains, in most areas, pyramidal cells that are fewer in number but larger in size than those in the external pyramidal layer. These cells project to distal structures (eg, brain stem and spinal cord). The fusiform (multiform) layer (VI) consists of irregular fusiform cells whose axons enter the adjacent white matter.

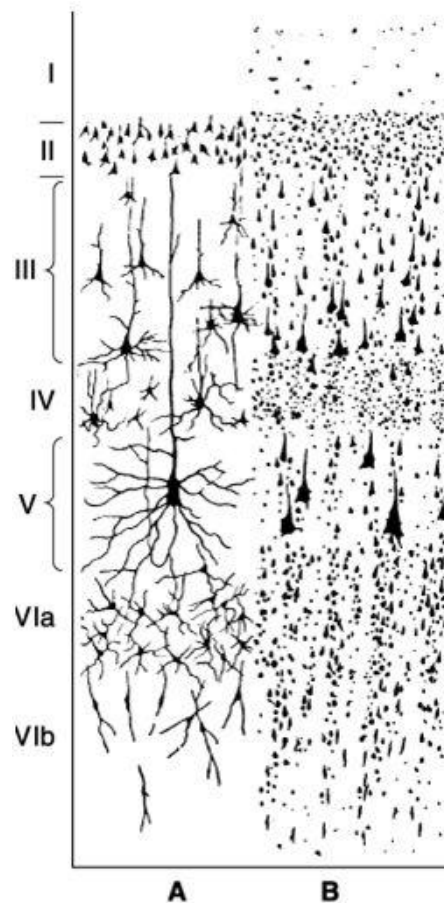


Figure 1.5: Schematic diagram of cytoarchitecture of a developed cortex. This represents the cells types that are located in each cortex layer. A) A typical Golgi neuronal stain B) A typical Nissl cellular stain. Taken and adapted from McGraw-Hill (2006).

### 1.2.1.2 Pyramidal cells

Pyramidal cells are the output neurons of the cerebrum (Elston *et al.*, 2009). They transmit signals to other parts of the central nervous system (CNS). Pyramidal cells are tall and conical, triangular in tissue sections. Their apex points toward the brain surface and has a thick dendrite with many branches, and small, knobby dendritic spines. The base gives rise to horizontally oriented dendrites, and an axon that passes into the white matter. Their axons have collaterals that synapse with other neurons in the cortex or in deeper regions of the brain.



### **1.3 Fetal brain development**

The fetal period of human development extends from gestational week (GW) 9 through to the end of gestation (Stiles, 2008). The gross morphology of the developing brain undergoes drastic changes during this time. The human brain begins as a smooth structure (lissencephalic) and gradually develops the characteristic mature pattern of gyral and sulcal folding. The formation of gyri and sulci follows an orderly sequence. Primary sulci are first seen as grooves positioned in specifically targeted brain regions, secondary branches then begin to form off the primary sulci, followed later by the tertiary branches (Morange, 2001). The first fissure to form is the longitudinal fissure that separates two cerebral hemispheres. Its development begins in rostral regions as early as GW 8 and proceeds caudally until it is complete at GW 22 (Stiles and Jernigan, 2010). Different populations of neurons form gray matter structures in many regions of the brain including hindbrain and spinal column, cerebellum, midbrain structures, deep subcortical nuclei and the neocortex.

#### **1.3.1 Prenatal period**

Much of brain development in the fetal period centres on the processes of neuron production, migration and differentiation. The changes that occur in the gross anatomy of the fetal brain reflect dramatic changes occurring at the cellular level.

##### **1.3.1.1 Neuron production**

Neuron production begins in the embryonic period at GW 6, and extends through mid-gestation in most brain areas (Stiles and Jernigan, 2010). At the very centre of the brain are a series of interconnected cavities that form the ventricular system of the brain. The ventricular system is filled with cerebral spinal fluid (Bering, 1955). The ventricular system has a number of important functions including cushioning and protection of the brain, removal of waste material, and transport of hormones and other substances (Brodal, 2010). During brain development the walls of the ventricles are the site of most neuron production. Soon after neurons are produced, neurons migrate away from the proliferative regions of the ventricular zone (Clancy *et al.*, 2001).

The first step in neuron production involves increasing the size of the neural progenitor cell population. Neural progenitors are a mitotic population of cells,

that is, they can divide to form new cells. Neurons are post-mitotic cells; once formed they are no longer capable of dividing and producing new cells (Stiles, 2008)

### **1.3.1.2 Neuron migration**

#### **1.3.1.2.1 The cerebellum**

The cerebellum develops over a long time, from the early gestational period until the first postnatal years in sheep (Petratos *et al.*, 2000), however ovine cerebellar development is not as well characterised as humans, rodents and primates. In general, the three key steps involved in the development of the cerebellum are: the formation of two proliferative compartments (the ventricular zone, VZ, and rhombic lip), inward migration of granule cells from the external to the internal granule cell layer, and the formation of cerebellar circuitry (Luo *et al.*, 2001). The main cell types of the cerebellum appear at different times of development and at different locations. Purkinje cells and the deep cerebellar nuclei arise from the VZ of the metencephalic alar plate (Chan-Palay *et al.*, 1981), Figure 1.6, whereas granule cells are generated from the rostral part of the rhombic lip, known as the upper rhombic lip (Fink, *et al.*, 2006).

Before migration begins, projections known as parallel fibres arise from granule cells. The parallel fibres synapse with Purkinje cells and mossy fibres (the axons of the dorsal spinocerebellar, cuneocerebellar, vestibulocerebellar and pontocerebellar tracts) and extend further with time as the molecular layer forms from the deep external granule layer (EGL) and pre-migratory zone. Another type of cerebellar input comes from climbing fibers which arise from the inferior olivary nucleus. In the cerebellum, terminally differentiating granule cell neurons express the genes required for migration to the internal granule layer (IGL), such as *GABRA6* (Wang *et al.*, 2004). Similar genes may be involved in determining the cell fate of cortex cell precursors such as *Unc5H3*, and *Netrin-1* (Wingate, 2001).

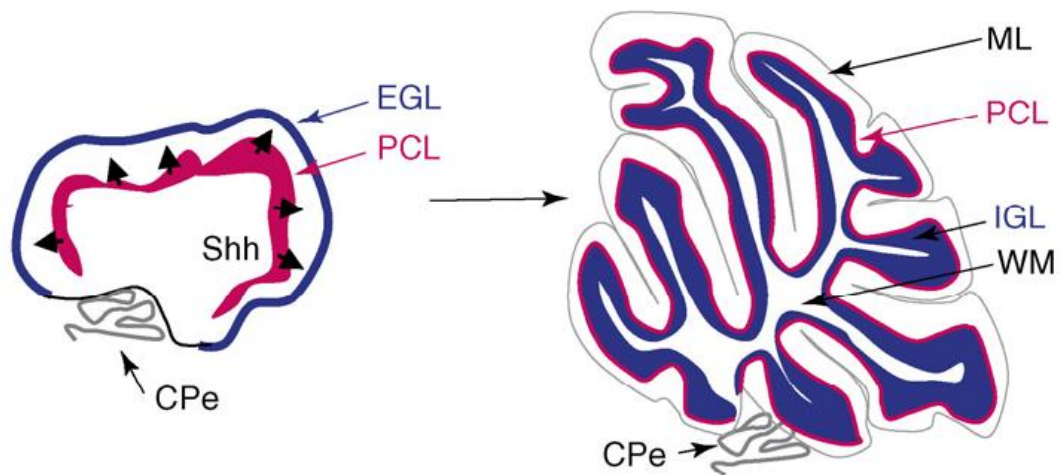


Figure 1.6: Mouse cerebellar neurogenesis. A schematic mid-sagittal section of mouse cerebellum at day of birth. The Purkinje cell layer (PCL) is located underneath the EGL and secretes *Sonic hedgehog* (Shh) which is received by EGL cells and drives their extensive proliferation such that granule cells become the most abundant neurons in the cerebellum and in the entire brain. Upon differentiation, EGL cells migrate through the PCL layer to form the IGL of the mature cerebellum. Their trailing axons form, the molecular layer (ML). Purkinje cells project into the cerebellar white matter (WM). choroid plexus (CPe) is a nerve complex localized in the ventricular system of the brain and form one of the interfaces between the blood and the CNS .Taken and adapted from (Millen and Gleeson, 2008).

After birth, between the second and fourth post-natal days (PND 2–PND 4) in mice, a number of signalling pathways promote granule cell precursor proliferation (Wechsler-Reya and Scott, 1999). Between birth and the end of the second postnatal week, granule cell precursors exit the cell cycle and move into the inner regions of the EGL where they extend parallel fibers and migrate along the radial fibres of Bergmann glial cells (Edmondson and Hatten, 1987) to a position beneath the Purkinje cells, where they form the IGL.

The development of the cerebellum is a complex process that requires the rather precise orchestration of multiple biological processes, such as the proliferation, migration, apoptosis, and differentiation of multiple cell types (Hatten and Heintz, 1995). The proliferation of external granule cells (EGCs) is promoted by factors secreted from Purkinje cells such as sonic hedgehog (*Shh*) (Wechsler-Reya and Scott, 1999) and the migration of Purkinje cells is regulated by factors secreted from the EGL, such as Reelin (Jensen *et al.*, 2002). Therefore, the accurate migration and survival of a subset of Purkinje

cells is essential for normal cerebellar development, including the generation of sufficient numbers of granule cells. The importance of Purkinje cells in these events was demonstrated by Yuasa *et al.*, (1993) and Miyata *et al.*, (1997) whereby Reelin null mice exhibited a reduced proliferation of EGCs in the face of aberrant Purkinje cell migration.

#### **1.3.1.2.1.1 Synaptogenesis**

Cerebellar synaptogenesis begins on embryonic day 19 in rodents (Altman and Bayer, 1997). The cerebellar cortex receives two classes of excitatory inputs: mossy fibres and climbing fibres (Ito, 2006). Mossy fibres originate in the spinal cord and brainstem whereas climbing fibres are the axons of inferior olive neurons. Mossy fibres convey sensory information from various body parts and motor commands from the upper centres. These signals are transferred to granule cells via glutamatergic (excitatory) synapses. The axons of granule cells (parallel fibres) give off their branches running laterally in the molecular layer of the cerebellar cortex (Ito, 2002). Purkinje cells spread huge, flat dendritic arbors along the parasagittal planes in the molecular layer. Each Purkinje cell receives excitatory synaptic inputs from more than 100,000 parallel fibres. Purkinje cells integrate sensory information and motor commands through summation of parallel fibre inputs. Parallel fibres also form glutamatergic synapses on interneurons in the cerebellar cortex, such as the Golgi cell, stellate cell and basket cell (Goto *et al.*, 2006). Golgi cells form inhibitory synapses on the synaptic terminals of mossy fibres. The parallel fibre-Golgi cell-mossy fibre loop circuitry imposes feedback inhibition, which attenuates glutamate release by the parallel fibres. Stellate and basket cells form inhibitory synapses on Purkinje cells (Kano *et al.*, 2002). Deep cerebellar nuclei integrate excitatory synaptic inputs from mossy fibres and inhibitory synaptic inputs from Purkinje cells, and send the integrated signals directly to the nuclei and indirectly to the primary motor cortex and premotor cortex, via the thalamus. The cerebellum influences the regulatory actions of these nuclei and cortices on the descending motor system, and thereby contributes to motor coordination and motor accuracy. Plasticity at synapses upstream of Purkinje cells changes the transferring efficacy of sensory information and motor commands to Purkinje cells. Plasticity of synapses on Purkinje cells and deep cerebellar nuclei changes the manner of signal integration in these neurons (Ito, 2001). Cerebellar synaptic plasticity may thereby further improve motor

coordination and motor accuracy, and contribute to the acquisition of new motor skills.

#### **1.3.1.2.2 The cortex**

The cerebral cortex arises from a sheet of undifferentiated neuroepithelial cells that line the lateral ventricle of the dorsal telencephalon. Most neurons are produced in the VZ and migrate radially from the VZ in the centre of the brain out to the developing cortex (Clancy *et al.*, 2001). Very early in neocortical development the distances the neuron must cross are small. Thus the earliest produced neurons can use a mode of migration referred to as somal translocation (Nadarajah and Parnavelas, 2002). In somal translocation the neuron extends a long basal process, which is an extension of the cell body, just beyond the edge of the VZ into the outer region of the brain compartment. The basal process attaches to the pial surface, the outer surface of the developing brain (Miyata *et al.*, 2010). The nucleus of the cell then moves through cytoplasm of the basal process. As the nucleus moves up the process becomes shorter and thicker but remains attached to the pial surface. At the end of somal translocation the nucleus of the cell has moved out of the VZ and into the embryonic cortex (Figure 1.7, Stiles, 2008).

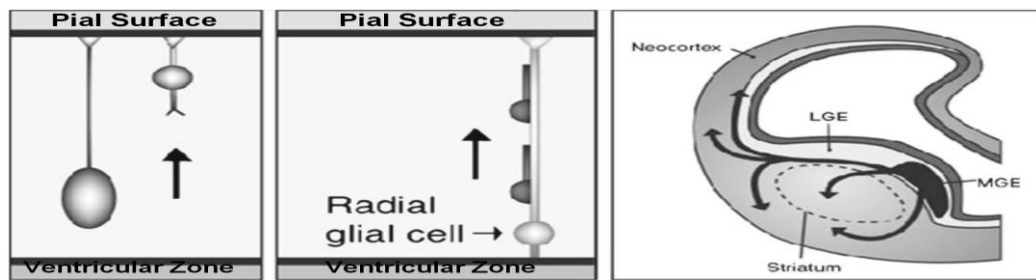


Figure 1.7: Different modes of neuronal migration to the neocortex. A) Neuron migration by somal translocation where cell extends a cytoplasmic process and attaches to the outside of the brain compartment (pial surface), and then the nucleus moves up into the brain area. B) Neuron migration radial glial guide. Radial glial provides scaffold for neuron to migrate along. C) Neuron migration from second proliferative zone in ganglionic eminences by tangential migration (arrows indicate direction of migration for different neuron populations). A and B taken and adapted from Nadarajah *et al.*, (2003). C taken and adapted from Stiles (2008).

The earliest produced neurons migrate to the deepest cortical layers. Subsequently migrating neurons migrate to successively more superficial layers creating an inside out order of migration (Cooper, 2008). The first neurons migrate from the ventricular zone (VZ) to form the preplate (PP). The next neurons split the PP into the marginal zone (MZ) and the subplate (SP), which are both transient brain structures. The mature brain has six well developed cortical layers (I-VI), but none of the embryonic structures (MZ, SP, VZ). The intermediate zone (IZ) has become a mature white matter layer (WM). The neurons that form the neocortex migrate in an orderly fashion forming the six-layered neocortical mantle. Once positioned in the cortex, neurons begin to differentiate producing neurotransmitter and neurotrophic factors, and extending the dendritic and axonal processes that form fibre pathways of the brain neural networks.

On arrival in the cortical plate, cells assume progressively more superficial positions to form the six layers of the adult cortex in an inside-first, outside-last sequence. The laminar identity of deep layer neurons is determined by cues in the ventricular zone, just prior to final mitotic division (McConnell and Kaznowski, 1991). In humans, the primary sulci of the prefrontal cortex develop during GW 25–26 (Stiles and Jernigan, 2010). In the dorsolateral and lateral prefrontal cortex, basic features of the dendritic arbors of pyramidal neurons emerge during GW 17–25 (Mrzljak *et al.*, 1988). From GW 26 to 34, layer III and

V dendrites continue to mature, as spines develop, basal dendritic length increases, and interneurons differentiate in layer IV (Mrzljak *et al.*, 1992).

#### **1.3.1.2.2.1 Synaptogenesis**

The process of synaptogenesis, where new synapses are formed, begins *in utero* at around the 20th gestational week in humans (Stiles and Jernigan, 2010). The formation and organisation of synapses in the cortex increases after birth, reaches a peak, and is followed by pruning and decline. Neurons are born, migrate to their appropriate cerebral region, mature, and form synapses. Throughout childhood and adolescence, brain development is characterized by both growth and then decline in gray matter volume, and increases in white matter volume.

Synaptogenesis occurs later in the cortex than it does in other areas. The middle frontal gyrus of the cortex reaches peak synaptic density late in infancy at 3.5 years, while auditory and visual cortex attains peak density at 3 months (Huttenlocher and Dabholkar, 1997). At the age of 3 months, synaptic density in the cortex is less than half of what it will eventually reach, and synapse elimination persists throughout adolescence. In humans, peak synapse density occurs in mid-childhood around 5 years of age (Huttenlocher and Dabholkar, 1997), with the pruning of synapses extending into the third decade of life (Petanjek *et al.*, 2011). In contrast, in macaques, synapse density peaks during infancy at around 3 months of age, and pruning of excess synapses into more efficient synaptic configurations, is completed by the end of adolescence (Liu *et al.*, 2003). There appears to be no literature that examines this development in sheep to date.

#### **1.3.1.3 Neuron differentiation**

Once the neurons have reached their target region of the brain, the young neurons need to become part of information processing networks. In order to become integrated into neural networks, the neurons need to develop neuronal processes (axons and dendrites) that allow them to communicate with other neurons. Axons are the principal means of sending signals from the neuron, while dendrites are major sites for receiving input from other neurons. Each cell has many dendrites that form dense “arbors” in the immediate vicinity of the cell, and a single axon that can extend for some distance away from the cell. During development, the tip of each axon is a structure called a growth cone. The

growth cone is the site of axon elongation and extension (Brown, 2001). As the axon is extended, the growth cone inspects the local environment for guidance molecules that direct the axon toward its target. Some guidance cues are attractive and signal movement toward a source, whereas others are repulsive and guide movement away. Once the axon has reached its target, connections called synapses are formed with the target cell. Synapses allow for the transmission of electrochemical information which is the essential means of communication in the brain.

#### **1.3.1.4 Regressive events – fetal period**

Neuronal numbers are influenced by two opposing processes: early proliferation in germinal centres and apoptosis throughout development and aging. Neuron production is adjusted during development via neuronal interactions that select a subset of precursor neurons for proper functional development of the central nervous system. Evidence of naturally occurring neuronal death during nervous system development has been reported in numerous populations of neurons (Oppenheim *et al.*, 1999; Pettmann and Henderson, 1998; Williams and Herrup, 1988). Variations in programmed neuronal cell death patterns have been observed within homologous populations in different species and even within individual groups of neurons. In addition, environmental, aging, and nutritional factors influence the patterns of naturally occurring cell death in the nervous system.

While most neurodevelopmental events involve the proliferation of neurons, two important processes involve substantial loss of neural elements. In humans, the first process is apoptosis (programmed cell death) involves the normal loss of 50% or more of the neurons within a brain region (Stiles and Jernigan, 2010). The second process in humans involves synaptic exuberance and pruning, in which there is massive excess production of connections followed by the systematic elimination of up to 50% of those connections. This process tends to occur post-natally (Bourgeois and Rakic, 1993). Both of these processes reflect non-pathological events that play an essential role in establishing the complex networks of the developing brain.



#### **1.3.1.4.1 Apoptosis**

Apoptosis is a distinct form of cell death that comprises a highly regulated sequence of physiological events. It involves a cascade of gene expression that ultimately results in the breakdown of nuclear chromatin (DNA and support proteins) and the fragmenting of the cell (Roth and D'Sa, 2001). All neurons and neural progenitor cells have this intrinsic "suicide" program. The set of genes involved in the apoptotic cascade is vast, but very specific, with each molecular signal triggering the next step in the cascade (Blomgren *et al.*, 2007). A wide variety of cell intrinsic and environmental factors can influence the apoptotic process. Some trigger cell death, while others protect the cell by preventing the cascade. Apoptosis has been documented within all of the neuronal and neural progenitor cell compartments in the human brain (Rakic, 2000).

Apoptosis is a common feature in the development of many types of neurons. In vertebrate species, neuronal cell death mechanisms have been associated with a variety of developmental roles, including the elimination of errors of axonal projection or cell position (Cowan *et al.*, 1984; Denton *et al.*, 1985; Lamb, 1979, 1981), the target-dependent matching of the sizes of the pre- and postsynaptic populations (Lanser and Fallon, 1984, 1987; Tanaka and Landmesser, 1986; Vogel *et al.*, 1989), and sculpting of neuronal circuits, both during development and in the adult (Bottjer and Johnson, 1992; Finlay, 1992).

One factor that protects cells against apoptosis is the uptake of neurotrophic substances (Levi-Montalcini, 1964; Oppenheim, 1989). Neurotrophic factors are produced by target neurons at synaptic sites, and are taken up by the afferent neurons that make effective connections with the targets (Huang and Reichardt, 2001). During development, it is thought that neurons compete for neurotrophic resources. Neurons that establish effective connections are able to obtain more neurotrophic factor and are more likely to survive (Oppenheim, 1989). Thus, one important function of cell death in brain development is its role in regulating the establishment of effective and functional neural circuits (Buss, *et al.*, 2006).

Apoptosis may play as vital a role in the shaping of the adult organism by cell division, migration, and differentiation. Furthermore, cell death mechanisms must be regulated throughout life as failure to inhibit or promote cell death may lead to degenerative diseases, cancer or autoimmune diseases (Bowen 1993;

Kerr, *et al.* 1994). Considerable progress has been made in understanding the molecular mechanisms of apoptosis, with the isolation of a number of genes associated with apoptosis (Boise *et al.*, 1993; Farrow *et al.*, 1995; Gagliardini *et al.*, 1994; Hengartner and Horvitz, 1994; Oltvai *et al.*, 1993; Yuan *et al.*, 1993) Because postmitotic neurons cannot replicate, the developing nervous system has only two general mechanisms for controlling neuronal cell number, detailed by Williams and Herrup (1988):- 1) the regulation of the number of neurons that are generated or committed to a particular phenotype or 2) the regulation of the extent of programmed cell death.

### **1.3.1.5 Postnatal period**

Though the production and migration of neurons are largely prenatal events, proliferation and migration of glial progenitors continues for an extended period after birth, and the differentiation and maturation of these cells continue throughout childhood. There is little doubt that these processes play a critical role in the functional maturation of developing neural circuits. Ongoing research continues to uncover additional molecular interactions between neurons, oligodendrocytes, and astrocytes. The existence of these interactions implies that the late maturation of glial populations probably has widespread functional implications.

#### **1.3.1.5.1 Postnatal proliferation and migration**

In the postnatal period, neurogenesis continues to only a very limited degree. The subventricular zone (SVZ) serves as a source of neural stem cells in the process of adult neurogenesis. This zone harbors the largest population of proliferating cells in the adult brain of rodents, monkeys and humans (Gates *et al.*, 1995).

Abraham, *et al.* (2001) examined cell proliferation in the human cerebellar cortex between the 24th gestational week (approximately GD 85 in sheep) and the 12th postnatal month. Intensive cell formation has been found in the EGL of the human cerebellum, where the highest cell proliferation rate occurs between the 28th and 34th gestational weeks (approximately GD 95-110 in sheep). This was followed by a gradual decrease that lasts up to the eighth postnatal month. As late in development as the fifth postnatal month, 30% of cells of the EGL are still proliferating. The width of the EGL remained unchanged from the 28<sup>th</sup> gestational week to the end of the first postnatal month, when it starts to

decrease and completely disappears by the 11th postnatal month. A large number of cells labelled with Ki-67, a marker of proliferation, occur in the IGL between the 24th and 28th gestational weeks (approximately GD 85-105 in sheep). From the 36th week onwards (approximately GD 120 in sheep), the labelling index was less than 1%, although a few labelled cells have always been found in this layer even in the late postnatal period. Labelled cells are distributed in the entire width of the IGL. However, from the 34<sup>th</sup> gestational week (approximately GD 110 in sheep), almost all labelled cells are found among and directly below the Purkinje cells. This study has provided insight into the typical proliferation of cerebellar cells during development and beyond.

The development of some peripheral and central nervous system tissue does not appear to include a major role for cell removal via apoptosis. In many brain subregions, including the cerebellum, the contribution of neuronal cell death during maturation remains to be accurately characterized. The central role of Purkinje cells in cerebellar development raises questions about the mechanisms regulating Purkinje cell organisation and number. Vogel *et al.* (1991) suggested that Purkinje cell number is fixed by a genetic program that controls the number of Purkinje cells generated by a small number of founder progenitor cells. There is little knowledge about whether prenatal steroidal exposure affects apoptosis and/or proliferation in a Purkinje cell population.

#### **1.3.1.5.2 Myelination**

The efficiency of information transmission in neural pathways is greatly enhanced by myelin which acts as a sheath that covers and electrically insulates the axons. Myelin is a fatty substance that is white in appearance, hence the name white matter (Deoni *et al.*, 2011). In humans, myelination begins in the 14th week of fetal development, although little myelin exists in the brain at the time of birth. During infancy, myelination occurs quickly, leading to a child's fast development, including crawling and walking in the first year. Myelination continues through the adolescent stage of life (Dangata & Kaufman, 1997). Myelination enables nerve cells to transmit information faster and allows for more complex brain processes. Thus, the process is vitally important to healthy central nervous system functioning. During adolescence, myelination mainly occurs in the cortex and aids in cognitive development and in particular, it enables better executive functioning (Sowell *et al.*, 2003). Schwann cells are a

type of glial cells in the peripheral nervous system that wrap some neuronal axons to form the myelin sheath. Oligodendrocytes and Schwann cells indirectly assist in the conduction of impulses as myelinated nerves can conduct impulses quicker than unmyelinated ones (Bhatheja & Field, 2006). Jung-Testas & Baulieu (1998) suggested that Schwann cells, and the other types of glial cells, are targets for steroid hormones as they expression steroid hormone receptors and have the ability to synthesise steroidal precursors and enzymes.

### **1.3.1.6 Regressive events occurring post-natally**

#### **1.3.1.6.1 Apoptosis – glial populations**

Brain development involves overproduction of neurons and glial cells, neural processes, and synapses. Although neural apoptosis peaks during prenatal life in humans, apoptosis in glial cell populations has a time course corresponding to the protracted differentiation from glial precursors. During the period of initial myelination, many excess oligodendrocytes undergo apoptosis a few days after differentiating, and there is evidence that this process depends on signals from nearby axons, such that the number of surviving oligodendrocytes matches the local axonal surface area (McTigue and Tripathi, 2008).

#### **1.3.1.6.2 Synaptic pruning**

The major mechanism for generating diversity of neuronal connections beyond their genetic determination is the activity-dependent stabilisation and selective elimination of the initially overproduced synapses (Changeux and Danchin, 1976). The largest number of synapses has been recorded in the cerebral cortex of human and nonhuman primates. It is generally accepted that synaptic pruning in the cerebral cortex occurs at puberty and is completed during early adolescence in humans (Huttenlocher, 1979).

Two features of cerebral development are very important for understanding how experiences can modify cortical organisation. Firstly, dendritic spine density, which is an approximate surrogate for the number of excitatory synapses on a neuron, is two to three-fold greater in childhood than in adulthood. This overproduction of synapses is reversed beginning in late childhood, and in the cortex, it continues well into the third decade of life (Petanjek *et al.*, 2011). Secondly, the overproduction of spines is greatest in the cortex, which then shows the slowest rate of synapse elimination (Elston *et al.*, 2009). This extraordinarily long period of synapse elimination in the cortex has implications for understanding the environmental influences in puberty on adult cognitive capacities. Nonetheless, the period of synapse production may be as important in understanding adult cognitive and emotional capacities as the later adolescent pruning. It has been argued that it is in the period from early childhood to kindergarten in which the cortex forms the basic neural circuitry that will later underlie higher cognitive functions (Tsujimoto, 2008). Experiences early in life can therefore lay down the basic circuitry that is modified in

adolescence. Early experiences (aversive or other) set up the cortex trajectories and have lifelong consequences on behavioural regulation (Kolb, 2012).

## 1.4 Steroids

### 1.4.1 Steroidogenesis and hormone receptor signalling

Estradiol, progesterone, and testosterone are all steroid hormones synthesised from cholesterol. These hormones are best known for their role in sexual behaviour in adult humans and animals (Roselli *et al.*, 2006). However, they also play an active role in the normal development of the CNS (Tsutsui, 2006). Steroidogenesis begins when cholesterol is transferred from the outer mitochondrial membrane to the inner mitochondrial membrane by the steroidogenic acute regulatory protein (StAR) which is the rate-limiting step in steroid production (Dean and McCarthy, 2008). StAR is expressed in Purkinje cells of adult rats (Furukawa *et al.*, 1998), suggesting a role in steroid biosynthesis in the cerebellum. Once inside the mitochondrion, cholesterol is then cleaved to form pregnenolone, a steroid hormone precursor, by the enzymatic actions of P450<sub>scc</sub> (*CYP11A1*, Stocco and Clark, 1996). P450<sub>scc</sub> is expressed in Purkinje cells of fetal sheep (Petratos *et al.*, 2000), further evidence that steroidogenesis occurs in the cerebellum.

Steroid hormone receptors are known to regulate neuronal function and act as transcription factors. The classical signalling pathway involves binding of hormone to its receptor, resulting in the formation of a hormone-receptor complex. This complex then binds to specific DNA sequences known as a response element, which in turn regulates the expression of steroid responsive genes (Chang *et al.*, 1995).

#### 1.4.1.1 The aromatase pathway

Testosterone is the ligand for the androgen receptor (AR). AR signalling has been shown to occur in the hypothalamus, and in the granule and Purkinje cells of the cerebellum (Chang *et al.*, 1995). An example of AR signalling occurring within the cerebellum was detailed by Ahlbom *et al.* (2001), who found that when testosterone binds to AR in cultured cerebellar granule cells, these cells were protected against oxidative stress. Testosterone decreased cell death induced by hydrogen peroxide, a reactive oxygen species, by increasing catalase activity, the enzyme which breaks it down. Ahlbom *et al.* (2001) also examined estrogen treatment, which demonstrated no protective effects against oxidative stress and went on to show that flutamide, an AR antagonist, blocked the AR-mediated protective effect. Therefore, this study concluded that the protective action of testosterone was due to the AR-mediated direct action of testosterone.

In the brain, androgens can be metabolised by two different major pathways. First is the aromatase (*CYP19*) pathway (Figure 1.8). This pathway involves the conversion of testosterone into estradiol via the actions of aromatase (Balthazart and Foidart, 1993), encoded by *CYP19* gene. Aromatase is expressed in neuronal cells of the cerebellum, amongst other areas of the rodent brain (Lephart *et al.*, 1996).

Estradiol acts on the estrogen receptor (ER). There are two isoforms of ER – alpha and beta (*ER $\alpha$*  and *ER $\beta$* ). *ER $\alpha$*  is weakly expressed in the developing rat cerebellum with no expression found in the adult (Beyer, 1999). This indicates that *ER $\alpha$*  may have a role in the regulation of early prenatal brain differentiation and development (Belcher, 1999). *ER $\beta$*  is also expressed in the developing fetal sheep cerebellum (Schaub *et al.*, 2008). Both isoforms bind to estradiol with high affinity however they appear to have different functions. *ER $\alpha$*  mediates estradiol-dependant activation of transcription whereas *ER $\beta$* , caused inhibition of transcription (Paech *et al.*, 1997), thus suggests important differences in the gene regulatory functions of these isoforms. Estradiol also impacts on brain regions involved in non-sexual motorised and cognitive functions *i.e.* the cerebellum (Beyer, 1999).



#### 1.4.1.2 The 5 $\alpha$ -reductase pathway

Androgens are also metabolised via the 5 $\alpha$ -reductase pathway (Figure 1.8). Here testosterone (T) is converted into dihydrotestosterone (DHT) and progesterone into dihydroprogesterone (DHP). Two isoforms have been discovered for 5 $\alpha$ -reductase – 5 $\alpha$ -R type 1 and 5 $\alpha$ -R type 2. *SRD5a1* (the gene encoding 5 $\alpha$ -reductase 1) mRNA expression has been found in the cerebellum of adult humans (Thigpen *et al.*, 1993) and there is evidence that *SRD5a2* (the gene encoding 5 $\alpha$ -reductase 2) is expressed in fetal sheep cerebellum (Petratos *et al.*, 2000).

#### 1.4.1.3 The progesterone pathway

Progesterone is synthesised from pregnenolone by *HSD $\beta$ 3* (Figure 1.8; Payne and Hales, 2004) which is expressed in granule and Purkinje cells of the cerebellum in adult male rats (Guennoun *et al.*, 1995). Progesterone may be metabolized further by 5 $\alpha$  reductase to 5 $\alpha$ -dihydroprogesterone (Compagnone and Mellon, 2000). Progesterone and 5 $\alpha$ -dihydroprogesterone can mediate effects on the brain through the nuclear progesterone receptor (Belelli and Lambert, 2005) and it has been shown that progesterone stimulates cerebellar neuronal circuit formation (Tsutsui, 2006).

The progesterone receptor (PR) has two isoforms encoded by a single gene – *PR-A* and *PR-B*. *PR-B* is the full length form (155 kDa) whereas *PR-A* is an N-terminally truncated, 80 kDa form of the protein (Mulac-Jericevic *et al.*, 2000). These are functionally distinct in terms of their ability to activate target genes (Tora *et al.*, 1988): *PR-B* is known to function as a transcriptional *activator* of progesterone responsive genes whereas *PR-A* is known to function as a transcriptional *inhibitor* of progesterone responsive genes (Tsutsui *et al.*, 2000). PR mRNA expression has been detected in the cerebellum of neonatal rats (Sakamoto *et al.*, 2001). Progesterone is thought to act directly on Purkinje cells through intranuclear receptor-mediated mechanisms to promote dendritic growth, spinogenesis and synaptogenesis (Sakamoto *et al.*, 2003). The expression of PR is under the control of estrogen, which ultimately increases PR expression. PR protein levels are increased after exogenous estrogen administration in the mammalian uterus (Milgrom *et al.*, 1973). PR show

sensitivity to progestins and evidence has illustrated that progesterone decreases *PR* expression in most target tissues (Leavitt *et al.*, 1977).

#### **1.4.1.3.1 Non-classical progesterone signalling – GABA**

In addition to classical signalling via nuclear receptors, neurosteroids, such as progesterone, exert non-classical rapid actions as allosteric agonists of the GABA<sub>A</sub> receptor, a ligand-gated ion channel.

GABA is the endogenous ligand for GABA<sub>A</sub> receptor and is also the major inhibitory neurotransmitter in the central nervous system (Compagnone and Mellon, 2000). Progesterone can enhance GABA<sub>A</sub>-induced chlorine currents and therefore modulates the inhibitory actions of GABA<sub>A</sub> (Nicol *et al.*, 1999). However, during fetal development and the first post-natal week in rodents, GABA, acting via GABA<sub>A</sub> receptors often exerts *excitatory* actions on immature cortical neurons of the hippocampus (Cherubini *et al.*, 1991). These excitatory effects of GABA are due to an elevated intracellular chloride concentration and depolarized values of the reversal potential of the GABA<sub>A</sub> receptor activated responses (Owens *et al.*, 1996).

GABA is the main inhibitory neurotransmitter in the CNS and its action is mediated by GABA receptors (GABAR). There are three types of GABARs – GABA<sub>A</sub>, GABA<sub>B</sub> and GABA<sub>C</sub>. GABA<sub>A</sub> and GABA<sub>C</sub> are ligand gated chloride channels (Sieghart and Sperk, 2002) whereas GABA<sub>B</sub> belongs to the G-coupled protein receptor superfamily (Chen, *et al.*, 2005) which open or close ion channels via intermediaries. The GABA<sub>A</sub> receptor binds two GABA molecules at the interface between  $\alpha$  and  $\beta$  subunits which open the ligand-gated channel and produces a chloride current across the membrane (Colquhoun and Sivilotti, 2004). 19 different subunits for GABA<sub>A</sub> have been identified -  $\alpha_{1-6}$ ,  $\beta_{1-3}$ ,  $\gamma_{1-3}$ ,  $\delta$ ,  $\epsilon$ ,  $\pi$ ,  $\theta$  and  $\rho_{1-3}$  (Barnard *et al.*, 1998). GABA<sub>A</sub> $\alpha_1$ , GABA<sub>A</sub> $\alpha_6$ , GABA<sub>A</sub> $\beta_2$ , GABA<sub>A</sub> $\beta_3$  and GABA<sub>A</sub> $\delta$  are the most abundantly expressed in the cerebellum (Sieghart and Sperk, 2002). Laurie *et al.* (1992) demonstrated that *GABRA6* mRNA is exclusive to postmigratory granule cells in the IGL of rats, which indicates a highly cell-specific control of gene expression. This mRNA could not be detected anywhere else in the developing brain or embryo. Therefore, in the present study, *GABRA6* gene expression was used as a unique indicator for cerebellar granule cell development.

In long-gestation species such as humans and sheep, the majority of brain development occurs before birth. In these species, GABAergic currents are excitatory for the first two thirds of gestation before becoming inhibitory by the last trimester (Hirst *et al.*, 2009). In the mature brain, GABA<sub>A</sub> receptor activation causes an influx of chlorine ions (Cl<sup>-</sup>) and membrane hyperpolarization, which is due to the electrochemical gradient for Cl<sup>-</sup>. This results in the inhibition of the cell (Wang and Kriegstein, 2009). The excitatory effect of GABA in developing neurons is due to an outflow rather than influx of Cl<sup>-</sup> ions following activation of GABA<sub>A</sub> receptors. This is due to the intracellular Cl<sup>-</sup> concentration being higher than in mature cells.

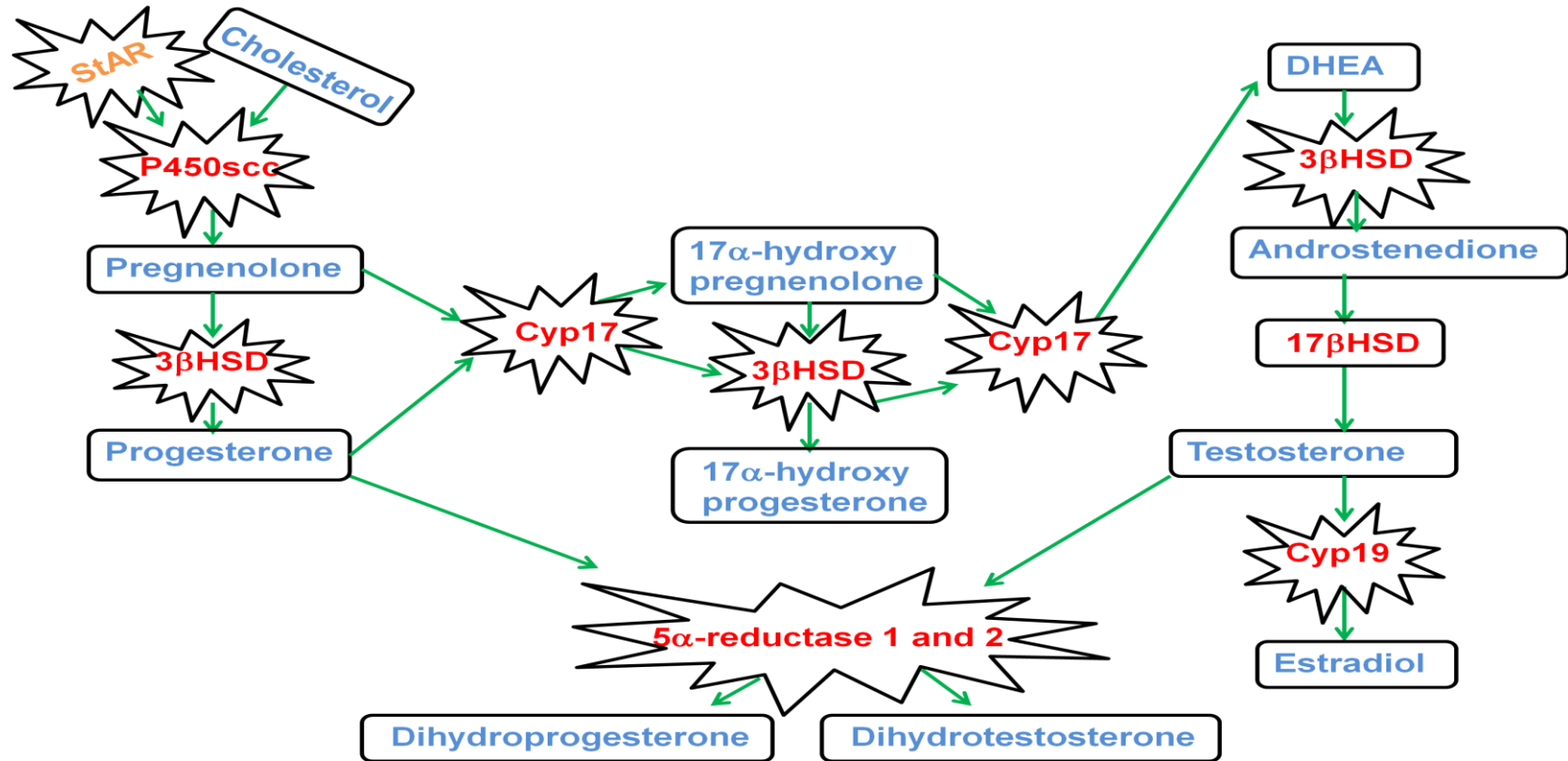


Figure 1.8: The steroid synthesis pathway contains the transporter at the rate limiting step of neurosteroidogenesis (in orange), the steroidal precursors (in blue) and the enzymes (in red), involved in this process. This is the pathway through which peripheral steroids are metabolised and how steroids are made *de novo* in the brain. The spike bubbles indicate the GOIs investigated in this study.

### **1.4.2 Peripheral steroids**

Peripheral steroids are gonadal hormones that are synthesised in the adrenal glands, gonads and placenta (Williams and Meck, 1991). These steroids exert biological effects on the nervous system and play a major role in the development, growth, maturation and differentiation of the CNS and the peripheral nervous system (Mensah-Nyagan *et al.*, 1999). Peripheral steroids act on brain tissue through receptor-mediated mechanisms in order to regulate the important neuronal functions mentioned above. The metabolism of peripheral steroids in brain tissues can result in biotransformation and the production of biologically active metabolites (Arnold and Gorski, 1984).

### **1.4.3 Neurosteroids**

The brain is capable of responding to steroid hormones synthesised locally (neurosteroids) and peripherally (via steroid gland secretion). Baulieu (1991) showed that in rat brain, steroids were being synthesised *de novo* and so coined the term “neurosteroids”. Neurosteroids are defined as steroids that are synthesised in the CNS from cholesterol or steroid precursors, such as pregnenolone and progesterone that accumulate in the nervous system. These steroids are produced separately from steroidogenic gland secretion. The metabolism of peripheral steroids in brain tissues can result in biotransformation and the production of biologically active metabolites (Arnold and Gorski, 1984). This, together with evidence of the expression of steroid receptors in the brain demonstrates why the brain is considered to be a steroid target site.

#### **1.4.3.1 The cerebellum**

The Purkinje cells of the cerebellum have been identified as a major site for neurosteroid formation in the brain (Tsutsui, 2006). These neurons produce several different neurosteroids, enzymes and precursors, therefore Purkinje cells have served as an excellent cellular model for the study of neurosteroid actions in the cerebellum (Tsutsui, 2008). During development, Purkinje neurons synthesise steroidogenic enzymes (Compagnone and Mellon, 2000). Furthermore, steroid hormone receptors have been shown to be localised within these cells (Tsutsui *et al.*, 2011). Although steroidogenic enzymes and receptors have been shown to be expressed in the cerebellum, the impact of steroid exposure on cerebellar development has yet to be investigated.

## **1.5 The impact of steroids on brain development**

Animal studies have shown that exposure to steroids during fetal brain development can affect neuroendocrine and behavioural functions. This occurs via intracellular and cell-surface receptors that regulate changes in protein synthesis (Mensah-Nyagan *et al.*, 1999). It has been suggested that steroidal hormones play a variety of regulatory functions in the CNS. In particular, during prenatal brain development gonadal androgens and estrogens appear to modify events related to neuronal survival and synapse formation (Breedlove, 1992). The effects of gonadal steroids on neuronal differentiation and survival vary with brain region and developmental state (Lauber *et al.*, 1997; MacLusky *et al.*, 1987).

Steroids exert many effects on the nervous system and play a major role in the development of the central nervous system, for example in the hypothalamus and pituitary gland. These brain areas are involved in sexual behaviour and have been the focus of most neuroendocrine studies to date. However other areas of the brain, including the cerebellum, express proteins that are necessary for local steroidogenesis and steroid signalling. In this study, a fetal steroid programming model was utilised in order to investigate how gene expression in the cerebellum is affected by steroid exposure during development - and how this effect subsequently changes the brain's architecture and function. This approach may yield insight into some of the mechanisms that underlie neurodevelopmental disorders.

### **1.5.1 The role of androgen in brain development**

Fetal testosterone has been shown to affect the anatomy of the brain, including the hypothalamus, limbic system, and neocortex (Breedlove, 1994). Sexually dimorphic cognitive skills, such as spatial navigation (Williams and Meck, 1991) have been shown to be influenced by fetal testosterone. In the fetal primate brain, significant androgen receptor binding occurs in the cerebral cortex, cerebellum, mediobasal hypothalamus and corpus callosum in both sexes (Naftolin, 1994). There is sexually dimorphic AR binding in males in the cortex (Chapman *et al.*, 2006).

There are two peaks of gonadal testosterone production in human male development – during weeks 8 to 24 of gestation and the first six months of infancy. The first surge influences behavioural and physical development.

However, not a lot is known about the effect of the second surge (Hines and Alexander, 2008). The variability in individual prenatal androgen exposure influences post-natal behaviour in humans (Hines and Alexander, 2008). For example, when pregnant non-human primates and rodents are treated with testosterone prenatally, their female offspring demonstrate an increase in behaviours associated with males (Herman, *et al.*, 2000).

Testosterone, and hormones metabolised from this steroid, plays an important role in brain development, directing neural development during early life and also influencing the underlying organisation of the brain (Arnold and Gorski, 1984). One of the best known examples of the neural influence of gonadal steroids is the sexually dimorphic nucleus of the preoptic area (SDN-POA), a sub region of the anterior hypothalamic/preoptic area (AH/POA) (Arnold and Gorski, 1984). Research on the SDN-POA has demonstrated that the early hormone environment can influence brain development by determining which cells survive, what other neural regions they connect to anatomically and which neurotransmitters they use (De Vries and Simerly, 2002).

Androgens alter the morphology, survival, and axonal regeneration of both sexually and non-sexually dimorphic motor neurons. Furthermore, testosterone increases expression of nerve growth factor (Tirassa *et al.*, 1997) and mediates promotion of neurite growth and interneural communication through branching and arborisation during development (Kujawa *et al.*, 1991). There is little literature on how testosterone affects cerebellar development but it has been suggested that flutamide, a non-steroidal AR antagonist, administered at PND 5 in mice, does not significantly affect Purkinje cell numbers (Biamonte *et al.*, 2009), which argues against a role for testosterone and DHT in Purkinje cell survival. Another possibility is that this effect is not mediated by AR. During the rodent perinatal critical period, there is a significantly higher level of estradiol in males compared to females, which is thought to act on male brain development (Wright *et al.*, 2010). Estradiol has been shown to regulate apoptosis (to produce sexually dimorphic cell numbers), dendritic spine formation, neuronal migration, and synaptic organization in hypothalamic regions, most of which are key regions for regulating male and female sexual functions in the adult brain (Zhang *et al.*, 2000; Sakamoto *et al.*, 2003). Another steroid hormone that influences brain development is progesterone. This steroid, but not its

metabolite allopregnenolone, is thought to act directly on Purkinje cells through intranuclear receptor-mediated mechanisms to promote dendritic growth, spinogenesis and synaptogenesis (Sakamoto *et al.*, 2003).

#### **1.5.1.1 How testosterone influences development of different brain regions**

Neonatal treatment with testosterone has been reported to alter morphological as well as functional development in various regions of the brain and spinal cord. For example, testosterone and DHT have been shown to promote survival of neurons in the male hippocampus (Spritzer and Galea, 2007). (Wright and Smolen, 1983) have illustrated that neonatal testosterone treatment increases neuron and synapse numbers in male rat superior cervical ganglion.

Experiments in male rodents suggest that testosterone is linked to an increase in neuron somal size, neuritic growth, plasticity and synaptogenesis in motoneurons of the spinal nucleus of the bulbocavernosus (Forger *et al.*, 1992; Matsumoto, 1997). Kurz *et al.*, (1986) demonstrated that a decrease in androgen levels after castration produced dramatic structural changes, decreasing the dendritic length and soma size of motoneurons in male rats. These changes were reversed by androgen replacement whereby dendritic arbor and soma size were restored to typical levels. This suggests that androgens may regulate neuronal function in adulthood by influence on length and size of dendrites of motoneuronal somas. These studies contrast the findings of Biamonte *et al.*, (2009), who implied that testosterone and DHT do not influence Purkinje cell survival.

#### **1.5.2 Clinical implications**

##### **1.5.2.1 Sexual differentiation**

Sexual differentiation is the process by which the central nervous system becomes structurally and functionally male or female. Structural sexual dimorphisms are typically manifested as sex differences in cell group volume, neuron or glial cell number, soma size, dendritic arborizations, dendritic spine density, degree of myelination, or a combination of these features (Juraska *et al.*, 2013). Phoenix *et al.* (1959) was the first to demonstrate that the expression of male and female sexual behaviour in adulthood could be masculinised and defeminised by manipulating exposure to testosterone during prenatal development. This group had suggested that these early developmental effects



of androgens on later adult behaviour must be based on organisational effects of these hormones on the structure of the developing nervous system.

From investigations in rodents, three different paths of sexual differentiation have been proposed. First, the critical window for hormonally mediated sexual differentiation of brain extends through to the adolescent period of development. Second, ovarian hormones, *i.e.* estrogens, play an active role in the feminisation of the brain during puberty. Lastly, hormonally mediated sex differences in the addition of new neurons and glial cells and sex differences in neuronal survival, contribute to sexual differentiation of hypothalamic, cortical and limbic regions during adolescence (Juraska *et al.*, 2013).

Structural sex differences in the cerebral cortex are not as visually prominent or as large in magnitude as those in the hypothalamus. Nevertheless, there are sex differences in the cortex and in its connections. Notably, many of the sex differences emerge during puberty as the result of exposure to ovarian hormones in females. Reid and Juraska (1992) found that adult male rats had a thicker cortex than adult females. The cortical thickness was examined in four locations: the primary motor cortex, the forelimb area of the sensorimotor cortex and the monocular and the binocular areas of the visual cortex and each showed sex differences. The investigation on the cellular basis for this indicated that it was not due to the number of synapses per neuron, as the number of synapses per neuron was not different between the sexes (Reid and Juraska, 1995) but was due to a 17% increase in the number of neurons in the in adult male rats, when compared to females (Reid and Juraska, 1992).

The sex difference in neuron number in the adult rat cortex occurs during puberty and is probably due to cell death-promoting influences of ovarian hormones in females. Similarly, synaptic pruning occurs during adolescence in the cortex, but less pruning occurs in female rats ovariectomised at PND 30 compared with intact female rats (Munoz-Cueto *et al.*, 1990). Thus, the findings for the cortex are similar to those sexually dimorphic regions such as anteroventral periventricular nucleus (AVPV) and SDN-POA, in the way that ovarian hormones affect the organisational effects of the female brain during puberty.

#### **1.5.2.1.1 Gene expression and sexual dimorphism**

Dewing,, *et al.*, (2003) explored the hypothesis that genes, by directly inducing sexually dimorphic patterns of neural development, can influence the sexual differences between male and female brains. The group performed microarray analysis on GD 10.5 male and female mice to evaluate any differences in gene expression between genders prior to gonadal formation. The majority of the genes that exhibit gender differential expression are known to be involved in cellular differentiation and proliferation, transcriptional regulators and cell signalling. This suggests that genetic factors may play a part in influencing brain sexual differentiation in the identification of genes differentially expressed between male and female brains prior to gonadal formation.

#### **1.5.2.2 Congenital adrenal hyperplasia**

Findings have arisen from clinical syndromes during early life. Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder that is caused by a 21-hydroxylase (21-OH) deficiency. This enzyme is needed to produce cortisol, also synthesised from cholesterol, and 21-OH deficiency results in cortisol precursors being shunted into the adrenal androgen pathway, and so testosterone is over produced. Males appear normal but they display virilised behaviour. However in females the testosterone level is elevated which results in masculinised or ambiguous genitalia (Meyer-Bahlburg *et al.*, 2004).

There is evidence to suggest that prenatal androgen exposure also influences brain development and therefore behaviour. For example 3-8 years old CAH female children show increased interest in toys associated with males and a reduced interest in dolls. These females also prefer rough, active play states found in young males (Hines and Kaufman, 1994). Maternal testosterone levels during gestation are correlated positively to masculine behaviours seen in their young female offspring (Hines *et al.*, 2002). This implies that prenatal androgen exposure can have a profound effect on behaviour and personality later on in life.

#### **1.5.2.3 Neurodevelopmental disorders**

Neurodevelopmental disorders are a group of conditions with onset during early childhood. The disorders typically manifest early in development and are characterized by developmental deficits that produce impairments of personal, social, academic, or occupational functioning. The range of developmental

deficits varies from very specific limitations of learning or control of executive functions to global impairments of social skills or intelligence. For some disorders, the clinical presentation includes symptoms of excess as well as deficits and delays in achieving expected milestones. For example, autism spectrum disorder is diagnosed only when the characteristic deficits of social communication are accompanied by excessively repetitive behaviours and restricted interests (Karlsson, 2014).

#### **1.5.2.3.1 Structural cerebellar abnormalities in neurodevelopmental disorders**

The cerebellum has a role in motor function e.g. voluntary limb movements and there is also evidence to suggest that the cerebellum contributes to cognitive function e.g. executive function (planning, abstract reasoning and working memory) and spatial cognition (visual spatial organization and memory; Diamond, 2000). Purkinje cells of the cerebellum play a key role in modulating the output from the cerebellum to the cerebral cortex, because they provide input to the deep nuclei such as the dentate nucleus. The deep nuclei in turn provide the sole output from the cerebellum to the cerebral cortex. It has been repeatedly shown that the cerebellum is activated in a variety of mental activities, even when motor activity is well-controlled, including facial recognition, emotion attribution, directed attention, and many types of memory (Allen *et al.*, 1997; Decety and Ingvar, 1990; Kim and Thompson, 1997; Seidler *et al.*, 2002).

There has been extensive research into the pathology of the cerebellum in autism. In general, there seems to be a consensus that there is a reduction in the number of Purkinje cells in adult male humans that suffer from autism (Bauman and Kemper, 1985; Fehlow *et al.*, 1993; Kemper and Bauman, 1993; Ritvo *et al.*, 1986). Fatemi *et al.* (2002) also illustrated that the size of Purkinje cells was reduced by 24% in autistic adult male humans, as did Bauman and Kemper (1985).

The growing recognition that the cerebellum is engaged in basic cognitive functions such as timing and associative learning, has led to an emerging interest in the role of the cerebellum in schizophrenia. However, there is less-well documented evidence that there are cerebellar alterations in patients with schizophrenia. Reyes and Gordon (1981) demonstrated that the linear density of Purkinje cells was reduced in schizophrenic males and Tran *et al.*, (1998)

reported that Purkinje cells were smaller by 8.3%. These findings are similar to the ones found in autistic males (Fatemi *et al.*, 2002; Bailey *et al.*, 1998), which may suggest that Purkinje cell alterations play a role in the onset of neurodevelopmental disorders. The literature points to a sex bias for both autism and schizophrenia, which suggests androgens play a role in the aetiology of neurodevelopmental disorders.

There is mounting evidence that gender and the level of steroid hormones during development, in particular androgens, are risk factors and may contribute to neurodevelopmental disorders such as autism and schizophrenia (Baron-Cohen, 2002; Rees and Harding, 2004). Previous literature has further demonstrated that in neurodevelopmental disorders such as autism and schizophrenia, there is abnormal cytoarchitectural development in the cerebellum (Bailey *et al.*, 1998; Bauman and Kemper, 1985; Reyes and Gordon, 1981; Tran *et al.*, 1998). This suggests that defects in neuronal maturation and cortical organisation may be responsible for some of the neurological problems seen in autism. A candidate gene is one which is thought to cause or contribute to a particular disorder or disease. Candidate genes can be identified by several methods, including prior knowledge of the biological pathway, linkage studies, expression studies, and genome wide association studies (GWAS). The identification of single nucleotide polymorphisms (SNPs) within a gene can also be used to narrow the region of investigation (Weiss *et al.*, 2009).

#### **1.5.2.3.2 Autism**

Autism is a severe neuropsychiatric disorder that predominantly affects male individuals with a 4:1 male:female ratio (Manning *et al.*, 2001). It has been suggested that the second to fourth digit ratio (the second being the index finger and fourth being the ring finger), is affected by prenatal exposure to excess testosterone and that this ratio can be considered a crude measure for prenatal androgen exposure. Indeed, Manning *et al.*, (1998) showed that low second to the fourth digit ratio negatively correlates with a high prenatal androgen exposure. Further evidence to suggest that excess prenatal testosterone levels are a contributing factor in the occurrence of autism is that the second to the fourth digit ratio is low in autistic individuals (Manning *et al.*, 2001). Baron-Cohen (2002) described the “extreme male brain” theory in which the behaviour

seen in autism is an exaggeration of typical sex differences, and that exposure to high levels of prenatal testosterone may be a risk factor.

Several candidate genes have been identified in autism. One of these genes is the *RELN* gene, which encodes for the Reelin protein, and has a major role in neuronal migration and in prenatal development of neuronal connections (D'Arcangelo *et al.*, 1995). Reduced Reelin expression in the cerebellum of adult humans has been found to be associated with autism (Fatemi *et al.*, 2005). Another candidate gene is *RORA* (retinoic acid-related orphan receptor alpha) which activates genes in the cerebellum that stimulate proliferation of granule cell precursors in the external granule layer whilst also regulating a set of genes in Purkinje cells involved in processing neurotransmitter signalling (Gold *et al.*, 2003). Sarachana *et al.* (2011) also demonstrated that *RORA* expression was increased in the human neuroblastoma cell line, SH-SY5Y, upon estradiol treatment but down-regulated with DHT treatment. This suggests that *RORA* is responsive to steroid hormone levels.

#### **1.5.2.3.3 Schizophrenia**

Schizophrenia is thought to be a neurodevelopmental disorder with its origin during the critical phases of gestational life (Lewis and Levitt, 2002) when neurons fail to grow or migrate to their appointed sites or are unable to make the right synaptic connections. There has been extensive research on structural abnormalities of the frontal and temporal cortex in schizophrenia. Examples include: decreased dendritic spine density in the prefrontal cortex (Glantz and Lewis, 2000); increased ventricular volume and decreased hippocampal volume in affected children (Gogtay *et al.*, 2004); and a reduced volume of the rostral hippocampal formation in the affected twin in most discordant monozygotic twin sets (Suddath *et al.*, 1990), which also implies that an environmental factor may be involved.

Several candidate genes have also been identified in schizophrenia. Brain-derived neurotrophic factor (BDNF) is a nerve growth factor that enhances neuronal survival (Beyer, 1999). BDNF is abnormally regulated in the CNS of animal models of schizophrenia (Fiore *et al.*, 2002) and human schizophrenic patients have reduced number of BDNF-positive neurons and BDNF in the serum (Toyooka *et al.*, 2002). Durany *et al.*, (2000) also demonstrated that

there was a significant decrease in BDNF concentrations in cortical areas and the hippocampus of human post-mortem tissue from schizophrenic patients. Therefore, all this evidence taken together would imply that BDNF may be a candidate gene for schizophrenia and so BDNF mRNA levels were measured and evaluated in the present study, in order to determine whether there were any changes in gene expression after prenatal androgen exposure.

*DISC1* has been identified as a susceptibility gene for schizophrenia due to the link between major psychiatric disease in a large Scottish cohort and being a carrier of the *DISC1* gene that has been mutated by a translocation (Millar *et al.*, 2000). Schurov *et al.*, (2004) demonstrated that *DISC1* expression is developmentally regulated with two major peaks at GD 13 and PND 35 (approximately GD 20 and one postnatal month in sheep, respectively) in the cortex and hypothalamus of mice. These time periods are critically important in the development of rodents and correspond to the period of neurogenesis in the developing brain (Schurov *et al.*, 2004). The cortex is strongly implicated in schizophrenia and it has been previously reported that the density of *DISC1* positive neurons was significantly reduced in certain layers of the cortex of schizophrenics (Millar *et al.*, 2000). It was proposed that the appearance of diagnostic symptoms was linked to the normal maturation of brain areas affected by the early developmental pathology, particularly the dorsolateral cortex (Buchanan *et al.*, 1998).

Pickard *et al.*, (2005) demonstrated that *NPAS3* expression is disrupted in schizophrenia. Further evidence to support this came from Pieper *et al.*, (2005) who saw reduced neurogenesis in the hippocampus of *NPAS3 knockout* mice and went on to suggest the possibility that impaired hippocampal neurogenesis may be relevant to some aspects of hippocampal pathology that are associated with schizophrenia. The genes mentioned above have been selected as genes of interest in our study and their mRNA expression was determined and then compared between the control and treated groups.

## 1.6 Gene expression

### 1.6.1 Nuclear receptor signalling

Nuclear receptors may be classified into four types based on their dimerisation and deoxyribonucleic acid (DNA) binding properties. Steroid hormone receptors bind to DNA as homodimers either to simple response elements comprising short palindromic sequences or to composite response elements with other transcription factors. Estrogen response elements consist of inverted repeats of the sequence A/ GGGTCA separated by three nucleotides, whereas the receptors for androgens, glucocorticoids and progestins bind to inverted repeats of the sequence AGA/GACA, also separated by three nucleotides.

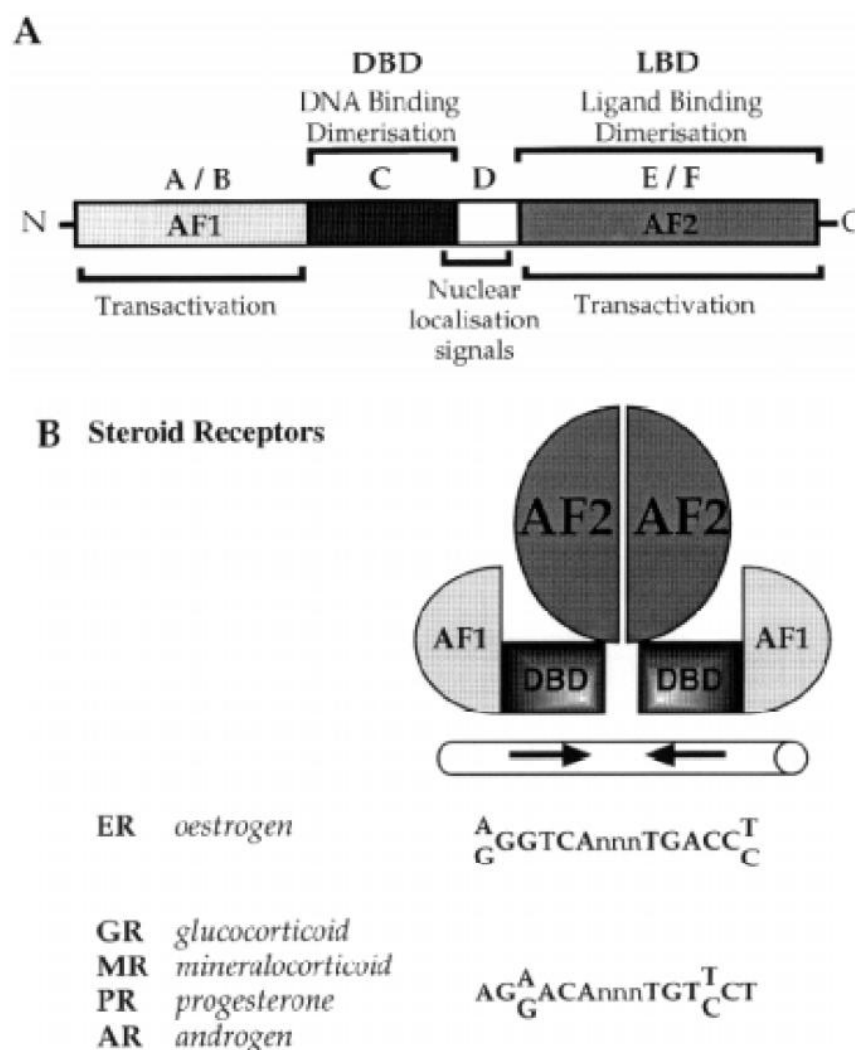


Figure 1.9: A) Organisation of functional domains in nuclear receptors. DBD = DNA binding domain. LBD = ligand binding domain. AF1 and AF2 = transactivational domains. B) Schematic representation of steroid hormone receptor binding to DNA. The receptors bind as homodimers to palindromic sequences. The specific sequences of simple response elements for steroid hormone receptors are shown. Taken from (White and Parker, 1998)

Receptors are characterised by a highly conserved DNA binding domain and a moderately conserved ligand binding domain which also functions in dimer formation and transcriptional activation, Figure 1.9 A (Mangelsdorf *et al.*, 1995; Parker, 1993). Transcriptional activation by steroid receptors is mediated by at least two distinct activation functions; AF1, is located in the N-terminal domain and is a strong regulator of transcription and AF2, is in the hormone binding domain which is dependent on ligand-binding (Lee, *et al.*, 1989). A number of studies have shown that this AF1 is a target for phosphorylation by other signalling pathways (Bunone *et al.*, 1996). The second activation function, AF2, is induced by hormone binding and a short sequence encoding an amphipathic  $\alpha$ -helix in the C-terminal part of the ligand binding domain, which is conserved in all transcriptionally active nuclear receptors, has been shown to be essential in receptor function (Durand *et al.*, 1994).

When activated by hormone binding, the receptors may act directly as transcription factors by binding to specific DNA sequences, termed hormone response elements, found in the vicinity of target genes, Figure 1.9 B, (Beato, 1989). Composite response elements have also been identified which bind receptors in addition to other transcription factors such that the binding of one influences, either positively or negatively, the activity of the other (Diamond *et al.*, 1990). In addition, it is evident that nuclear receptors are also capable of regulating the transcription of genes that lack hormone response elements by modulating the activity of other transcription factors such as AP-1 (Webb *et al.*, 1995).

In the absence of hormone, steroid hormone receptors exist as inactive oligomeric complexes with a number of other proteins including chaperone proteins, namely the heat shock proteins Hsp90 and Hsp70 and cyclophilin-40 and p23 (Smith and Toft, 1993). The role of Hsp90 and other chaperones may be to maintain the receptors folded in an appropriate conformation to respond rapidly to hormonal signals. Whether different types of receptor complexes occur in cells, each containing a subset of chaperones and able to respond to different types of signal, or whether receptors are folded into a mature complex containing chaperones in an assembly line type of process is as yet unclear (Bohen *et al.*, 1995). Following hormone binding, the oligomeric complex dissociates allowing the receptors to function either directly as transcription



factors by binding to DNA in the vicinity of target genes or indirectly by modulating the activity of other transcription factors.

## 1.7 Fetal programming

Barker postulated the hypothesis that exposure of a fetus to environmental insults at critical periods of development could programme long term changes in gene expression that manifest in adulthood (Barker, 1998; Barker *et al.*, 1990). This mode of inheritance may therefore be transferred from mother to fetus through nutrition, stress, hormonal imbalances, exposure to environmental toxins and so forth that could have adverse effects on fetal development (Symonds *et al.*, 2009).

There is a vast amount of literature that highlights fetal programming. One of the most well-known studies examined the long-term metabolic effects and cardiovascular disease in offspring of women that were pregnant during the Dutch hunger winter in 1944-45 (Schulz, 2010). The low calorie intake and under nutrition during pregnancy resulted in a cohort of offspring that were born with low birth weights and were susceptible to diabetes, pulmonary disease and cancers in adult life, which was particularly prevalent if starvation occurred during the third trimester of pregnancy (Roseboom *et al.*, 2006). Interestingly, obesity was more common in those individuals whose mothers were starved during early pregnancy and early exposure to undernutrition has also been associated with schizophrenia and personality disorders (Hoek *et al.*, 1996; Neugebauer *et al.*, 1999). Other studies have indicated that fetal adaptation to an unfavourable environment *in utero* can result in poor sensitivity to a plentiful post-natal environment. This is commonly reflected by rapid post-natal catch-up growth which itself has been associated with the onset of obesity, diabetes and cardiovascular disease (Hales and Barker, 2001).

### 1.7.1 PCOS

Polycystic ovary syndrome (PCOS) is a complex endocrine disorder that affects women in their reproductive years. PCOS affects the endocrinic, reproductive and metabolic systems. This syndrome is characterised by the cessation of maturation of the ovarian follicle prior to complete development which ultimately leads to an increased number of larger follicles in the ovary (Franks *et al.*, 2008). Other associated reproductive alterations include increased risk of miscarriage and hyperandrogenism, which includes hirsutism and acne (Ehrmann *et al.*, 1999). The metabolic disorders are typically insulin resistance, obesity and

impaired pancreatic  $\beta$ -cell insulin secretion that results in type II diabetes (Abbott *et al.*, 2002).

It has been suggested that there is a genetic basis of PCOS as there is a high prevalence of family members afflicted with symptoms that are characteristic to PCOS (Sam *et al.*, 2008). Familial aggregation of PCOS has been evidenced by sisters of women with PCOS. Approximately 40% of these sisters also have hyperandrogenaemia and metabolic disorders (Legro *et al.*, 1998). The male phenotype of PCOS has been evaluated and Legro (2000) suggested that premature balding and insulin resistance were characteristics. Recabarren *et al.*, (2008) performed a longitudinal study and observed that sons born to women with PCOS exhibited a greater body weight and insulin resistance in adulthood, compared to sons born to women without PCOS.

Intrauterine growth retardation (IUGR) in humans has been linked to the development of hyperandrogenism (Ibanez *et al.*, 2002) and PCOS (Cresswell *et al.*, 1997). Sir-Petermann *et al.*, (2002) showed that pregnant women with PCOS had an increased level of testosterone, compared to normal, pregnant women. Prenatal androgen excess exposure has been recognised as a factor in the re-programming of organ systems in PCOS animal models (Abbott *et al.*, 1997; Hogg *et al.*, 2011; Connelly *et al.*, 2013; Rae *et al.*, 2013). There are already established PCOS sheep models known (Hogg *et al.*, 2011; Connelly *et al.*, 2013; Rae *et al.*, 2013), which have ultimately lead to a firmer understanding of fetal programming in the gonads, pancreas, liver and adrenal glands. Two models have been designed – one whereby the pregnant ewe is injected by testosterone propionate (TP, an androgen) which would allow the placental aromatisation of androgen to estrogen to be passed onto the fetus. The second is a unique fetal programming model by which the fetus is directly injected, thus bypassing any placental aromatisation and any maternal effects of androgens (Hogg *et al.*, 2011; Connelly *et al.*, 2013; Rae *et al.*, 2013).

### **1.7.2 Maternal stress**

In addition to nutritional stress, maternal emotional stress, anxiety or depression can have adverse effects on fetal neurodevelopment (Nicoletto and Rinaldi, 2011). Maternal stress is a risk factor for ADHD (Talge *et al.*, 2007) and depression (Markham and Koenig, 2011). A consequence of these types of

environmental stress is fetal overexposure to glucocorticoids (Pryce *et al.*, 2011). Fetal over-exposure to glucocorticoids may also occur as a result of treatment for gestational disease of the mother or fetus and is a routine treatment for those at risk of preterm delivery (Pryce *et al.*, 2011). Dexamethasone (DEX) is a synthetic glucocorticoid, and the behavioural consequences of prenatal dexamethasone administration are similar the effects of prenatal stress, causing a reduction in social play and skilled motor behaviour in marmosets (Pryce *et al.*, 2011). Prenatal exposure to DEX also delays neuronal maturation and leads to degeneration of hippocampal volume in adolescent rats, this correlates with hippocampal changes seen in adult primates subjected to prenatal stress and with changes seen in psychiatric patients (Pryce *et al.*, 2011).

The literature in regards to stress and cortical development is vast, however there is little evidence for androgens affecting cortical development. Chronic stress is associated with increased risk of illness, the development of a variety of psychological disorders, and changes in cognition. Stress has also been hypothesized to play a causal role in several psychological disorders. For instance, stressful life events appear to increase the probability of a psychotic episode in schizophrenics (Ventura *et al.*, 1989). Animal studies have also demonstrated detrimental effects of stress on cognitive function such as stress-induced deficits in a variety of cognitive tasks, including shuttle escape (Seligman and Maier, 1967), water maze (Altenor and DeYoe, 1977), food-motivated operant conditioning (Rosellini *et al.*, 1984), and radial maze tasks (Luine and Rodriguez, 1994).

The cortex is a target for glucocorticoids, which are involved in stress response - DEX binds to receptors in the cortex. In addition, DEX binding in the cortex is altered by both corticosterone treatment and adrenalectomy, which indicates the presence of endogenously regulated corticosterone receptors (Meaney and Aitken, 1985). Furthermore, the cortex is also involved in many of the tasks that are influenced by chronic elevations of circulating glucocorticoids. Lesions of the cortex in rats impair spontaneous alternation, radial maze performance, and passive avoidance (Dias *et al.*, 1996). Therefore, potential alterations in the cortex may mediate some corticosterone-induced behavioural changes. Wellman (2001) demonstrated that chronic corticosterone administration

dramatically reorganised apical arbors of layer II-III pyramidal cells of the cortex, with an increase of 21% in dendritic size proximal to the soma, along with a decrease of up to 58% in dendritic size distal to the soma.

### **1.7.3 Alterations in the *in utero* endocrine environment**

Exposure to an atypical endocrine environment during fetal development leads to an alteration in behaviours such as empathy and aggression presumably because of the effect on brain organisation and development (Hines, 2008; Meyer-Bahlburg, 2011). Indeed, there is a correlation between the levels of testosterone in maternal blood or amniotic fluid samples and the masculinity of behaviour of young girls and boys (Chapman *et al.*, 2006; Hines *et al.*, 2002).

Another important source of information concerning the effects of abnormal hormone exposure on human physiology are studies on a group of people born between 1948 and 1971 whose mothers had been administered diethylstilbestrol (DES) during pregnancy to prevent abortion and treat morning sickness. In addition to reproductive disorders, female offspring also had disorders of the immune system and depression (Colborn *et al.*, 1993). Similar studies into people exposed to endocrine disruptors during development indicate that exposure results in delayed psychomotor development, poor visual recognition, problems with short-term memory and growth retardation (Colborn *et al.*, 1993). Interestingly, it was estimated in the 1990s that at least 5% of the infants born in the United States were exposed to sufficient quantities of endocrine disruptors to cause neurological effects (Tilson, 1990). In rat models, it has been shown that a single exposure to an endocrine disruptor at a critical developmental time can affect the offspring (Colborn *et al.*, 1993).

### **1.8 Why sheep are a good model for human pregnancy**

Most neurological studies have been performed on rodents, but this has its limitations as they are short lived animals. The life expectancy of rodents is between 2 to 3.5 years (Pass and Freeth, 1993). Rodents also lack a developed forebrain which is a major anatomical characteristic of the human brain (Morton and Avanzo, 2011). The cognitive function of sheep is poorly characterised yet sheep are a model for research into brain function and behaviour. Morton and Avanzo (2011) demonstrated that sheep could perform well in sensitive tests of cognitive dysfunction in humans which implies that sheep do have a capacity for executing cognitive tasks (e.g. discrimination learning, reversal learning, attentional set-shifting, intradimensional and extradimensional set-shifting tasks). Sheep have large brains with human-like basal ganglia, and well developed convoluted cerebral cortices (Jacobsen *et al.*, 2010). These animals are longer living (than rodents) and so they have a more human-like biological timeframe. Like humans, sheep demonstrate a good capacity for learning and memory with the ability to remember faces of other sheep (Kendrick *et al.*, 2001). Therefore to some degree, sheep have similar cognitive abilities to humans.

The gestation period for sheep approximately 145 days and for humans, is around 270 days (nine months), whereas for rodents, the gestational period is between 20 and 22 days (Barry and Anthony, 2008). Sheep make a better pregnancy model for humans for a variety of reasons. Rodents do not have a breeding season and females of breeding age come into their estrus cycle all year-round, approximately every four to five days. On the other hand, sheep do have a breeding season, typically in autumn and ewes enter their estrus cycle every seventeen days (Thompson and Meyer, 1994). The typical litter for a ewe is one or two lambs, which is a stark contrast to the ten to twelve pups in a rodent litter. In sheep, the placenta is attached to the wall of the ewe's uterus by about 80 small buttons, the cotyledons. It is through these and the placenta that the developing lamb has received nutrients from the ewe's blood supply. The placenta with the cotyledons will be expelled as the afterbirth. Each lamb has a separate placenta, which is the same as rodent pups (Gimenez and Rodning, 2007). One limitation of using sheep as model is that the whole ovine genome was not yet fully sequenced and annotated until after the experiments in this project were performed (Jiang *et al.*, 2014). Figure 1.10 shows a comparison of

the sheep, rat and human cerebellar development timeline. No animal model truly recapitulates human pregnancy; however the pregnant sheep are an established model to investigate maternal-fetal interactions (Hogg *et al.*, 2012; Hogg *et al.*, 2011) and fetal programming (Rae *et al.*, 2013).

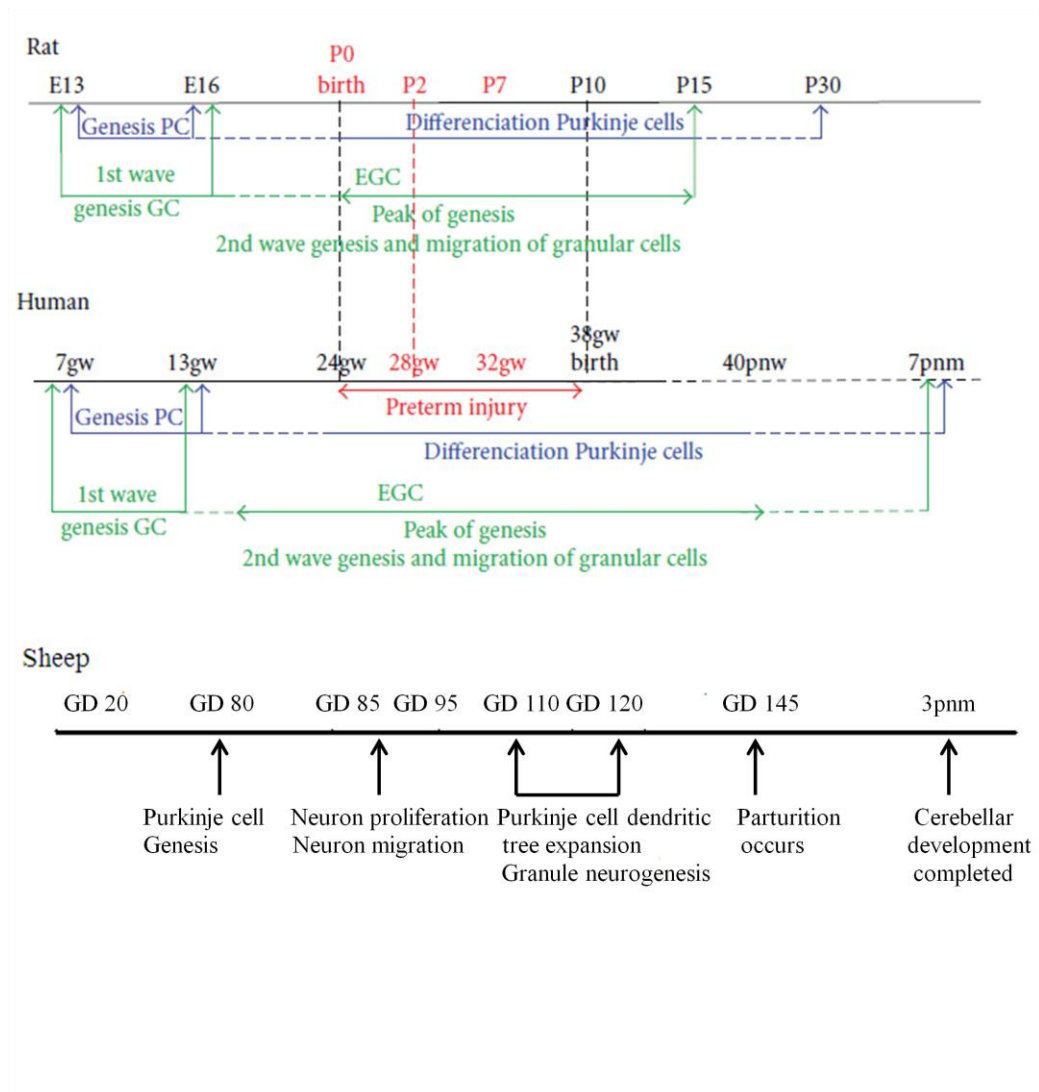


Figure 1.10: Comparison of timing of cerebellar development for rat, human and sheep. Key - gestational day (GD), embryonic day (E), postnatal day 0 (P0), gestational weeks (gw), postnatal weeks (pnw), Purkinje cells (PC), granule cells (gc), external granule cells (egc). . Taken and adapted from (Biran, 2011). The sheep cerebellar development timeline is incomplete due to lack of literature. Sheep timeline composed from findings from: (Potter *et al.*, 1982), Rees and Harding (1988) and Roselli *et al.* (2006).

The fetal sheep brain has the capacity to aromatize androgens to estrogens on GD 64 (Roselli *et al.*, 2003). This is the time point that falls in the middle of the critical period of sexual differentiation of the brain and gonads (Roselli *et al.*, 2003). The samples used in the present study were injected with 20mg TP at

GD 62 and 82, which is well within the GD 50-80 critical window for the effects of androgen on sheep behaviour, yet after the critical window for gonadal differentiation (GD 30-35; Roselli *et al.*, 2006), before being sacrificed at GD 90.



## **1.9 Focus of study**

Previously literature has shown that fetuses are exposed to elevated levels of androgens *in utero* conditions such as polycystic ovary syndrome and congenital hyperplasia (Abbott, *et al.*, 1997; Hogg, *et al.*, 2011; Connelly, *et al.*, 2013; Rae, *et al.*, 2013; Recabarren, *et al.*, 2008; Ibanez, *et al.*, 2002; Meyer-Bahlburg, *et al.*, 2004; Hines and Kaufman, 1994) and that the effects of this can be detrimental in later life. Elevated levels of androgens have also been implicated in neurodevelopmental disorders such as autism and schizophrenia (Baron-Cohen 2002; Lewis and Levitt, 2002).

Steroids exert effects on the nervous system and play a major role in the development of the CNS, *e.g.* in the hypothalamus and pituitary gland (Feist and Schreck, 1996; Mong *et al.*, 1999; Toran-Allerand *et al.*, 1980). These brain areas are involved in sexual behaviour and have been the focus of most neuroendocrine studies to date. However, it is known that other areas of the brain, *e.g.* the cerebellum and cortex, the brain regions involved in cognitive function, express those proteins necessary for local steroidogenesis and steroid signalling. A steroidal fetal programming model was utilised in order to investigate how gene expression in these two brain regions is affected by steroid exposure during development, and how this may lead to a change in brain architecture and function.

### **1.9.1 The cerebellum**

The cerebellum expresses proteins for local steroidogenesis and steroid signalling (Baulieu, 1998), however the impact of steroids on cerebellar development has yet to be investigated. Cerebellar Purkinje cells are a major site of neurosteroid formation (Tsutsui *et al.*, 2003), implying that these cells may be affected by developmental androgen exposure. How cerebellar gene expression is affected by androgen exposure during development - and how this subsequently changes the brain's cytoarchitecture and function are discussed in Chapters 3, 4 and 5.

- Aim 1 - identify changes in the expression of genetic markers of steroid metabolism and signalling, neuronal activity and cell markers in the cerebellum of male sheep that results from exposure to testosterone propionate (TP) during development, using quantitative real-time

polymerase chain reaction (qRT-PCR).

Many enzymes involved in steroidogenesis have been described in the cerebellum but the impact of steroids on its development has yet to be identified.

- Aim 2 - detect any alterations in the cerebellar cytoarchitecture, in response to androgen over-exposure.

The cerebellum has a clear developmental pathway and an established cytoarchitecture that will enable us to more easily identify effects of TP treatment on the development of the brain. To verify the mRNA expression data at the protein level, histological and immunohistochemical techniques were performed in order to determine whether there are any structural effects of TP treatment of the cerebellum, such as differences in cell numbers expressing a particular protein which may result from the gene expression changes outlined. Western blotting was attempted to verify and quantify protein expression alteration as a result of TP administration.

- Aim 3 - ascertain if there is a difference in EGL:IGL ratio between the control and TP-treated groups using Nissl staining (Chapter 4).

The cell density thickness of the EGL and IGL layers will be calculated (using Image J software) for the control and TP-treated samples and then the ratio of EGL:IGL was investigated to confirm if TP administration has an effect on granule cell migration in the cerebellum. Also in, Chapter 4, further examination of structural differences in Purkinje cell morphology was performed using immunohistochemistry. The Purkinje soma diameter, number, and Purkinje cell layer width were measured and compared between the control and TP-treated groups, so that any dysregulation in Purkinje cell development and migration due to prenatal androgen over-exposure, could be established.

Due to the vast amount of literature that suggests androgen levels can influence the likelihood that a fetus will have a neurodevelopmental disorder, several neurodevelopmental candidate genes for autism and schizophrenia were examined by means of qRT-PCR (Chapter 5).

- Aim 4 - determine whether these candidate genes were altered due to the *in utero* androgen over-exposure in this unique fetal programming

model.

If any significant changes were observed, this would explain the role of testosterone in brain development and may ultimately give insight into a variety of neurodevelopmental disorders.

### **1.9.2 The cortex**

Similar to the cerebellum, the cortex expresses proteins involved in steroidogenesis.

- Aim 5 - identify changes in the expression of 15 000 genes in the cortex of male sheep that results from exposure to testosterone propionate (TP), diethylstilbestrol (DES) and dexamethasone (DEX) during development, using microarray analysis.

Chapter 6 focuses on the results between the comparison of the control and TP-treated groups. Microarray data was generated in order to detect gene expression alterations in the cortex, between the control and TP-treated groups. This data allowed for, with the aid of bioinformatics, the determination of which genes have been affected as a result of *in utero* TP administration.

- Aim 6 – Verify the microarray data using qRT-PCR.

Histology was performed to determine whether these changes can be validated at the mRNA and protein level.

We would like to establish if there is a connection between the changes observed in the cerebellum and the prefrontal cortex.

## **CHAPTER 2**

### **Methodology**

## **2.1 Methodology**

This chapter details the general materials and methods that were employed in this thesis. The animal husbandry, the administration of injections was performed by collaborators from the University of Edinburgh whereas as tissue collection was performed by Dr. Claire Garden or Miss Lisa-Marie Wilson. Any work that was carried out in conjunction with others is duly acknowledged in the chapters in which the specific work was presented.

### **2.1.1 Animal husbandry**

A flock of Scottish Greyface ewes were purchased for these studies and housed and cared for at the Marshall Building, Roslin, Midlothian under the regulations authorised by UK Home Office Project Licence Number 60/3744. Ethical approval was reviewed by the University of Edinburgh Animal Research Ethics Committee. The current study specifically focuses on the fetal injection cohort GD 90, outlined below and in Connolly *et al.*, (2013). The current study was part of a much larger study and therefore various cohorts were utilised, with pregnant ewes randomly assigned to control and treatment groups.

#### **2.1.1.1 Mating and pregnant ewe husbandry**

Ewes with good body conditioning scores of 2-3 (Thompson and Meyer, 1994 ) were mated with Texel rams under natural seasonal breeding conditions from November to January. Prior to mating, estrous cycles were aligned in two separate groups that were staggered over two weeks to achieve pregnancy at the same time for the majority of animals within each group. Chronogest CR sponges impregnated with 20mg flugstone acetate (Intervet U.K. Ltd., Buckinghamshire, U.K.) were inserted into the vagina for 10-12 days. At sponge withdrawal animals were given 0.5ml prostaglandin estrumate (intramuscular; Schering Plough Animal Health, Welwyn Garden City, U.K.) to terminate the luteal phase. Ewes not carrying a reference paint marker on their hind to indicate ram-ewe contact, were considered not mated, and were returned for mating a second time. Pregnancy was confirmed by ultrasound scan and ewes placed into treatment groups according to the number of fetuses (singleton, twin or triplet pregnancies). Pregnant ewes were housed in groups in spacious enclosures and fed hay *ad libitum*, supplemented with Excel Ewe Nuts (0.5-1.0 kg daily; Carrs Billington, Lancashire, U.K.) and Crystalyx Extra High Energy Lick (Caltech Solway Mills, Cumbria, U.K.). To avoid dominant females from

over-feeding at the expense of smaller ewes, these animals were housed in separate pens. All ewes were boosted against Clostridial diseases and Pasteurellosis 4-6 weeks before lambing by treatment with Heptavac P Plus (Intervet U.K. Ltd.).

### 2.1.1.2 Treatment regimes

Treatment regime began at GD 62 of an average 147 day gestation and repeated at GD 82. TP (AMS Biotechnology (Europe) Ltd., Abingdon, U.K.) was dissolved in vegetable oil (Sainsbury's SO organic range) and 5% ethanol to a concentration of 100mg/ml and stored in an incubator at 37°C, to prevent crystal formation, until ready to use. DES (Sigma Aldrich, U.K.) was dissolved in vegetable oil and 5% ethanol to a concentration of 250µg/ml and vehicle controls (C) consisted of vegetable oil and 5% ethanol and was stored in the same way. The final injection dose for TP and DES was 20mg and 50µg, respectively (Connolly *et al.*, 2013). Fetal injections were carried out following anaesthesia of the mother. Ewes were sedated using 10mg Xylazine (intramuscular; Rompun; Bayer Plc Animal Health Division, Berkshire, U.K.), left to settle for 10 min, then given 2.0mg/kg ketamine (intravenous; Ketaset; Fort Dodge Animal Health, Southampton, U.K.). Ewes were additionally administered a post-operative dose of antibiotics (1ml/25kg; intramuscular; Streptacare; Animalcare Ltd., York, U.K.). Under surgically sterile conditions, injections were performed with the guidance of ultrasound using a vaginal probe fitted with a needle guide at GD 62 and then again at GD 82. This is within the GD 50-80 critical window for the effects of androgen on sheep behaviour, yet after the critical window for gonadal differentiation (GD 30-35; Roselli *et al.*, 2006). A 20G Quincke spinal needle (BD Biosciences, Oxford, U.K.) was inserted through the uterine wall and into the flank of the fetus. Delivery of fluid into the peritoneal cavity (200µl) was monitored by ultrasound and any anomaly such as leakage into amniotic fluid or uptake into the fetal circulation was recorded. Ultrasound was performed by Dr Colin Duncan and fetal injection carried out by Dr. Mick Rae, Dr. Kirsten Hogg or Dr. Fiona Connolly, according to the licensing conditions permitted by the UK Home Office.

Fetal sex	Treatment Groups (sacrificed at GD 90)		
	Control	TP	DES
Male	11	6	11
Female	6*	6	4*

Table 2.1: The resulting offspring from fetal injections at GD 62 and 82. This table shows a breakdown of various treatment cohorts. (\*differences in numbers of fetuses used are attributable to constraints of tissue available for each analysis).

In the current study, the ovine fetuses were subjected to two bolus injections of 20mg TP (one at GD 62 and another at GD 82) which meant that the androgen levels in the blood were significantly elevated (23093 IU), compared to the control samples (192.5 IU, Connolly *et al.*, 2013). The dose of TP (20mg) was chosen to reflect androgen levels of a polycystic ovarian syndrome (PCOS) model. The level of peripheral androgen that crosses the blood-brain barrier to the cerebellum remains unclear. It would have therefore been useful to the current study to measure testosterone levels by utilising techniques such as radioimmunoassay or luminescence immunoassay. However, this was beyond the scope of the study.



### **2.1.1.3 Animal sacrifice and collection of specimens**

#### **2.1.1.3.1 Fetal, lamb and adult animal sacrifice**

Pregnant ewes were euthanised at GD 90 of and their fetus(es) immediately collected. Euthanasia was carried out in accordance with the regulatory outline of Schedule 1: Appropriate Methods of Humane Killing of the Animals (Scientific Procedures) Act 1986. A single lethal injection of pentobarbitone sodium (150mg/kg; intravenous.; Euthetal; Merial Animal Health Ltd., Essex, U.K.) was administered to the animal.

#### **2.1.1.3.2 Tissue collection**

A range of tissues were collected from animals for ongoing studies in Dr. Colin Duncan's laboratory, as well as for the studies outlined in this thesis. Weights of postnatal cerebellar tissue were recorded immediately following dissection. The brain was bisected along the midline and one half (a hemisphere and half the cerebellum) were stored in neutral buffered formalin to prevent tissue degradation and maintain tissue integrity. The other half which included the cerebellum, hippocampus and prefrontal cortex, were snap frozen in dry ice followed by storage at -80°C, to prevent RNA degradation. Other tissues dissected included fetal ovaries, testes, adrenal glands, liver and fat, were stored in Bouins solution and also snap frozen.

## 2.1.2 RNA

### 2.1.2.1 RNA isolation

RNA from fetal ovine cerebellar tissue was isolated from frozen tissue from four male control animals and four TP-treated animals using the Trizol method (Chomczynski, 1993). RNA from fetal ovine cortex tissue was also isolated using this method but the sample numbers were different - six male control animals and six TP-treated animals. DES-treated cerebellar RNA isolation was performed by an Honour's student (n=4). The variance in sample numbers for cerebellar and cortex tissue is due to poor quality RNA in two cerebellar samples. Each tissue sample was ground into a fine powder in liquid nitrogen, then homogenised in TRIsure (100mg tissue powder per ml, Bioline, U.K.) and then transferred into 1ml eppendorf tubes. 200µl of chloroform per 100ml of tissue (Sigma Aldrich, U.K.) was then added to each tube and centrifuged at 4°C for 10 minutes at 10 000 x g. This left a clear, aqueous phase at the top which contained total RNA. The interphase contained proteins and genomic DNA. The aqueous phase was transferred into clean tubes and 500µl of cold isopropanol (Sigma Aldrich, U.K.) was added. This was centrifuged as before. The supernatant was discarded and the pellets were left to air dry, ensuring the pellets were not over-dried. The pellets were then solubilised in 50µl of RNase free water (Bioline, U.K.). 5µl of 4M sodium chloride (NaCl) and 150 µl of 100% ethanol (Sigma Aldrich, U.K.) was added to the pooled pellets and incubated at -20°C for 1 hour. Meanwhile, the tubes containing the interphase had 300µl 100% ethanol added to them and stored at -20°C for future protein studies. The total RNA was then centrifuged as previous, the supernatant discarded and the pellet left to air dry. 50µl of RNase free water, 5µl of 4M sodium chloride (NaCl) and 150 µl of 100% ethanol was added to the pooled pellets and incubated at -20°C for 1 hour. This incubation occurred in total, three times. After the final centrifugation, the pellet was washed in 300µl 75% ethanol and centrifuged at 10 000 x g for 5 minutes. This ethanol precipitation method is a widely used technique to purify or concentrate nucleic acids. This is accomplished by adding salt (in this instance, 4M NaCl) and ethanol to a solution containing RNA. In the presence of NaCl, ethanol efficiently precipitates nucleic acids, such as total RNA. The supernatant was discarded and once the pellet had dried, 50µl of RNase free water was added to solubilise the pellet and then stored at -80°C.

### **2.1.2.2 Determining RNA integrity**

Denaturing paraformaldehyde gel electrophoresis was used to monitor the integrity of the RNA isolated. A 1% paraformaldehyde gel was made using 1g of agarose (Bioline, U.K.), 72ml RNase free water, 10ml MOPS-EDTA-sodium-acetate buffer (Sigma Aldrich, U.K.), 18ml formaldehyde (Sigma Aldrich, U.K.). 5  $\mu$ l of SafeView (NBS Biologicals, U.K.) was added to the mixture just before pouring. This was used instead of ethidium bromide. 6  $\mu$ l of loading buffer (Sigma Aldrich, U.K.) was added to 3  $\mu$ l of RNA ladder (Transcript RNA Markers 0.2-10 kb, Sigma Aldrich, U.K.) and 3  $\mu$ l of total RNA (in separate tubes). All samples and markers were denatured at 65°C for 10 minutes, placed on ice and then loaded onto the gel. The gel was electrophoresed at 50 volts for 3-4 hours then visualised using the BioRad Chemidoc XRS image analyser (Bio Rad, U.K.).

### 2.1.2.3 DNase treatment and determining RNA concentration

The samples were DNase treated using the DNase I amplification grade kit (Sigma Aldrich, U.K.) to eliminate any DNA contamination in the RNA sample. In short, 1 unit/ $\mu\text{l}$  of DNase I and 1  $\mu\text{l}$  10X reaction buffer was added to 1  $\mu\text{g}/\text{ml}$  RNA and incubated at room temperature for 15 minutes. 1  $\mu\text{l}$  of stop solution (binds calcium and magnesium ions to inactivate the DNase I) was then added to the tubes and incubated at 70°C for 10 minutes. This denatured the DNase I and RNA. The samples were then placed on ice for 5 minutes. Spectrophotometer readings were used to determine the concentration of total RNA for each sample. The RNA was diluted 1:1000 in RNase free water and read at 260nm and 280nm to obtain an absorbance measurement on the BioMate 3 Thermo Spectronic spectrophotometer (U.K.). These measurements were used in the Beer-Lambert Law to give the RNA concentration;-

$$A = \epsilon CL$$

Where A= absorbance,  $\epsilon$ = molar extinction coefficient ( $(\mu\text{g}/\text{ml})^{-1} \text{cm}^{-1}$ ), C= concentration (M) and L= path length (cm). RNA has a molar extinction coefficient of  $0.025 (\mu\text{g}/\text{ml})^{-1} \text{cm}^{-1}$  and the path length for the instrument used was 1cm.

RNA was stored in 4  $\mu\text{g}$  aliquots at -80°C for cDNA synthesis. 2  $\mu\text{g}$  of RNA was used to synthesise cDNA using a cDNA Synthesis reverse transcriptase kit according to the manufacturer's instructions (Bioline, U.K.). Each sample also had a negative control (water substituted for reverse transcriptase enzyme – thereafter referred to as 'no RT'), where no reverse transcriptase was added. This controls for any residual genomic DNA contamination in a given sample.

### **2.1.3 qRT-PCR optimisation**

#### **2.1.3.1 Genes of Interest identification**

The genes of interest (GOI) that were scrutinised in GD 90 male ovine cerebellar tissue were *AR*, *CYP19*, *ER $\alpha$*  and *ER $\beta$* , *Cyp11A1*, *Cyp17*, *HSD3B1*, *STAR*, *BDNF*, *DISC1*, *GABRA6*, *GAD1*, *FOS*, *NPAS3*, *RELN*, *RORA*, *GFAP*, *PR*, *SRD5a1* and *SRD5a2* (see Table 2.2). This panel of genes were chosen for examination as they are involved in steroid signalling and metabolism, candidate genes for neurodevelopmental disorders or are markers for specific cell types or neuronal activity.

Gene Name	Classical Name	Reason for being GOI	Reference
<i>AR</i>	Androgen receptor	To investigate T signalling	Chang <i>et al.</i> , (1995)
<i>CYP19</i>	Aromatase	Enzyme that converts T into E	Naftolin (1994)
<i>BDNF</i>	Brain-derived neurotrophic factor	Modulated by steroids, associated with disease	Numakawa (2010)
<i>DISC1</i>	Disrupted in schizophrenia 1	Altered in schizophrenia	Millar <i>et al.</i> , (2000)
<i>ER<math>\alpha</math></i>	Estrogen receptor $\alpha$	To investigate E signalling	Belcher (1999)
<i>ER<math>\beta</math></i>	Estrogen receptor $\beta$	To investigate E signalling	Belcher (1999)
<i>GABRA6</i>	Gamma-aminobutyric acid A receptor, alpha 6	Altered in autism, target for schizophrenia treatment.	Dean <i>et al.</i> , (2005); Fatemi <i>et al.</i> , (2010)
<i>GAD1</i>	Glutamate decarboxylase 1	Purkinje cell marker.	Westmoreland <i>et al.</i> , (2001)
<i>FOS</i>	Immediate early gene	Marker for neuronal activity in neuroendocrine systems	Hoffman <i>et al.</i> , (1993)
<i>NPAS3</i>	Neuronal PAS domain protein 3	Altered in schizophrenia and regulates glucose metabolism	Pickard <i>et al.</i> , (2005)
<i>CYP11A1</i>	P450scc	Converts cholesterol to pregnenolone.	Baulieu (1998)
<i>PR</i>	Progesterone receptor	To investigate progesterone (P) signalling	Sakamoto <i>et al.</i> , (2003)
<i>RELN</i>	Reelin	Autism marker	Serajee <i>et al.</i> , (2006)
<i>RORA</i>	Retinoid-related orphan receptor alpha	Autism marker modulated by steroids	Sarachana <i>et al.</i> , (2011)
<i>SRD5a1</i>	5 $\alpha$ - reductase 1	Converts T and P into DHT and DHP	Mensah-Nyagan <i>et al.</i> , (1999)
<i>SRD5a2</i>	5 $\alpha$ - reductase 2	Converts T and P into DHT and DHP	Mensah-Nyagan <i>et al.</i> , (1999)
<i>STAR</i>	Steroidogenic acute regulatory protein	Rate-limiting step in steroidogenesis	Stocco and Clark (1996)
<i>GFAP</i>	Glial fibrillary acidic protein	Microglial marker	Eng <i>et al.</i> , (2000)
<i>HSD3B1</i>	3 $\beta$ hydroxysteroid dehydrogenase	Key enzyme in steroidogenesis	Payne and Hales (2004)
<i>Cyp17</i>	17 $\alpha$ hydroxylase	Key enzyme in steroidogenesis	Payne and Hales (2004)

Table 2.2: A list of all the initial genes of interest that were tested. These were selected for three suitable categories that will allow the hypotheses of this study to be tested – cell marker, steroidal genes and disorder markers. The primary focus of this study is to identify changes in the cerebellar expression of genetic markers of steroid metabolism and signalling that result from exposure to testosterone propionate (TP) during development in sheep.

### 2.1.3.2 Primer design

Initially for each GOI, the ovine DNA sequence (if available) was obtained using CLC sequence viewer software (<http://www.clcbio.com/index.php?id=28>). Because the whole ovine genome was not yet fully sequenced and annotated during this part of the project, the bovine DNA sequence was utilised where applicable. There were some ready-made primers on the market available to be tried e.g. TaqMan by Applied Biosystems (ABI, U.K.). If the ready-made primers were unsuitable, e.g. no amplification in qRT-PCR, custom-made primers were designed. This was done for AR, BDNF, DISC1, ER $\alpha$ , ER $\beta$ , GABRA5, NPAS3, PVALB and ROR $\alpha$ . If there was no ovine sequence, then the bovine, human and murine sequences were compared and primer sets were designed over an exon boundary that had the best homology using Primer Express software (ABI, U.K.). When designing custom primers, it was essential that the GC content was between 50 and 60%, amplicon length was 50 to 150 bases long and there were minimal secondary structures e.g. primer dimers and hairpin loops. The melting temperature of primers is also a crucial factor in primer design. Primers with melting temperatures in the range of 52-58°C generally produce better results than primers with lower melting temperatures. While the annealing temperature can go as high as 72°C, primers with melting temperatures above 65°C have a higher potential for secondary annealing (Bustin *et al.*, 2009). These sequences were then put through NCBI's BLAST website (U.S.A) to ensure that the primer pairs are specific to the GOI. The custom-made primers were synthesised by Eurofins MWG Operon (Germany).

### 2.1.3.3 Optimising Custom-Made Primers

According to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al.*, 2009) all custom-made primers must be optimised and validated. One way to do this was to create a standard curve using DNA copy number (Wong and Medrano, 2005). The PCR product for AR, was ligated into the vector pCR2.1 (Sigma Aldrich, U.K.) and then transformed into  $\alpha$ -select chemically competent cells (Bioline, U.K.). These cells were then plated onto a Luria broth (LB) plates with 50 $\mu$ g/ml kanamycin and incubated at 37°C overnight. Then, colony PCR was performed to see if any of these PCR products had successfully been cloned into the vector. For the successful colonies, a Miniprep of plasmid DNA (Sigma Aldrich, U.K.) was carried out, as per manufacturer's instructions, and then the plasmid DNA concentration was determined. From this, the DNA copy number was established using a calculation program (<http://endmemo.com/bio/dnacopynum.php>). In summary and a brief example, the DNA concentration (e.g. 0.04 $\mu$ g/ $\mu$ l) was calculated and converted to M (e.g. 0.000946M). These figures were entered into the above website and converted into the DNA copy number (e.g. 569, 940, 828, 402). The next step was to calculate the concentration of DNA that would hold 10 000 000 DNA copies. This was achieved as follows:-

1. 0.04 $\mu$ g/ $\mu$ l = 40ng/ $\mu$ l = 569, 940, 828, 402 DNA copies
2. 1/1000 = 40pg/ $\mu$ l
3. 1/57 = 0.7pg/ $\mu$ l = 10 000 000 DNA copies

Serial dilutions were prepared in order to obtain a standard curve that ranged from 10 million DNA copies to 100 DNA copies. qRT-PCR was then performed in triplicate. The cloned vector was sequenced (by Eurofins MWG Operon, Germany) to ensure that the appropriate PCR product was indeed cloned.



#### 2.1.3.4 Optimising Primer Concentration

With all primers used, it was imperative that the optimal concentration for each primer for qRT-PCR was established.

FORWARD PRIMER				
75nM	150nM	300nM		
			75nM	REVERSE PRIMER
			150nM	
			300nM	

Figure 2.1: Shows a primer concentration matrix.

Concentrations of the AR forward and reverse primers were set up as Figure 2.1 SYBR green qRT-PCR was performed with 25ng of cDNA (from a male control sample) and the Ct values and melt curves were compared to establish the reaction that yields the lowest Ct value and the best curve.

### 2.1.3.5 Melt curve analysis

Using SYBR green qRT-PCR assay means that melt curve analysis must be performed. The purpose of this analysis is to check that primers are giving a specific PCR product and there is no non-specific amplification e.g. primer dimers. All SYBR green assays were checked in this way and the assays that displayed a single peak were used for further qRT-PCR analysis and the assays that demonstrated multiple peaks were discarded and their use was discontinued and the primers were redesigned. Figure 2.2 A is a typical image for primer sets that had an optimal melt curve pattern whereas any melt curve patterns that resembled Figure 2.2 B were discarded.

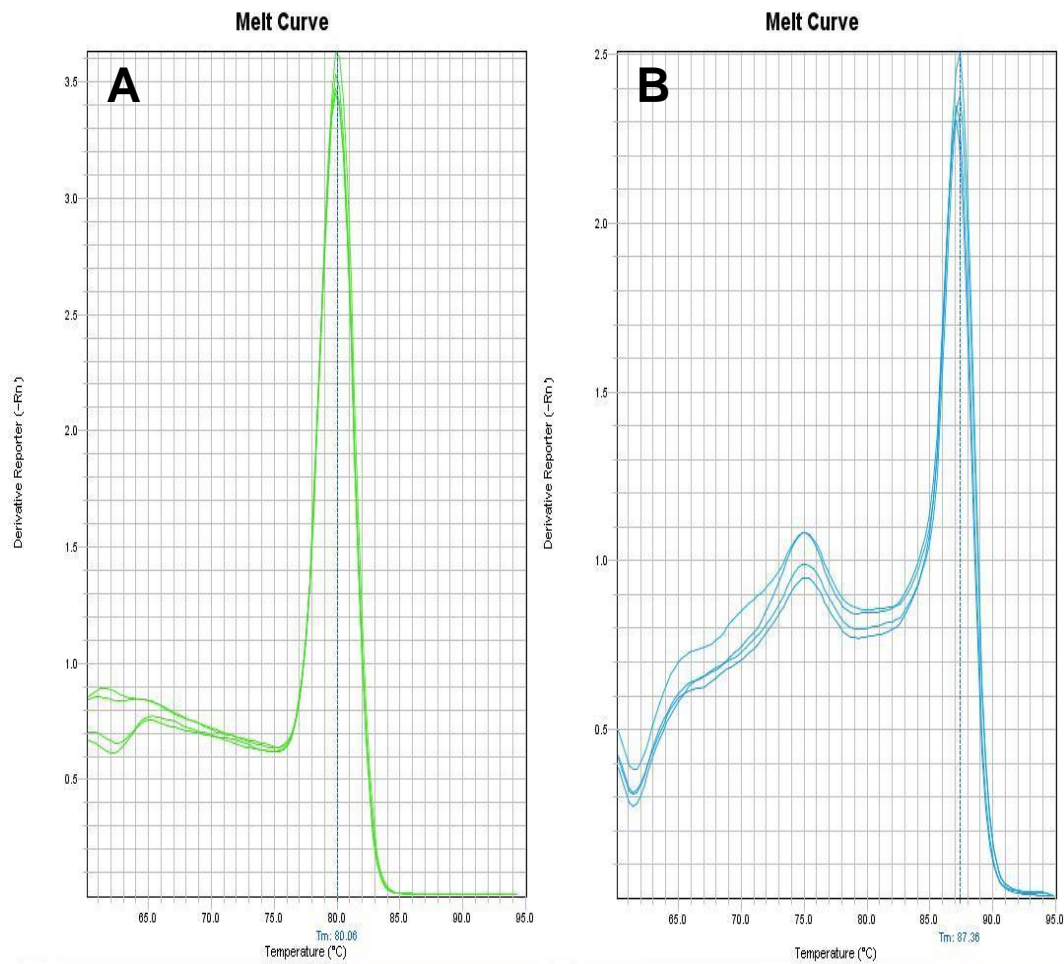


Figure 2.2: A) A melt curve with one single peak shows a good affinity between primer and cDNA. B) A melt curve with multiple peaks. This shows that secondary structures have occurred.

### **2.1.3.6 Optimising and validating custom-made primer sets**

Due to the large number of genes chosen for investigation, it was not possible to design, optimise and validate each set of primers due to time constraints. Therefore certain primers sets were synthesised from previously published and validated sequences (Hogg *et al.*, 2011). The remainder of primer sets used in the current study were designed by PrimerDesign (U.K.), who optimised each primer set.

Confidence of the specificity of a primer set can be gleaned from analysis of the melt curve that can be generated after SYBR green qRT-PCR (Tindall *et al.*, 2009). Figure 2.2 A depicts a melt curve with one large peak. This shows good design of a particular primer set as the peak represents product with no other inferences. Figure 2.2 B illustrates a primer set that has been designed with poor consideration of the forward and/or reverse primers as more than one peak in a melt curve would imply that there are secondary structures being created during qRT-PCR, as well as product. Melt curve analysis was performed on all the primer sets used in this study and confirmed product specificity, giving confidence that each primer set was amplifying the correct amplicon. The primer sets (*DISC1* and *RORA*) that were designed within our lab were discarded due to the observation of secondary structures during melt curve analysis and because the cloning and sequencing of these PCR products showed the amplicon was not specific for these genes alone.

## 2.1.4 qRT-PCR

### 2.1.4.1 Pre-validated assays for cerebellar gene expression studies

Due to time constraints, there was difficulty validating every GOI primer set. Pre-validated SYBR green primers were therefore made by PrimerDesign (Table 2.3) or published primer sequences, that have been used on sheep tissue and been successful, were used (Hogg *et al.*, 2011). Melt curve analysis was also performed on these primers in order to detect and artefacts or secondary structures. For the GOI GFAP, a Taqman probe was used (ABI, U.K.).

Gene (accession number)	Forward sequence (5' – 3')	Reverse sequence (5' – 3')
<i>BDNF</i> (XM_004016358)	CCAGCGAGGAAAACAATAAAG AC	GCGGCATCCAGGTAATTTTTG
<i>DISC1</i> (XM_004021602)	CTTGTCGGTTGCTTATCCAGAG	GCCAGAGCCATTGTATAAGTACC
<i>GABRA6</i> (XM_004009039)	CCTTGCCAAAAGTGTCTTATGC	TGTGTCTGAAGATTGGTAAAGTAG T
<i>GAD1</i> (XM_004004607)	AATGCTTGGAAGTGGCTGAATA C	CCCCTGAGGCTTTGTGGAATAT
<i>FOS</i> (NM_001166182)	ACTCAAGTCCTTACCTCCTCTG	CTCCACATGCTACTAACTACCA
<i>NPAS3</i> (XM_004018112)	ATCGTCGGCGTTTTTCGTTTA	AGAATGAGTCCAGTGAAAGAAAG A
<i>PR</i> (XM_004016530)	TCTCTGCTGACAAGTCTGAATC	ATCAATATGTAAGTTCCGAAAACC T
<i>RELN</i> (XM_004007830)	GCAGTCACGATGGAAGAGAAG	TTCAGACACTTTACATCCAACAGA
<i>RORA</i> (XM_004011005)	TCACCGAGAAGATGGAATACTA AC	GTCGCACAATGTCTGGGTATAT
<i>SRD5a1</i> (XM_004017153)	ATGTTCCCTCGTCCACTATGC	GTAGCCATTATAGGTGCAGAAGA
<i>SRD5a2</i> (XM_004006044)	GCCGTTTCCAGTTGTATTCCT	AGCAGGGTATTCAGCACAGTA

Table 2.3: Custom-made primer sequences designed by PrimerDesign (U.K.)

<b>Gene (accession number)</b>	<b>Forward sequence (5' – 3')</b>	<b>Reverse sequence (5' – 3')</b>
<i>AR</i> (XM_001253942)	GCCCATCTTTCTGAATGTCC	CAAACACCATAAGCCCCATC
<i>CYP19</i> (NM_001123000)	AATCCAGCACTCTGGAAAGC	ACGTCCACATAGCCCAAGTC
<i>ESR1</i> (NM_001001443)	GAATCTGCCAAGGAGACTCG	CCTGACAGCTCTTCCTCCTG
<i>ESR2</i> (NM_001009737)	GAGGCCTCCATGATGATGTC	GGTCTGGAGCAAAGATGAGC
<i>CYP11a1</i> (NM_001093789)	CAACGTCCCTCCAGAACTGT	CAGGAGGCAGTAGAGGATGC
<i>STAR</i> (NM_001009243)	GCATCCTCAAAGACCAGGAG	CTTGACACTGGGGTTCCACT
<i>HSD3B1</i> (NM_001135932)	GGAGACATTCTGGATGAGCAG	TCTATGGTGCTGGTGTGGA
<i>CYP17</i> (NM_001009483)	AGACATATTCCTGCGCTGA	GCAGCTTTGAATCCTGCTCT

Table 2.4: Primer sequences that have been published for use in ovine tissue. All primers sets were published in Hogg *et al.*, (2011).

### 2.1.4.2 Pre-validated assays for cortex gene expression studies

Published primer sequences, that have either been used on sheep tissue, or shared close homology with the ovine sequence and been successful, were used. Table 2.5 shows the GOI, its primer sequences and the publication where it was first published.

Gene (accession number)	Forward sequence (5' – 3')	Reverse sequence (5' – 3')	Publicatio n
<i>ROBO1</i> (NM_133631)	TTGAATTCAGGAGCAACTCC	ATTAGCTGCCCTCACAAGGC	Hogg <i>et al.</i> , (2011)
<i>ROBO2</i> (NM_0011289 29)	CTGAGAATCGGGTTGGAAA A	AGGTTCTGGCTGCCTTCTTT	Hogg <i>et al.</i> , (2011)
<i>ROBO4</i> (NM_019055)	TGTGAGGCCAGCAACCGGC T	GTGGGCTCTGGGTGGCCCCA	Hogg <i>et al.</i> , (2011)
<i>SLIT2</i> (NM_004787)	TGGAGGTGTCCTCTGTGAT G	TTATCCTTTCCCCTCGACAA	Hogg <i>et al.</i> , (20110)
ERBA BETA 1 (NM_0011903 91)	GAAGCTCGTGGGAATGTCT	GCCTTTGCACTTCTTCTCCT	Johnsen <i>et al.</i> , (2013)
COL4A2 (XM_0040123 56)	CTCATCCGCACGCACATC	TCACAGGTTCTTCATGCACAC C	Hecht <i>et al.</i> , (2006)
POMC (NM_0010092 66)	CCGGCAACGGCGATGAG	GGAAATGCCCATGACGTAC T	Adapted from Ali <i>et al.</i> , (2005)
APP (XM_0040028 06)	CACCACGGAGTCTGTGGAA G	AGGCGTCTCAAGATACTTGT	Adapted from Burton <i>et al.</i> , (2002)
CXCR4 (NM_0012771 68)	CCGTAGAAAAGGGAAGTGA ACATC	CAGGAAAAAGAAAATGTTCCA CTGG	Adapted from Mansouri- Attia <i>et al.</i> , (2009)

Table 2.5: Primer sequences that have been published for the gene of interests examined for microarray validation.

### 2.1.4.3 qRT-PCR Assay set up

SYBR green Quantitative PCR (qRT-PCR) was performed on the cDNA samples from the male control group and the TP-treated male group. One Taqman qRT-PCR primer and probe set was used for one gene of interest (GFAP). The cDNA concentration that was used for all qRT-PCR experiments after optimisation was 12.5ng per reaction.

Gene expression of each GOI was evaluated using qRT-PCR analysis. There were eight samples in total for the cerebellar tissue - four in the control group and four in the TP treated group whereas for the cortex samples, there were six in each group (twelve in total). Each sample was analysed in quadruplicate and had two negative controls – a no template (water only) and a no reverse transcriptase (no RT). When a master mix was prepared, it was prepared for fifty reactions to take into account potential pipette accuracy deviations and ensure sufficient mix for the analysis. PrecisionBlue Precision MasterMix with ROX with SYBRgreen (Primer Design, U.K.) was used in all SYBR green assays whereas TaqMan Universal PCR Master Mix (without UNG, Life Technologies, U.K.) was used for the Taqman assay. SYBR green assays made by PrimerDesign (U.K.) and the one Taqman assay were performed in the following way:-

<b>Mastermix</b>	<b>For 1 reaction (µl)</b>
Primer set	1
Mastermix	10
Water	4
<b>TOTAL VOLUME</b>	<b>15</b>

Table 2.6: This table shows the volumes needed for mastermixes for PrimerDesign and Taqman assays. The PrimerDesign primers had a stock concentration of 30 micromolar ( $\mu\text{M}$ ) and the Taqman primer and probe set had a stock concentration of 25  $\mu\text{M}$ . Therefore the final concentrations for each primer by PrimerDesign was 300nm and by Taqman, 250nm.

For the above assays (Table 2.6), the forward and reverse primers were combined prior to arrival (Table 2.3).

In the case of assays where primer sets were separate forward and reverse (Tables 2.4 and 2.5), Table 2.7 details the reaction mix constituents:

<b>Mastermix</b>	<b>For 1 reaction (μl)</b>
Forward primer	1
Reverse primer	1
SYBR green Mastermix	10
Water	3
<b>TOTAL VOLUME</b>	<b>15</b>

Table 2.7: This table shows the volumes needed for the mastermixes for the published assays. Each primer had a stock concentration of 25μm and a final concentration of 250nm.

Once the mastermix was prepared, 15μl was added to the correct number of wells of a 96-well plate. Then, 5μl of water, no RT or 2.5ng/μl cDNA was added into the appropriate wells. The StepOnePlus Real-Time PCR system (ABI, U.K.) was used as follows; the holding stage (95.0°C for ten minutes) and the cycling stage (95.0°C for fifteen seconds then 60.0°C for one minute) for forty cycles. With the SYBR green assays, melt curve analysis was also performed at the end of the cycling profile by heating to 60.0°C for one minute, then gradually increasing the temperature in 0.3°C increments until 95.0°C. At each temperature increment, a measurement was taken permitting a curve to be formed. C<sub>T</sub> values were obtained in order to calculate the fold difference for each sample. Threshold setting was automatically generated by the machine software, but checked visually in each case. The fold difference was calculated by the 2-ΔΔC<sub>T</sub> method.



#### **2.1.4.4 Reference gene selection**

The expression levels of a panel of 12 candidate reference genes from Primer Design were determined using geNorm analysis (Vandesompele, 2002) in order to select the most appropriate (stable over all treatments) reference genes. This is a software program that establishes which genes are the most stable to use as a reference gene(s) in the sample tissue (Bustin *et al.*, 2009). For cerebellar gene expression studies, beta-2 microglobulin (*B2M*) and ribosomal protein L19 gene (*RPL19*) were selected as the reference genes as they were the most stable in the male control and TP treated groups. For cortex gene expression studies, malate dehydrogenase 1 (*MDH1*) and ribosomal protein S2 gene (*RPS2*) have been selected as the reference genes. Reference gene selection was performed using SYBR green qRT-PCR as Table 2.6.

#### **2.1.4.5 Melt curve analysis**

With the SYBR green assays, melt curve analysis was also performed. This allowed the determination of whether there were secondary structures *i.e.* primer dimers. A good melt curve should have one single peak (Figure 2.2 A) whereas a melt curve with multiple peaks indicates secondary structures were formed (Figure 2.2 B).

#### **2.1.4.6 Calculating $\Delta\Delta C_T$**

Each assay was done in quadruplicate for each sample and the three closest  $C_T$  values were used for the  $2^{-\Delta\Delta C_T}$  method (Table 2.8). Reference genes are needed in qRT-PCR as 'no change' comparators for the purposes of normalisation. The geometric reference mean was calculated using reference genes  $C_T$  values (Vandesompele *et al.*, 2002) This was achieved by taking a mean of three  $C_T$  values for each of the two reference genes and then multiplying the two means together. Then the square root of that was calculated to derive the geometric reference mean. This was done for each sample.  $\Delta C_T$  was calculated by subtracting the mean  $C_T$  value for each particular GOI away from the geometric reference mean value. Then,  $\Delta\Delta C_T$  could be calculated by subtracting the  $\Delta C_T$  value from one of the samples *i.e.* the reference sample. For this study, sample number 204/1 was chosen as the reference sample. This was because it was the first control sample when placed in numerical order. This sample was kept constant in all the calculations and was chosen because it was the first control sample in ascending numerical order (sample 204/1). The

fold difference was then calculated by  $2^{\Delta\Delta CT^{*(-1)}}$ . Fold changes that were less than two were considered to not illustrate any change in gene expression and not reported.

**A)**

	<b>Sample 204/3</b>
Mean B2M $C_T$	26.6633
Mean RPL19 $C_T$	20.904
Multiply means (26.6633 X 20.904)	557.36962
Square root of 557.36962 ( <b>Geo ref mean</b> )	23.608676

**B)**

<b>Sample</b>	<b>Treatment</b>	<b><math>C_T</math></b>	<b><math>C_T</math> Mean</b>	<b><math>C_T</math> S.D.</b>	<b>Geo. Ref. <math>C_T</math></b>	<b><math>\Delta C_T</math></b>	<b><math>\Delta\Delta C_T</math></b>	<b>Fold Difference</b>
204/3	Control	20.635	20.657	0.0197	23.609	-2.952	-0.152	1.111
204/3		20.673						
204/3		20.663						

Table 2.8: Worked example A) Geometric reference mean calculation B) The fold difference calculation for one sample in a StAR assay. Sample – Sample number of the sheep. Treatment – Whether the sample received control or TP vehicle.  $C_T$  – Threshold cycle, determined by qRT-PCR.  $C_T$  mean – The mean of the three  $C_T$  values per sample.  $C_T$  S.D. – Standard deviation of the three  $C_T$  values. Geo. Ref.  $C_T$  – Geometric reference mean  $C_T$  value.  $\Delta C_T$  – Normalisation of  $C_T$  obtained by subtracting the Geo. Ref.  $C_T$ : ( $\Delta C_T = C_T - \text{Geo. Ref. } C_T$ ).  $\Delta\Delta C_T$  – Identifies relative quantification between sample and a replicate sample ( $\Delta\Delta C_T = \Delta C_T - \Delta C_T$  sample 204/1). Fold difference – fold change between sample and replicate sample (Fold change =  $2^{\Delta\Delta CT^{*(-1)}}$ ).

#### 2.1.4.7 Statistical analysis

Each individual sample therefore generated a single data point, which was derived from a fold change over/below the reference. These data points are then the gene expression score for that particular sample. The results from the male control group and the male TP treated group were compared to determine differences in gene expression, if any, using a two tailed, unpaired t test (GraphPad Prism software, U.S.A.).

Fold change shift in TP-treated group was calculated using: - TP/Control mean fold change

Fold difference was calculated using: - TP/Control mean fold change= X then 1/X

## **2.1.5 Protein Preparation**

### **2.1.5.1 Protein Dialysis**

The protocol for optimising protein preparation from samples used to extract RNA was followed from Hummon *et al.*, (2007). Due to the limited number of samples, the protein fraction was obtained during RNA isolation. The sample was loaded onto a 2K molecular weight cut-off (MWCO) Slide-A-Lyzer dialysis cassette (Thermo Scientific, U.K.) with 1% *SIGMA FAST* protease inhibitor cocktail solution (Sigma Aldrich, U.K.) and dialysed through three changes of 0.1% sodium dodecyl sulphate (SDS, Sigma Aldrich, U.K.) in distilled water at 4°C. The supernatant was removed from the cassette and placed into a tube. The globular mass in the cassette was also placed into a tube and both were stored at -20°C. 500µl of the supernatant and the globular mass were placed into separate concentrator tubes (15ml, Thermo Scientific, U.K.) and 11.5ml 0.1% SDS was added to each tube and centrifuged at 6000 x g. The liquid that had not been drawn down into the collecting part of the tube *i.e.* the concentrated supernatant and globular mass, was used for the subsequent step.

### 2.1.5.2 Protein extraction optimisation

In order to allow further analysis to identify whether the protein required concentration and to determine the optimum buffer requirements for solubilisation, the globular mass concentrated protein fraction was added to either 1% SDS, 8M Urea and Tris or a combination of the two solvents (equal volumes) and stored at -20°C (Figure 2.3).

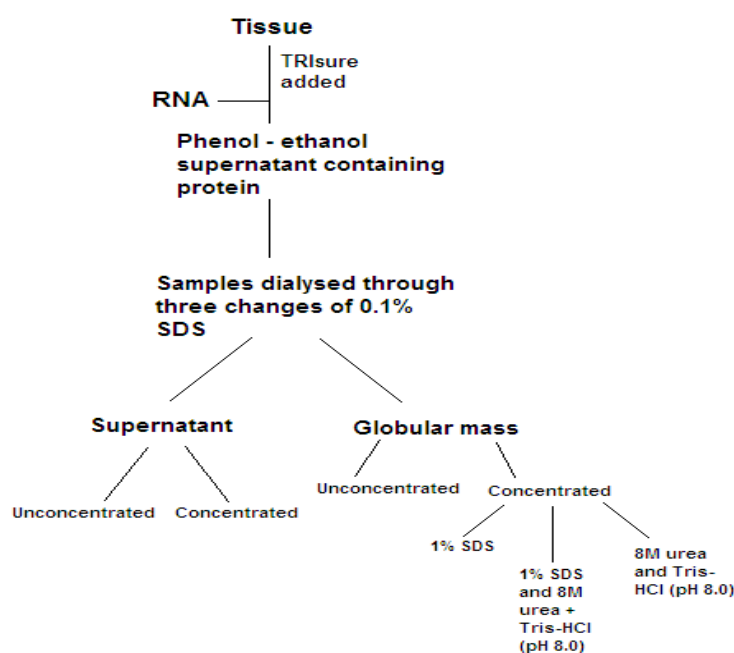


Figure 2.3: A diagram to show the process and conditions of protein preparation to find the optimal condition for protein analysis.

### 2.1.5.3 Determining Protein Concentration

The bicinchoninic acid (BCA) assay was used to determine protein concentration for all samples, using bovine serum albumin (BSA, Sigma Aldrich, U.K.) as the standard. BSA standards, made up from 10mg/ml BSA solution, were 0, 0.2, 0.4, 0.6, 0.8 and 1.0mg/ml. The working solution was made up with bicinchoninic acid and copper (II) sulphate pentahydrate 4% solution (solutions supplied together by Sigma Aldrich, U.K.). In a 96-well plate, each standard was added into the appropriate wells and the protein samples were diluted to a 1:5 dilution, using dH<sub>2</sub>O. The working solution was added to each well and incubated at 37°C for 30 minutes. The plate was then read by LT-5000 MS (Labtech, U.K.) at an absorbance of 595nm.

### **2.1.6 Western Blotting as a protein detection method**

Western blotting refers to the immunological detection of a protein, using specific antibodies. Proteins of different molecular weights (kDa) will separate accordingly via SDS-PAGE gel electrophoresis, the gel is then transferred to a more stable membrane *i.e.* nitrocellulose and then the membrane is probed for the protein of interest.

Enhanced Chemiluminescence (ECL) was used for signal detection because it is highly sensitive, so even the weakest band, if any, should be observed. A charged couple device (CCD) camera is used to capture the ECL signals on a blot and is highly dependent on timing and exposure. This method of imaging gives a broader linear dynamic range whereas using an alternative method such as X-ray film exposure is inaccurate for quantifying results, because of its lower dynamic range (Elbaggari, 2008). A broad, linear dynamic range allows comparison of weak and strong bands on the same blot. ECL exposure times can be cut drastically because CCD imaging is more sensitive than X-ray imaging (Elbaggari, 2008).

Western blotting is often used for semi-quantification of protein levels. This requires the digitisation of a probed immunoblot and subsequent analysis of the gray values of the bands in the resulting images. In principle, the quantification procedure consists of acquiring an image of the immunoblot, the bands detected are then selected and using computer software, the optical density (OD) can be determined (Gassmann, *et al.*, 2009). This would allow us to determine the amount of the protein of interest in the TP-treated group, relative to the control group but not the absolute amount of protein in a given sample.

### 2.1.6.1 SDS-PAGE electrophoresis

The absorbance read for each of the unknown protein samples was then converted into a concentration (mg/ml). From this, 50 $\mu$ g of each protein sample was used in for Western blotting analysis. 2X Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris hydrogen chloride, Sigma Aldrich, U.K.) was added to each sample, which were then boiled at 100 $^{\circ}$ C for 5 or 10 minutes (Kurien and Scofield, 2009), in order to denature the higher order structure, while retaining sulfide bridges. Denaturing the protein ensures that the negative charge of amino acids is not neutralised, enabling the protein to move in an electric field applied during electrotransfer (Mahmood and Yang, 2012). The samples were then loaded onto a SDS-PAGE gel, along with Page Ruler 10-250kDa ladder (Thermo Scientific, U.K.). Tables 2.9 and 2.10 refer to the volumes of reagents used to make one gel.

Reagent	Volume	Volume
	10%	12%
H <sub>2</sub> O	6ml	4.8ml
30% Acrylamide	3.8ml	3.9ml
1.5M Tris (pH8.8)	5ml	6ml
10% SDS	150 $\mu$ l	150 $\mu$ l
10% APS	150 $\mu$ l	150 $\mu$ l
TEMED	10 $\mu$ l	10 $\mu$ l

Table 2.9: The reagents and volumes used to make 10% and 12% resolving gels for SDS-PAGE.

Reagent	Volume
H <sub>2</sub> O	2.7ml
30% Acrylamide	500 $\mu$ l
1.5M Tris (pH8.8)	670 $\mu$ l
10% SDS	40 $\mu$ l
10% APS	40 $\mu$ l
TEMED	5 $\mu$ l

Table 2.10: The reagents and volumes used to make a 5% stacking gel for SDS-PAGE.

### **2.1.6.2 Primary antibody selection**

Primary antibodies were selected by determining which antibody was most suitable for use on ovine tissue. Some of the antibodies available on the market stated they were suitable for use on ovine tissue; others did not. Therefore we aligned the protein sequences for ovine, bovine, human and murine to determine the homology of a particular target, using CLC sequence viewer software (<http://www.clcbio.com/index.php?id=28>). The results for the sequence alignment, together with liaising with the antibody manufacturers (Abcam and Santa Cruz), allowed us to determine that all the antibodies initially chosen, were suitable for this investigation. To determine which was the most suitable dilution for each primary antibody, titration optimisation experiments were performed (Table 2.11). StAR and 5 $\alpha$ -reductase 1 antibodies were predicted to react to the bovine protein sequence, which is very similar to the ovine protein sequence. The PR and 5 $\alpha$ -reductase 2 antibodies did not specify whether they would react on ovine or bovine tissue but after contact with the suppliers, they suggested they could. Due to the limited demand and supply of antibodies reactive to ovine tissue, these were the most suitable antibodies to test. Lamb adrenal gland tissue was used as a positive tissue control in order to validate the antibodies, as it is known to express high levels of the proteins of interest (Nussey and Whitehead, 2001). IgG serum, specific to which species each antibody was raised in, was used instead of primary antibodies, as a negative control.

<b>Antibody</b>	<b>Company</b>	<b>Product code</b>	<b>Dilution(s) used</b>	<b>Secondary antibody used</b>
Actin mouse monoclonal	Abcam	ab3280	1:2500	Donkey polyclonal to mouse IgG (Abcam ab98799)
PR rabbit polyclonal	Santa Cruz	Sc-539	1:200	Donkey polyclonal to rabbit IgG (Abcam ab6802)
StAR rabbit polyclonal	Abcam	ab96637	1:500 1:1000	Donkey polyclonal to rabbit IgG (Abcam ab6802)
SRD5a1 goat polyclonal	Abcam	ab110123	1:500 1:1000	Rabbit polyclonal to goat IgG (Sigma A5420)
SRD5a2 rabbit polyclonal	Abcam	ab101896	1:500 1:750 1:1000	Donkey polyclonal to rabbit IgG (Abcam ab6802)

Table 2.11: A list of each antibody used for Western blot analysis.



### **2.1.6.3 Western Blotting**

50µg protein was loaded and resolved on a SDS-PAGE gel (see Tables 2.9 and 2.10) at 200V for 45 minutes and then transferred onto a nitrocellulose membrane (Thermo Scientific, U.K.) via two methods of transfer. For semi-wet transfer, three pieces of filter paper (the same size as the gel) were soaked in cold transfer buffer (100mM tris, 130mM glycine and 10% methanol which was added fresh each time) and placed onto the transfer apparatus, ensuring no air bubbles were formed. The nitrocellulose membrane was also soaked in transfer buffer and placed on top of the filter paper. Another three pieces of filter paper soaked in transfer buffer was added on top of the membrane, again removing any air bubbles. The Trans-Blot SD Semi-Dry Transfer Cell (Bio Rad, U.K.) was then set to 15 volts for 60 minutes. For wet transfer, the same gel sandwich was made, then fully submersed in cold transfer buffer in a transfer tank at 100 volts for 60 minutes.

To check for sufficient transfer, the protein bands were visualised with Memcode Reversible Stain kit (Thermo Scientific, U.K.) or 0.1% Ponceau S buffer (Sigma, U.K.). The membrane was then blocked in 5% non-fat milk (NFM) or 5% BSA in tris-buffered saline (TBS: 50mM Tris base, 150mM sodium chloride, pH 7.4) at 4°C for 2 hours on a rocker.

Each antibody (Table 2.11) was diluted in 5% NFM or 5% BSA in TBS buffer which was added to the blot and left at 4°C overnight on a rocker. The blot was then washed in TBS-0.1% Tween (TBS-T) four times for five minutes each. The blot was then incubated with the correct secondary antibody (see Table 3) for 1 hour at room temperature on a rocker and washed as before. Pierce Enhanced chemiluminescence (ECL, Thermo Scientific, U.K.) detection was used to visualise the bands on the BioRad Chemidoc XRS image analyser (U.K.). The substrate was applied to the blot for 5 minutes before exposure times began at 10 seconds and then every 15 seconds for 300 seconds so that images could be captured.

## 2.1.7 Histology

### 2.1.7.1 Sample Processing

Ovine cerebellar and cortex tissues from the control and TP treated groups (GD 90 fetal injection) were fixed and stored in neutral formalin buffer. Samples were processed in an automated Leica TP1050 tissue processor (Leica Microsystems, Milton Keynes, UK). In summary, the tissues were then dehydrated using standard protocols with ethanol and xylene and paraffin wax was used to stabilise the fixed tissue (Table 2.12). The tissue was then embedded in heated liquid paraffin wax and cooled into blocks for long term storage at room temperature.

Solution	Time	Temp	Photovoltaic (P/V) Temp	Drain (seconds)	Stir
Ethanol 70%	1hr 30 min	Ambient	Ambient	120	On
Ethanol 80%	1hr 30 min	Ambient	Ambient	120	On
Ethanol 90%	1hr 30 min	Ambient	Ambient	120	On
Ethanol 95%	1hr 30 min	Ambient	Ambient	120	On
Ethanol 95%	1hr 30 min	Ambient	Ambient	120	On
Absolute Ethanol	2hr	Ambient	Ambient	120	On
Absolute Ethanol	2hr	Ambient	Ambient	120	On
Xylene	1hr	Ambient	Ambient	120	On
Xylene	1hr	Ambient	Ambient	120	On
Xylene	1hr	Ambient	Ambient	120	On
Paraffin wax	1hr	60oC	P/V	120	On
Paraffin wax	1hr	60oC	P/V	120	On
Paraffin wax	1hr 30 min	60oC	P/V	120	On

Table 2.12: The protocol used for tissue processing.

The wax-embedded tissue was sectioned in 50 $\mu$ m intervals to 5 $\mu$ m thickness using a Leica RM 2135 microtome and wax ribbons were transferred to a pre-heated water bath (55°C), to float the sections to remove creases in the tissues and then mounted onto charged slides (Superfrost Plus Gold slides, Thermo Scientific, Germany). The slides were then placed in an incubator at 50°C overnight to dry. The slides were dewaxed in xylene (Sigma Aldrich, U.K.) and then gradually rehydrated in ethanol at concentrations of 100%, 95% and 70% and then in deionised water for five minutes each.

### **2.1.7.2 Haematoxylin and Eosin Staining**

The slides were immersed in haematoxylin (Harris modified, Vector Laboratories, U.K.) for four minutes with gentle agitation at room temperature and then rinsed with running tap water for one minute. Then the slides were placed in 0.1% acetic acid for one minute at room temperature and then washed in warm tap water with 1.5% lithium carbonate for 30 seconds. The sections were briefly rinsed again in tap water and then placed in 0.1% eosin (the counterstain) for five minutes at room temperature. The sections were then dehydrated in ascending graded ethanol solutions, cleared in xylene and then mounted in Pertex mountant (Vector Laboratories, U.K.). The slides were then ready for visualisation.

#### **2.1.7.2.1 Visualisation**

The slides were visualised under a light microscope (Leitz Laborlux S, Germany) using the X10 and X20 objectives. The sections were digitally captured and recorded using Spot 32 software (Diagnostic Instruments Inc., U.S.A.).

### **2.1.7.3 Nissl Staining**

The wax-embedded tissue was sectioned in 50 $\mu$ m intervals to different thicknesses ranging from 5 $\mu$ m to 40 $\mu$ m. For subsequent Nissl experiments, the cerebellar tissue was cut at a thickness of 5 $\mu$ m. 0.1% cresyl violet solution (Fisher Scientific, U.K.) was warmed to 65°C. The sections were immersed in this solution for ten minutes at room temperature, with gentle agitation. The slides were rinsed briefly in tap water and then placed in 95% ethyl alcohol for one hour at room temperature, with gentle agitation. The sections were then dehydrated in ascending graded ethanol solutions, cleared in xylene and then mounted in Pertex mountant (Vector Laboratories, U.K.). The slides were then ready for visualisation.

#### **2.1.7.3.1 Visualisation**

The slides for both brain tissues were visualised under light microscopy (Leitz Laborlux S, Germany) using the X10 and X20 objectives. The sections were digitally captured and recorded using Spot 32 software (Diagnostic Instruments Inc., U.S.A.). Using Image J software (N.I.H., U.S.A.), each captured image was examined in five random areas of each slide (four slides per sample). In each of the five random areas, five sections of the cerebellar tissue were measured so that five widths were obtained for the EGL, ML and IGL and the mean for each layer was then calculated.

#### **2.1.7.3.2 Statistical Analysis**

All statistics were performed as two-tailed unpaired t-tests (GraphPad Prism 6, GraphPad Software Ltd., U.S.A.). This allowed us to investigate if there were any alterations in granule cell migration between the control and TP-treated fetal cerebella.

## **2.1.7.4 Immunohistochemistry as a protein detection method**

### **2.1.7.4.1 Immunohistochemistry**

Immunohistochemistry is a technique that detects an antigen within a tissue section, by the use of a specific antibody (Ramos-Vara, 2005). There were many steps involved and begins with the deparaffinization and rehydration of the sections (see Rae *et al.*, 2013). Antigen retrieval was then performed to release the cross linking that the fixative (neutral formalin buffer) has caused and allowed the epitope of the antigen to be exposed. Antigen retrieval was achieved using 0.1M citrate buffer (0.1M citric acid monohydrate, pH 6). The slides, submerged in citrate buffer, were placed in a microwave for five minutes on high setting (750W) with one minutes rest, and then a further five minutes on high setting, followed by twenty minutes rest. Each slide was then blocked in 3% hydrogen peroxide (Sigma Aldrich, U.K.) solution in distilled water (dH<sub>2</sub>O) for twenty minutes at room temperature. The purpose of the hydrogen peroxide step was to quench any endogenous peroxidase activity in the tissue (Ramos-Vara, 2005). The slides were washed in distilled water followed by two washings in (Tris buffered saline) TBS for five minutes each. In a humidified chamber, the samples then underwent serum blocking using prediluted 2.5% horse serum (Vector Laboratories, U.K.) for thirty minutes at room temperature. This step is performed to reduce non-specific binding sites. Following the serum block, excess serum was blotted from each section, with care taken not to permit any drying of the tissue sections. 50µl of the appropriate primary antibody was added to each section.

Tables 2.13 and 2.14 refer to the antibodies and dilutions used in immunohistochemistry. Each primary antibody was diluted in commercially prediluted horse serum. The slides were then incubated overnight at 4°C in a humidified chamber. As with all primary antibodies, they need to react with the tissue species so in this case the antibodies used, detected antigen in or were predicted to react in ovine and/bovine tissue. Lamb adrenal gland tissue was used as a positive tissue control in order to validate the antibodies, as it is known to express high levels of the proteins of interest (Nussey and Whitehead, 2001). IgG serum, specific to which species each antibody was raised in, was used instead of primary antibodies, as a negative control.

The primary antiserum incubation was followed by two washes of TBS and 0.1% Tween (TBS-T) at five minutes each, with gentle agitation.

The sections were then incubated with biotinylated secondary antibody (1:200 dilution, prediluted multi-species secondary antibody, Vector Laboratories, U.K.) for thirty minutes at room temperature in a humidified chamber. The slides were then washed in TBS-T, twice for five minutes. Avidin-biotin-HRP (horse radish peroxidase) complex (ABC complex, Vector Laboratories) was applied to each section and incubated at room temperature for thirty minutes in a humidified chamber. The ABC complex is a complex of avidin mixed with biotin linked with appropriate label (in this case, horseradish peroxidase). The avidin and labelled biotin react together, resulting in the formation of a large complex with numerous molecules of label (Polak and Van Noorden, 2003) . The slides were washed again in TBS-T, twice for five minutes, with gentle agitation.

3,3'-diaminobenzidine (DAB) substrate solution was made up, as per manufacturer's instructions (Vector Laboratories, U.K.) and then added to the slides. DAB reacts by staining cells which are positive for the specific antigen. DAB binds to the ABC complex and oxidizes the horseradish peroxidase, thus producing a brown precipitate (as per manufacturer's instructions, Vector Laboratories). The colour development of the DAB substrate was carefully monitored by microscopy and once the colour development was sufficient, the slides were rinsed in dH<sub>2</sub>O for five minutes. The counterstain used was haematoxylin (Harris modified, Vector Laboratories, U.K.) so that cells that do not express the particular protein of interest are stained blue.

The slides were immersed in haematoxylin for 1 minute and then placed in running tap water. The sections were then dehydrated in ascending graded ethanol solutions, cleared in xylene and then mounted in Pertex mountant (Vector Laboratories, U.K.). The slides were then ready for visualisation.

#### **2.1.7.4.1.1 Visualisation for cerebellar tissue**

The slides were visualised using light microscopy (Leitz Laborlux S, Germany) using the X40 objective. The number of positive cells was counted for each slide using a grid eyepiece. The sections were digitally captured using CellSens 1.9 software (Olympus, U.S.A). Each slide was examined in five random areas using the grid eyepiece. The sections counted were also randomly allocated.

The eye grid was measured in conjunction with a stage micrometer, so that the real size of the grid can be calculated (in this study this number was 0.28mm). This number was then divided by 1mm to obtain the number, which is needed to be multiplied with 0.28mm, in order to achieve a conversion of mm into positive cells per mm<sup>2</sup>. This did not have any bearing on whether or not there were any differences in cell number due to TP-treatment, as all data was subjected to the same calculation. The mean of the five grids was calculated and then multiplied by 3.57 to convert the mean into positive cells/mm<sup>2</sup>. The value of each grid of positive cells was 0.28mm and by multiplying each grid by 3.57 converts the mean into mm<sup>2</sup>. This analysis allowed us to determine if androgen exposure had altered the number of cells expressing the antigen per unit area of cerebellum for each protein examined.



### 2.1.7.4.1.2 Primary antibody selection

The antibodies used in our study are all polyclonal antibodies. Polyclonal antibodies are a heterogeneous mix of antibodies that recognise several epitopes. This is in contrast to monoclonal antibodies, which show specificity for a single epitope and are therefore considered more specific to the target antigen than polyclonal antibodies. Therefore there will be more background staining of polyclonal antibodies, which is reflected in our findings. A way to minimise non-specific background staining, is to use highly diluted antibodies and so we have chosen these for all immunohistochemical analysis. Table 2.13 shows the antibodies and dilutions used for optimisation studies and the optimised dilution for cerebellar tissue.

Antibody	Company	Product code	Dilution titer experiments	Optimum dilution
PR rabbit polyclonal	Santa Cruz	Sc-539	1:100 1:1000	1:1000
StAR rabbit polyclonal	Abcam	ab96637	1:500 1:2000	1:500
SRD5a1 goat polyclonal	Abcam	ab110123	1:500 1:750 1:1000 1:2000	1:1000
SRD5a2 rabbit polyclonal	Abcam	ab101896	1:250 1:2000	1:2000
Calbindin	Sigma Aldrich	C9848	1:2000 1:3000	1:3000
Ki-67	Dako	M7240	1:100 1:150 1:200	1:100
Cleaved Caspase 3	Cell Signalling	9662S	1:100 1:200 1:250 1:300 1:400 1:750 1:1000	1:750

Table 2.13: A list of the antibodies used for immunohistochemistry.

### 2.1.7.4.1.3 Localisation studies

We investigated the colocalisation of AR and calbindin in the developing male cerebellum and attempted to do this in male cortex tissue (Table 2.14). We chose these proteins as we have previously shown that AR is located in the nuclei of Purkinje cells and calbindin is a known Purkinje cell marker (Dougherty *et al.*, 2013).

Protein of interest	Antibody	Company	Product code	Dilution titer experiments
AR – to investigate AR localisation	AR rabbit polyclonal	Santa Cruz	Sc-816	1:100 1:1000
T-box, brain, 1 – to investigate pyramidal cells of the prefrontal cortex	TBR-1 rabbit polyclonal	Merck Millipore	AB10554	1:50 1:100 1:400
Calbindin – to investigate Purkinje cells of the cerebellum	Calbindin mouse monoclonal	Sigma	C9848-2ml	1:500 1:750 1:1000 1:2000

Table 2.14: A list of the antibodies used in localisation studies on cerebellar and cortex tissue

### 2.1.7.4.1.4 Statistical Analysis

All statistics were performed as two-tailed unpaired t-tests (GraphPad Prism 6, GraphPad Software Ltd., USA). This allowed the investigation of potential differences in protein expression and Purkinje cell morphology between the control and TP-treated fetal cerebella.

### **2.1.7.5 Simultaneous Immunofluorescence**

Antigen retrieval was achieved using 0.1M citrate buffer (0.1M citric acid monohydrate, made up to 800ml in distilled water, pH 6). The slides, submerged in citrate buffer, were placed in a microwave for five minutes on high setting (750W) with one minute rest. This cycle was repeated a further two times and followed by twenty minutes rest. The slides were washed in (phosphate buffered saline) PBS (tablets, Sigma, U.K.) for five minutes. In a humidified chamber, the samples then underwent serum blocking using goat serum (20% NGS / 5% BSA in PBS) for thirty minutes. Following the serum block, excess serum was blotted from each section, with care taken not to permit any drying of the tissue sections. Avidin block was added to each section for 15 minutes and the slides were then briefly washed in PBS. Biotin block was then added to the sections for a further 15 minutes and briefly washed in PBS. 50 $\mu$ l of the primary antibody dilution was added to each section. The two primary antibodies (calbindin, 1:1000 dilution and AR, 1:50 dilution) were diluted in goat serum (20% NGS / 5% BSA in PBS). The slides were then incubated overnight at 4°C in a humidified chamber. The negative controls used in this study were goat serum in place of a primary antibody for one section and goat serum in place of a secondary antibody for another section.

The primary antiserum incubation was followed by two washes of PBS at five minutes each, with gentle agitation. The sections were then incubated with biotinylated secondary antibody (goat anti-rabbit antibody, Vector Laboratories, U.K.) at a 1:500 dilution in goat serum (20% NGS, 5% BSA, PBS) for 1 hour at room temperature in a humidified chamber. This was followed by two washes of PBS at five minutes each. The sections were further incubated with fluorescently-labelled StreptAvidin 594 (Vector Laboratories, U.K.) and Alexafluor goat anti-mouse 488 (Invitrogen, U.K.) which were both diluted to 1:100 in goat serum (2.0% NGS, 0.5% BSA in PBS). This incubation was followed by two washes of PBS in a light tight box, at five minutes each. The slides were mounted in Vectashield (aqueous mounting medium containing DAPI, Vector Laboratories, U.K.), air-dried in the dark and stored at 4°C. The slides were then ready for visualisation.

#### **2.1.7.5.1 Visualisation**

Images were captured using confocal microscopy.

#### **2.1.7.6 Sequential Immunohistochemistry**

Antigen retrieval was achieved using 0.1M citrate buffer (0.1M citric acid monohydrate, made up to 800ml in distilled water, pH 6). The slides, submerged in citrate buffer, were placed in a microwave for five minutes on high setting (750W) with one minutes rest, and then a further five minutes on high setting, followed by twenty minutes rest. Each slide was then blocked in 3% hydrogen peroxide (Sigma Aldrich, U.K.) solution in distilled water for twenty minutes. The slides were washed in dH<sub>2</sub>O followed by two washings in PBS for five minutes each. Avidin block was added to each section for 15 minutes and the slides were then briefly washed in PBS. Biotin block was then added to the sections for a further 15 minutes and briefly washed in PBS. 50µl of the primary antibody dilution was added to each section. 50µl of the primary antibody dilution was added to each section. The first primary antibody, AR, (1:50 dilution) was diluted in goat serum (20% NGS / 5% BSA in PBS). The slides were then incubated overnight at 4°C in a humidified chamber. The negative controls used in this study were goat serum in place of a primary antibody for one section and goat serum in place of a secondary antibody for another section.

The first primary antiserum incubation was followed by two washes of TBS-T at five minutes each, with gentle agitation. The sections were then incubated with biotinylated secondary antibody 1:500 dilution in goat serum (20% NGS, 5% BSA, PBS) for thirty minutes at room temperature in a humidified chamber. The slides were then washed in TBS-T, twice for five minutes. Avidin-biotin-HRP (horseradish peroxidase) complex (ABC complex, Vector Laboratories, U.K.) was applied to each section and incubated at room temperature for thirty minutes in a humidified chamber. The slides were washed again in TBS-T, twice for five minutes, with gentle agitation. NovaRED substrate solution (Vector Laboratories, U.K.) added to the slides. The colour development of the substrate was carefully monitored by microscopy and once the colour development was sufficient (approximately 5 minutes), the slides were rinsed in dH<sub>2</sub>O for five minutes. The counterstain used was haematoxylin (Harris modified, Vector Laboratories, U.K.). The slides were immersed in haematoxylin for 1 minute and then placed in running tap water.

These slides were then exposed to an Avidin block for 15 minutes and the slides were then briefly washed in PBS. Biotin block was then added to the

sections for a further 15 minutes and briefly washed in PBS. 50µl of the primary antibody dilution was added to each section. 50µl of the primary antibody dilution was added to each section. The second primary antibody, calbindin, (1:3000 dilution) was diluted in goat serum (20% NGS / 5% BSA in PBS). The slides were then incubated overnight at 4°C in a humidified chamber. The negative controls used in this study were goat serum in place of a primary antibody for one section and goat serum in place of a secondary antibody for another section.

The second primary antiserum incubation was followed by two washes of TBS-T at five minutes each, with gentle agitation. The sections were then incubated with biotinylated secondary antibody 1:500 dilution in goat serum (20% NGS, 5% BSA, PBS) for thirty minutes at room temperature in a humidified chamber. The slides were then washed in TBS-T, twice for five minutes. Avidin-biotin-HRP (horseradish peroxidase) complex (ABC complex, Vector Laboratories, U.K.) was applied to each section and incubated at room temperature for thirty minutes in a humidified chamber. The slides were washed again in TBS-T, twice for five minutes, with gentle agitation. 3,3'-diaminobenzidine (DAB) substrate solution was made up, as per manufacturer's instructions (Vector Laboratories, U.K.) and then added to the slides. The colour development of the DAB substrate was carefully monitored by microscopy and once the colour development was sufficient, the slides were rinsed in dH<sub>2</sub>O for five minutes. The counterstain used was haematoxylin (Harris modified, Vector Laboratories, U.K.). The slides were immersed in haematoxylin for 1 minute and then placed in running tap water. The sections were then dehydrated in ascending graded ethanol solutions, cleared in xylene and then mounted in Pertex mountant (Vector Laboratories, U.K.). The slides were then ready for visualisation.

#### **2.1.7.1.6.1 Visualisation**

The slides were visualised under light microscopy (Leitz Laborlux S, Germany) using the X10 and X20 objectives. The sections were digitally captured and recorded using CellSens 1.9 software (Olympus, U.S.A).

### **2.1.8 Microarray experiments**

Microarray was performed by the Oxford Gene Technology company (U.K.). GD 90 male cortex samples were used from four different treatment groups – control, TP, DES and DEX. Each treatment group comprised of six samples. The microarray was performed on an Agilent sheep transcriptome wide microarray (AGEOD-10778). In summary, TIFF image files were processed using Agilent's Feature Extraction software (v10.7.3.1) which carried out a number of normalization steps including; background correction, dye bias correction (via LOWESS normalization) and flagging outlier spots on each array. A pdf report was generated for each array to highlight whether any sample had failed a key QC metric. The 24 feature extracted files were then imported into GeneSpring GX v11.5 and  $\log_2$  ratios were calculated as  $\text{Cy3}(\text{sample})/\text{Cy5}(\text{reference})$  and sample annotation was added. Box plots were produced to examine the spread of data across all arrays.

GeneSpring has a quality control module that was run on the samples. The data was examined using Principal Component Analysis (PCA) which shows how closely samples are related to each other based on the variance in the signal intensity data. Ideally, samples in the same replicate group will cluster closely together. To keep the data set as clean as possible, probes were filtered out if they were flagged as control probes, non-uniform, saturated or an obvious outlier

Hierarchical clustering (HCL) was performed on the filtered data to see how well the samples cluster. Finally, boxplots of each group were produced to compare the signal distribution of the samples. Volcano analysis (which combines statistical T-Tests and fold change analysis) was used to compare the control and TP-treated. All groups were compared using ANOVA analysis with the Benjamini-Hochberg multiple testing correction applied. The significant result sets from the Volcano Analysis were analysed for over represented Gene Ontology terms (GO analysis).

To validate the results from microarray analyses, qRT-PCR was performed as previously discussed.

## **CHAPTER 3**

### **Steroid synthesis and signalling in the developing ovine cerebellum**

### **3. Steroid synthesis and signalling in the developing ovine cerebellum**

#### **3.1 Aims and Objectives**

##### **3.1.1 Aim 1 and objectives**

To identify whether genes associated with steroid signalling and metabolism are expressed in GD 90 male sheep and whether mRNA expression levels are altered as a result of androgen over-exposure.

Numerous genes, and the functional proteins they encode, are key to steroid synthesis and metabolism. These genes have been described in the cerebellum. However, the role of androgens in cerebellar development is unknown. The hypothesis for this study is that the full complement of steroid-associated genes are expressed in the cerebellum of male fetal sheep at GD 90. It is further hypothesised that prenatal excess androgen (2x20mg depot injections at GD 62 and GD 82) alters cerebellar steroid-associated gene expression in GD 90 male sheep, as measured by qRT-PCR and Western blotting. RNA integrity, the optimisation of primer sets utilised in qRT-PCR and of components utilised in Western blotting were performed.

##### **3.1.2 Aim 2 and objectives**

To ascertain the cell types expressing steroid-associated proteins in the cerebellum of GD 90 male sheep and whether prenatal androgen over-exposure affects the number of cells that produce these proteins.

Optimisation studies included: -

- The identification of the different cell types that the cerebellum is composed of and an investigation into cell migration through mid-late gestation utilised H&E staining. This aided in identifying the cerebellar layers of the cerebellum and the layers of the prefrontal cortex in control GD 90 male sheep (fetally injected) and GD 112 male sheep (maternally injected).
- Determining the optimal section thickness using Nissl staining on GD 90 male sheep.
- AR localisation in fetal cerebellar and prefrontal cortex tissues was established using immunohistochemistry. The colocalisation of AR and



calbindin, a cerebellar neuronal marker, in control and TP-treated GD 90 male sheep, simultaneous immunofluorescence and sequential immunohistochemistry were utilised.

- Antibody titration studies allowed us to ascertain the optimal antibody dilution to use in immunohistochemistry for each protein of interest. This further enabled the determination of any differences in the number of Purkinje cells expressing StAR, 5 $\alpha$ -reductase 1, 5 $\alpha$ -reductase 2 and PR, between the control and TP-treated groups. Each slide was examined in five random areas using the grid eyepiece. The sections counted were also randomly allocated. The mean of the five grids was calculated and then multiplied by 3.57 to convert the mean into positive cells/mm<sup>2</sup>. The value of each grid of positive cells was 0.28mm<sup>2</sup> and by multiplying each grid by 3.57 converts the mean into mm<sup>2</sup>.

## 3.2 Results

### 3.2.1 RNA integrity using denaturing paraformaldehyde gels

All frozen brain samples were processed to extract total RNA for qRT-PCR analysis. Before any such analysis could take place, the integrity of the RNA was checked. Denaturing gel analysis demonstrated that each RNA sample had two distinct bands which implies that our RNA was of good quality. Intact RNA (run on a denaturing gel) should have sharp, clear 28S and 18S ribosomal RNA (rRNA) bands and the 28S rRNA band should be approximately twice as intense as the 18S rRNA band, (Figure 3.1, Fleige and Pfaffl, 2006),. Partially degraded RNA will have a smeared appearance, will lack the sharp rRNA bands, or will not exhibit the 2:1 ratio of high quality RNA. Completely degraded RNA will appear as a very low molecular weight smear (Fleige and Pfaffl, 2006). In all downstream analyses, inclusion of samples with known high quality gave confidence in the data obtained. Where this criterion was not met, the RNA sample was discarded and not included in any further analysis. This accounts for differences in initial numbers of samples and numbers of samples analysed – in each group there was one sample that after repeated attempts would not yield RNA of sufficient quality for further downstream analysis

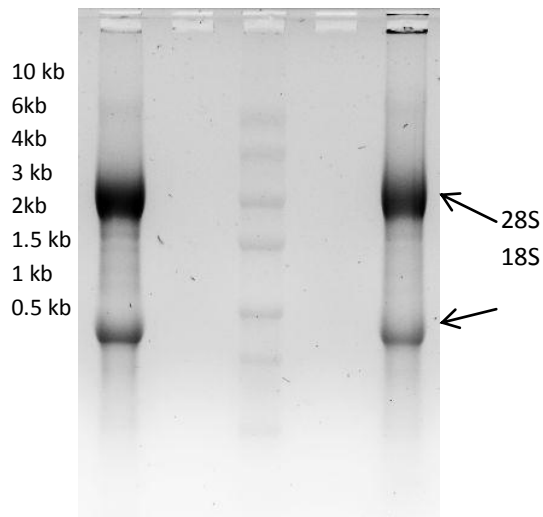


Figure 3.1: This gel is representative of a good quality total RNA sample. The upper band is 28S RNA and the lower band is 18S RNA. Good integrity RNA has a 28S band of approximately twice the intensity of the 18S band, as shown above.

### 3.2.2 qRT-PCR optimisation

#### 3.2.2.1 Determination of successful cloning of a PCR product for primer validation

Colony PCR is a convenient high-throughput method for determining the presence or absence of insert DNA in plasmid constructs. In this study, the insert DNA was the PCR product for AR, where the primer set was custom designed within our lab. Figure 3.2 shows successful cloning of AR and the absence of this particular gene in negative controls.

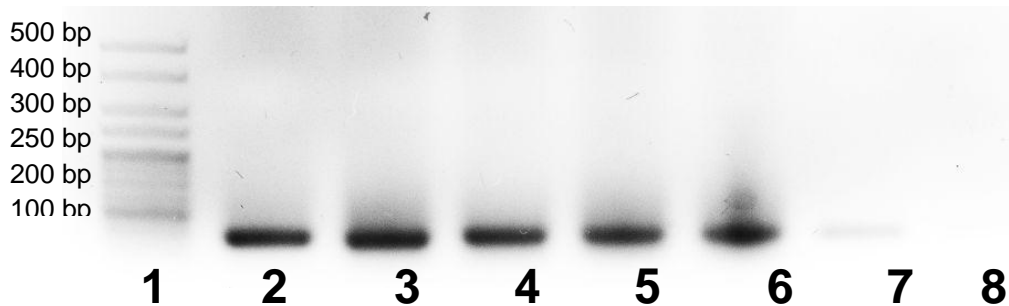


Figure 3.2: Colony PCR was performed to check AR PCR product had been successfully cloned into a vector. The bands shown are approximately 75bp which indicated that the AR PCR was successfully cloned. Lane 1 – ladder, Lane 2 – AR colony 1, Lane 3 – AR colony 2, Lane 4 – AR colony 3, Lane 5 – AR colony 4, Lane 6 – AR colony 5, Lane 7 – AR PCR product only, Lane 8 – negative control.

### 3.2.2.2 Standard curve of cloned AR PCR product to show validation of custom-made primers

According to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al.*, 2009) all custom-made primers must be optimised and validated. One way to do this was to create a standard curve using DNA copy number (Wong and Medrano, 2005).

Using a standard curve, the amplification efficiency of the AR primer sets was determined. Figure 3.3 illustrates the standard curve generated. A slope between  $-3.9$  and  $-3.0$  is acceptable for optimisation and our standard curve shows a slope of  $-3.152$ .  $R^2$  is the correlation coefficient. Our  $R^2$  value was  $0.985$  and a value of  $>0.985$  provides good confidence in correlating the two variables. The standard curve efficiency between  $80-110\%$  is generally satisfactory for optimisation and the results of our study show an efficiency of  $107.6\%$ . Therefore we can conclude that the primers designed for AR were suitable for our experiments. In order to then determine if the AR primers designed were specific for the AR sequence, a cloned vector containing the AR insert was sequenced (by Eurofins MWG Operon, Germany) to ensure that the appropriate PCR product was indeed cloned. The results were put through BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>) and confirmed that the DNA sequence was specific for AR.

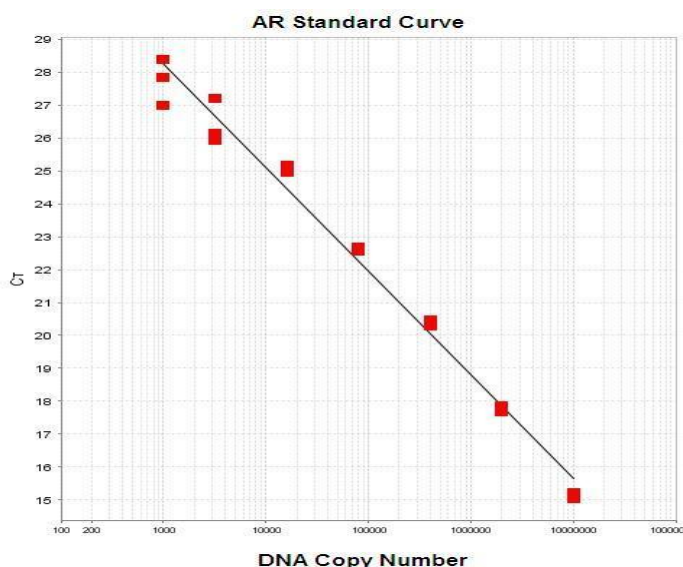


Figure 3.3: This figure shows a standard curve for a set of AR primers. Slope:  $-3.152$ ,  $R^2$ :  $0.985$ , Efficiency:  $107.6\%$ .  $R^2$  is the correlation coefficient.

### **3.2.3 Gene expression analysis**

The level of gene expression for each gene of interest (GOI) was evaluated using qRT-PCR analysis via the  $\Delta\Delta C_T$  method (Livak and Schmittgen, 2001). The fold difference relative to a single reference (control) sample was then calculated by  $2^{(-\Delta\Delta C_T)}$ . Relative gene expression from the male control group and the male TP-treated group were compared to determine differences in gene expression, if any, using a two tailed, unpaired t test (GraphPad Prism software, U.S.A.).

The GOIs were chosen because they are involved in neurosteroidogenesis (Table 3.1), which involves local transformation of cholesterol or circulating steroid precursors into neurosteroids in the brain. Steroid hormones from endocrine organs will also reach the brain and therefore contribute to the effects of neurosteroids and/or serve as precursors for metabolism into steroid receptor active metabolites (Sierra, 2004).

Gene Name	Classical Name	Reason for being GOI	Reference
<i>AR</i>	Androgen receptor	To investigate T signalling	Chang <i>et al.</i> , (1995)
<i>CYP19</i>	Aromatase	Enzyme that converts T into E	Naftolin (1994)
<i>ER<math>\alpha</math></i>	Estrogen receptor $\alpha$	To investigate E signalling	Belcher (1999)
<i>ER<math>\beta</math></i>	Estrogen receptor $\beta$	To investigate E signalling	Belcher (1999)
<i>CYP11A1</i>	P450scc	Converts cholesterol to pregnenolone	Baulieu (1998)
<i>PR</i>	Progesterone receptor	To investigate P signalling	Sakamoto <i>et al.</i> , (2003)
<i>SRD5a1</i>	5 $\alpha$ - reductase 1	Converts T and P into DHT and DHP	Mensah-Nyagan <i>et al.</i> , (1999)
<i>SRD5a2</i>	5 $\alpha$ - reductase 2	Converts T and P into DHT and DHP	Mensah-Nyagan <i>et al.</i> , (1999)
<i>StAR</i>	Steroidogenic acute regulatory protein	Rate-limiting step in steroidogenesis	Stocco and Clark (1996)
<i>HSD<math>\beta</math>3</i>	3 $\beta$ hydroxysteroid dehydrogenase	Key enzyme in steroidogenesis	Payne and Hales (2004)
<i>CYP17</i>	17 $\alpha$ hydroxylase	Key enzyme in steroidogenesis	Payne and Hales (2004)

Table 3.1: A list of all the initial genes of interest that were tested. The primary focus of this study was to identify changes in the cerebellar expression of genetic markers of steroid metabolism and signalling that result from exposure to testosterone propionate (TP) during development in sheep. T = testosterone, E = estradiol, P = progesterone, DHT = dihydrotestosterone, DHP = dihydroprogesterone.

All steroid-associated genes examined were found to be expressed in GD 90 male cerebellum (Table 3.2).

Gene Name	Previous Evidence For Cerebellar Expression	Reference for Previous Evidence	Cerebellar Expression In This Study
<i>StAR</i>	Adult rat	Furukawa <i>et al.</i> , (1998)	Yes
<i>SRD5a1</i>	Adult human	Thigpen <i>et al.</i> , (1993)	Yes
<i>SRD5a2</i>	Fetal sheep	Petratos <i>et al.</i> , (2000)	Yes
<i>CYP11A1</i>	Fetal sheep	Petratos <i>et al.</i> , (2000)	Yes
<i>CYP17</i>	Adult rat	Stromsedt and Waterman (1995)	Yes
<i>CYP19</i>	Neonatal rat	Lavaque <i>et al.</i> , (2005)	Yes
<i>HSD<math>\beta</math>3</i>	Adult rat	Guennon <i>et al.</i> , (1995)	Yes
<i>AR</i>	Adult rat	Takeda <i>et al.</i> , (1990)	Yes
<i>PR</i>	Neonatal rat	Sakomoto <i>et al.</i> , (2001)	Yes
<i>ER<math>\alpha</math></i>	Neonatal rat	Belcher (1999)	Yes
<i>ER<math>\beta</math></i>	Adult rat	Price <i>et al.</i> , (2000)	Yes

Table 3.2: This table depicts whether mRNA expression for each GOI has been identified in the literature. In the majority of literature, cerebellar gene expression studies have been carried out on neonatal or adult rats. Here it was ascertained that all GOI are expressed in male fetal sheep cerebellum.

### 3.2.3.1 The effect of excess fetal androgen on the mRNA expression of genes associated with steroidogenesis in the male cerebellum

The level of gene expression for each GOI was evaluated using qRT-PCR analysis via the  $\Delta\Delta C_T$  method, where the fold differences were calculated by  $2^{(-\Delta\Delta C_T)}$ . The results of the fold differences from the male control group and the male TP-treated group were compared first to determine differences in gene expression, if any, using a two tailed, unpaired t test (GraphPad Prism software).

Gene of Interest	P value (* <0.05)	Fold difference
<i>StAR</i>	0.0264*	5.88-fold reduction
<i>SRD5a1</i>	0.0770	2.93-fold increase
<i>SRD5a2</i>	0.0986	5.55-fold reduction
<i>CYP11A1</i>	0.1194	2.17-fold reduction
<i>CYP17</i>	0.1102	2.32-fold reduction
<i>CYP19</i>	0.1172	2.63-fold reduction
<i>HSD<math>\beta</math>3</i>	0.5111	2.09-fold increase
<i>AR</i>	0.2604	< 2
<i>PR</i>	0.0341*	5.01-fold increase
<i>ER<math>\alpha</math></i>	0.0721	7.14-fold reduction
<i>ER<math>\beta</math></i>	0.3435	< 2

Table 3.3: qRT-PCR analysis demonstrated significant changes in mRNA expression for *StAR* and *PR* in the cerebellum of TP-treated group compared to control of GD90 male sheep, (n=4; p = 0.0264 and 0.0341, respectively). Relative expression is the change in gene expression in a given sample, relative to reference sample (in our study the reference sample was always 204/1). This is calculated by determining a fold change ( $2^{(-\Delta\Delta C_T)}$ ). A fold change of less than 2 implies no change.



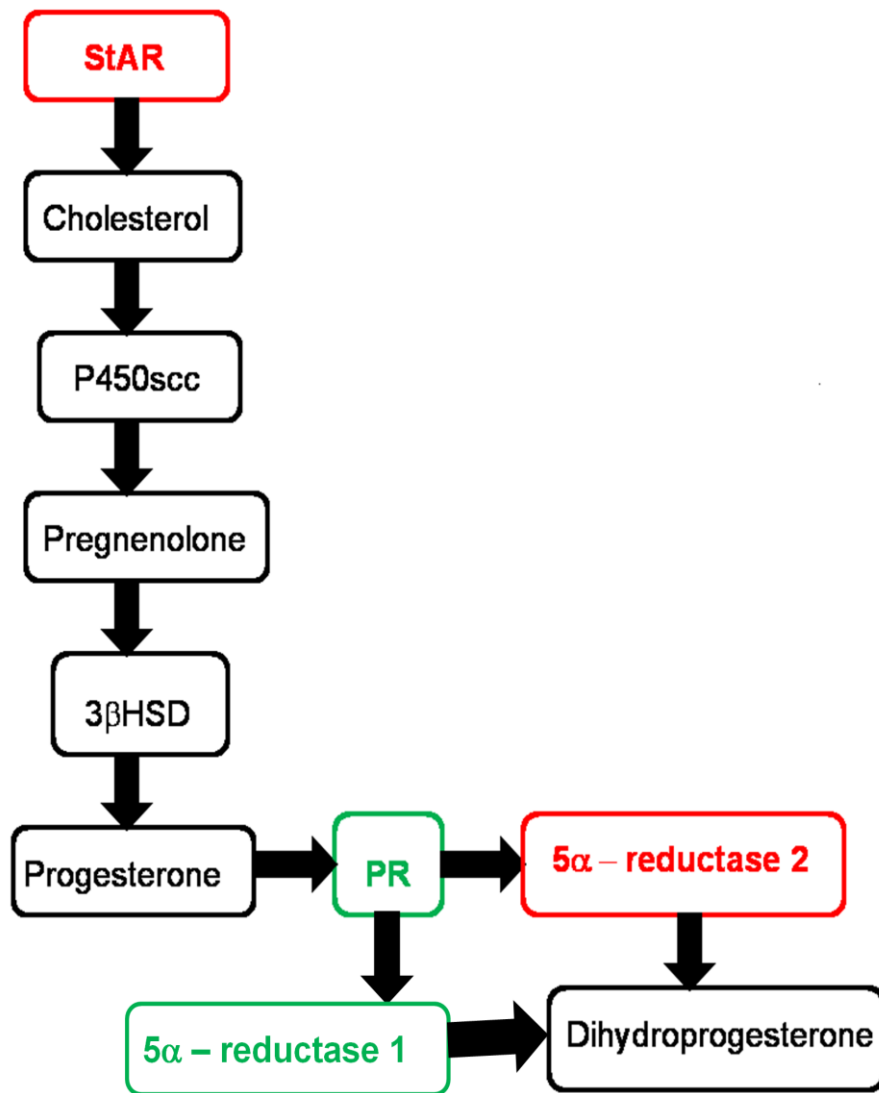


Figure 3.4: A summary diagram of the observed changes (significant or near significant) in gene expression in GD 90 male sheep in the TP-treated group. Red indicates a reduction at the mRNA level whereas green indicates an increase in mRNA expression (n=4).

### 3.2.3.1.1 Determination of whether the changes in gene expression observed are due to an androgenic or estrogenic effect

This study also investigated whether the observed alterations in mRNA expression are due to an androgenic or estrogenic effect. There is potential for the placental aromatisation of testosterone into estrogenic metabolites. Therefore, to define the effects of both sex hormones, fetal male sheep were directly injected with either TP or the potent estradiol receptor agonist, diethylstilbestrol (DES). There were no significant changes between the control and DES-treated groups at the mRNA level for *StAR*, *PR*, *SRD5a1* and *SRD5a2* (Table 3.4). Furthermore, Table 3.4 shows a comparison of p values and fold differences for TP-treated and control cerebellar samples and DES-treated and control cerebellar samples.

GOI	TP-treated vs control group p value (* < 0.05)	Fold difference	DES-treated vs control group p value (* < 0.05)	Fold difference	TP effect mediated by androgen or estrogen
<i>STAR</i>	0.0264*	5.88-fold reduction	0.726	< 2	Androgen
<i>SRD5a1</i>	0.0770	2.93-fold increase	0.323	< 2	Androgen
<i>SRD5a2</i>	0.0986	5.55-fold reduction	0.387	< 2	Androgen
<i>PR</i>	0.0341*	5.01-fold increase	0.372	< 2	Androgen

Table 3.4: qRT-PCR analysis demonstrated no significant changes in mRNA expression for *StAR*, *PR*, *SRD5a1* and *SRD5a2* in the cerebellum of DES-treated group compared to control of GD90 male sheep, (n=4). Relative expression is the change in gene expression in a given sample, relative to reference sample (in our study the reference sample was always 204/1). This is calculated by determining a fold change ( $2^{-(\Delta\Delta CT)}$ ). By determining the statistical significance of the TP-treated and control group fold changes and the DES-treated and control group fold changes, it can be deduced whether TP exposure is mediated by androgen or estrogen. A fold change of less than 2 implies no change.

### 3.2.3.2 Protein analysis

Western blotting was performed in order to determine whether the statistically significant changes in mRNA expression observed for *StAR* and *PR* (a decrease and an increase, respectively) using qRT-PCR, were recapitulated at the protein level. We further observed a trend toward an increase in *SRD5a1* mRNA expression and a trend toward a decrease in *SRD5a2* mRNA expression. Due to the reported developmental regulation of expression in the brain of these two isoforms (*SRD5a2* expressed almost exclusively in fetal life; *SRD5a1* is mostly expressed in adult life, Melcangi *et al.*, 1998), an investigation of both isoforms was performed, to try and understand this relationship within the developing cerebellum.

### 3.2.3.2.1 Visualisation of protein bands

Protein was extracted from samples that had been used for RNA isolation using the TRIsure method (Chapter 2). The first phenol/ethanol supernatants were dialysed approximately 3 months after RNA isolation. In Figure 3.5, the protein transferred to the immunoblot does not appear degraded for any of the different protein preparations, as it can be observed that each lane (with the exception of Lane 1, Ladder) shows clear bands with different molecular weights. Each lane had the same amount of protein loaded (50 $\mu$ g) but each lane has a different protein solubilisation buffer, so that the optimal one can be chosen for each protein of interest.

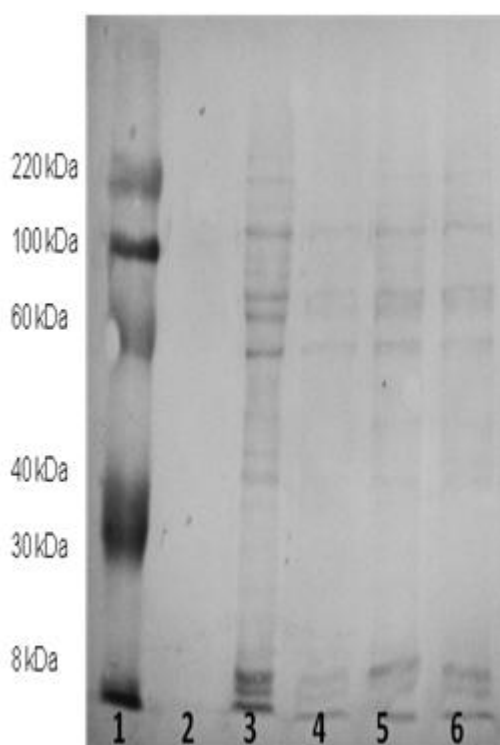


Figure 3.5: Protein bands stained with Memcode Reversible Stain (Thermo Scientific) to show the protein has transferred from gel to membrane. Lane 1 – Ladder, Lane 2 – Supernatant (Sn) unconcentrated, Lane 3 – Globular mass unconcentrated, Lane 4 – Glob mass in 1% SDS, Lane 5 – Glob mass in 8M urea and Tris, Lane 6 - Glob mass in 1% SDS and 8M urea and Tris,

### 3.2.3.2.2 Western blotting

Western blotting was attempted in order to quantify StAR and 5 $\alpha$ -reductase 2 protein expression in GD 90 male sheep cerebellum using 50 $\mu$ g protein, in order to determine whether there were any changes as a result of prenatal excess androgen exposure. This study also attempted to measure protein levels for PR and 5 $\alpha$ -reductase 1.

#### 3.2.3.2.2.1 Anti-actin as a positive control

$\beta$ -actin is abundant in the developing cerebellum (Micheva *et al.*, 1998). When each blot, containing four different protein solubilisation preparations, was probed for  $\beta$ -actin, there were strong signals for each of the Western blots (Figure 3.6), which indicates  $\beta$ -actin protein expression at GD 90 in the male sheep cerebellum. This immunoblot also demonstrated that the protein preparation (the protein was extracted from this sample three months after RNA isolation of the same sample) and Western blotting protocol were successful.

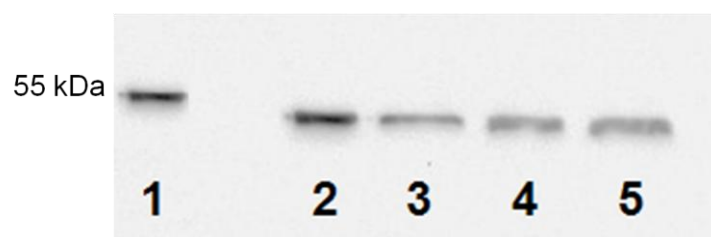


Figure 3.6: Protein bands show actin Ab (1 in 2000). The size of actin is 42 kDa. Lane 1 – Molecular weight ladder showing 55 kDa (Page Ruler, Pierce), Lane 2 - Globular mass, Lane 3 – Glob mass in 1% SDS, Lane 4 – Glob mass in 8M urea and Tris , Lane 5 - Glob mass in 1% SDS and 8M urea and Tris.

### 3.2.3.2.2 StAR protein expression in the developing cerebellum

When the Western blotting membrane was probed for StAR, there was no signal in the globular mass sample but there was a weak signal in the protein sample that contained 8M urea and tris (a solubilisation buffer), and the protein sample that included 1% SDS, 8M urea and tris (a solubilisation buffer, Figure 3.7 A, lanes 7 and 8). This therefore suggests that these are optimal protein fractions for StAR protein. These solubilising solutions, and others (Chapter 2), were added to the protein as demonstrated by (Hummon *et al.*, 2007). The molecular weight for StAR is 37 kDa and the faint bands seen were just under the 35 kDa marker, therefore it suggests that this is the mature form of StAR protein. There also appears to be non-specific binding occurring above the StAR signal which could be the precursor forms of StAR at 32 and 37 kDa. The blot was stripped and re-probed for  $\beta$ -actin (1:2500 dilution) and shows bands for the concentrated protein preparations (Figure 3.7 B). When the immunoblot was re-probed for  $\beta$ -actin protein, the protein preparations that were most abundant were the same samples as for StAR - the globular mass solubilised in 8M urea and Tris and the globular mass with 1% SDS, 8M urea and tris (Figure 3.7 B, lanes 7 and 8).

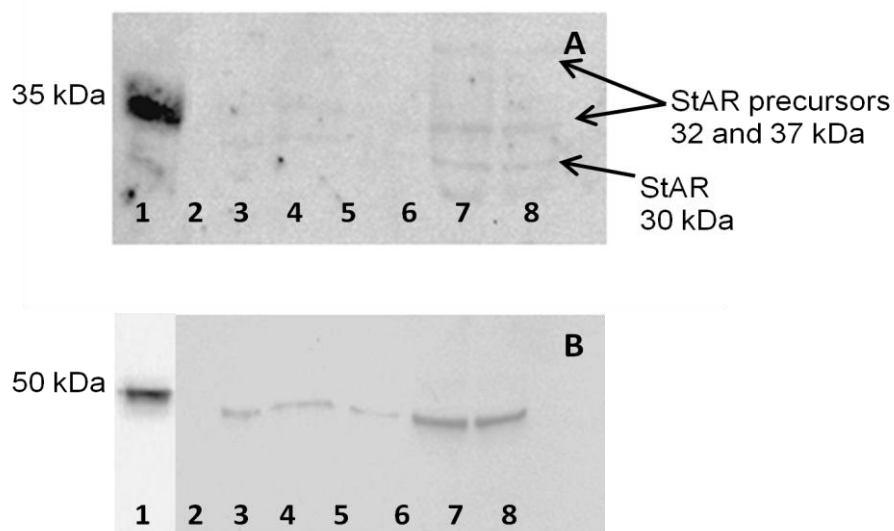


Figure 3.7: A) StAR (1:500) was probed on a nitrocellulose membrane (semi-wet transfer from 10% SDS-PAGE gel) at 180 second ECI exposure. 50 $\mu$ g of protein was loaded into each lane. B) The membrane was then stripped and re-probed for  $\beta$ -actin (1:2500). Lane 1 – Molecular weight ladder showing 35 and 50 kDa (Colour Burst, Sigma), Lane 2 – Sn unconcentrated, Lane 3 – Sn concentrated, Lane 4 – Glob mass unconcentrated, Lane 5 – Glob mass concentrated, Lane 6 – Glob mass in 1% SDS, Lane 7 – Glob mass in 8M urea and Tris.

### 3.2.3.2.3 5 $\alpha$ -reductase 2 protein expression in the developing cerebellum

Western blots probed with the 5 $\alpha$ -reductase 2 antibody (1:500 dilution) had a signal for the unconcentrated globular mass sample and a weak signal for the concentrated globular mass in 1% SDS (Figure 3.8, lanes 4 and 5 respectively). After protein dialysis, half of the protein was concentrated using centrifugal filter concentrator tubes and the other half was not treated. Concentration of the proteins should allow an enhanced yield in concentration however, here the unconcentrated protein, which had the same amount of protein loaded into the well as the concentrated one, had a strong signal for 5 $\alpha$ -reductase 2 (Figure 3.8, lane 4). The molecular weight for this protein is 28 kDa and the band appeared to be slightly above the 25 kDa marker, thus suggests that this is the correct band. The other bands could be due to non-specific background being detected or even protein that has been degraded.

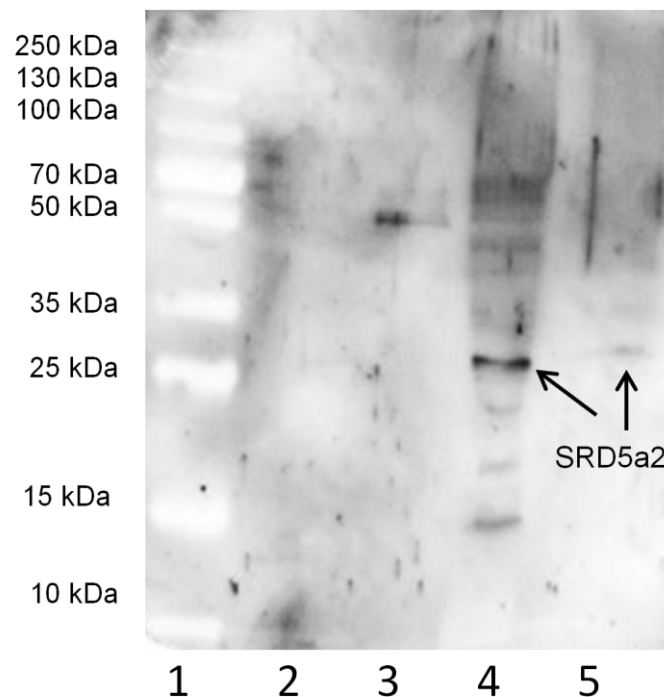


Figure 3.8: 5 $\alpha$ -reductase 2 (1:500) was probed on a nitrocellulose membrane (semi-wet transfer from 12% SDS-PAGE gel) at 300 second ECI exposure. 50 $\mu$ g of protein was loaded into each lane. Lane 1 – Molecular weight ladder (Page Ruler, Pierce), Lane 2 – Sn unconcentrated, Lane 3 – Sn concentrated, Lane 4 – Glob mass unconcentrated, Lane 5 – Glob mass concentrated.

#### 3.2.3.2.4 Using lamb adrenal gland as a positive control tissue

Initially, protein isolation and Western blotting optimisation were successful *i.e.* protein bands were visualised on stained gels and blots. Unfortunately, further into the study, Western blotting was no longer successful. Due to the limited sample number, each sample was used for both protein extraction and RNA isolation, together with prolonged storage at  $-20^{\circ}\text{C}$  for more than three months, may have degraded the protein within the sample. To confirm that there was no issue with the antibodies used, a positive control tissue was used. Figure 3.9 shows that there were weak bands for anti-StAR and PR, a strong band for anti- $5\alpha$ -reductase 1 and no signal for anti- $5\alpha$ -reductase 2 in the lamb adrenal gland.

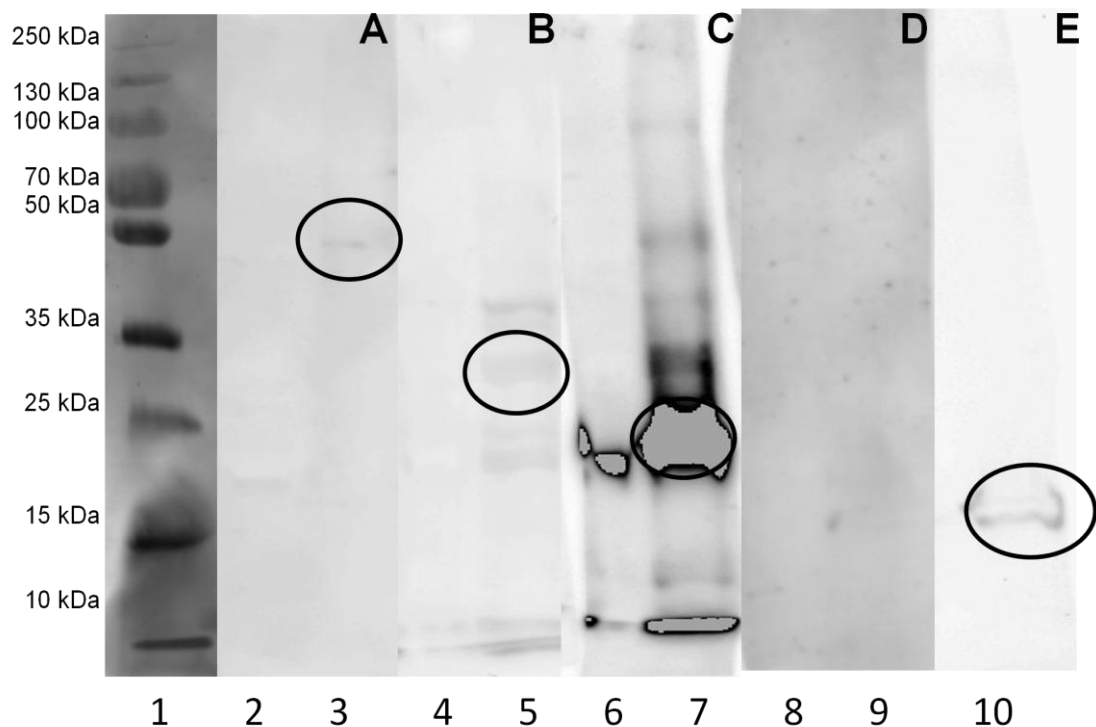


Figure 3.9: Western blots for the lamb adrenal gland. ECL exposure time was 30 seconds. A) PR, 1:250 dilution B) StAR, 1:500 dilution C) SRD5a1, 1:500 dilution D) SRD5a2, 1:500 dilution E) b-actin, 1:2500 dilution. Lane 1 – Ladder (Page Ruler, Pierce), Lane 2 – Ladder (Page Ruler, Pierce), Lane 3 – Globular mass unconcentrated, Lane 4 – Ladder (Page Ruler, Pierce), Lane 5 – Globular mass unconcentrated, Lane 6 – Ladder (Page Ruler, Pierce), Lane 7 – Globular mass unconcentrated, Lane 8 – Ladder (Page Ruler, Pierce), Lane 9 – Globular mass unconcentrated, Lane 10 – Globular mass unconcentrated.



### **3.2.3.3 Immunohistochemistry**

Immunohistochemistry was utilised to investigate protein expression in the fetal cerebellum, by enabling the visualisation of cell types in the developing cerebellum that express the proteins of interest for this study. The number of protein positive cells/mm<sup>2</sup> were counted to determine if there were any changes in the number of cells expressing a particular protein between the control and TP-treated groups. The same antibodies were utilised that were used for Western blotting and all were successful (*i.e.* positive staining).

The number of positive cells (stained brown) were counted using a grid eyepiece at five random areas of each cerebellar section (GD90 male sheep). The mean was calculated and then converted into positive cells/mm<sup>2</sup>. Immunohistochemical analysis of StAR, PR, 5 $\alpha$ -reductase 1 and 5 $\alpha$ -reductase 2 revealed a significant increase in the number of Purkinje cells that express each protein of interest ( $p = 0.0002, 0.0201, 0.0001$  and  $0.0001$ , Figures 3.16-19, respectively) in the TP-treated group compared to the control group.

### 3.2.3.3.1 Investigation of cell migration in the developing cerebellum

Two time points during ovine gestation were observed in order to look at the cell migration in the developing cerebellum of GD 90 (fetally injected) and GD 112 (maternally injected) control male sheep. Figure 3.10 demonstrates how the cytoarchitecture has changed within this period of time.

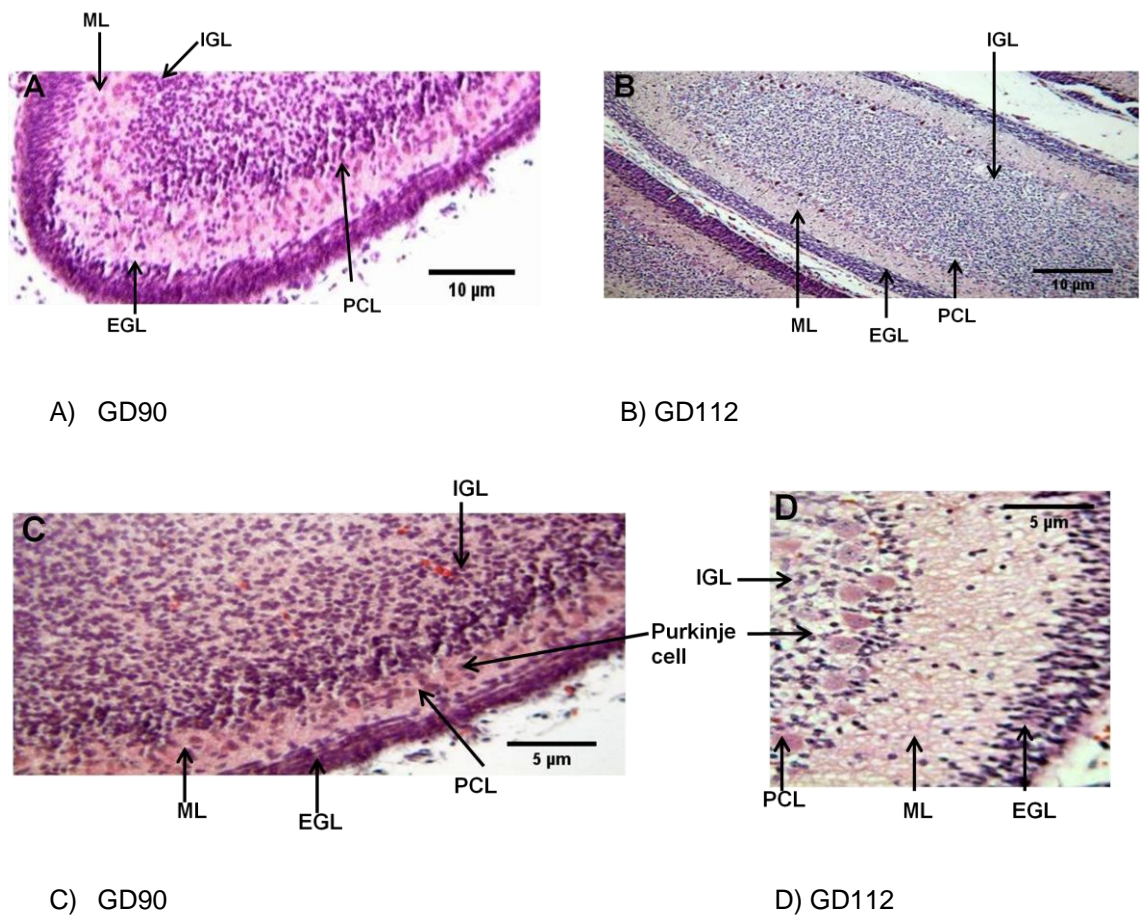


Figure 3.10: Haematoxylin and Eosin Staining of cerebellar tissue of control male sheep to distinguish the different layers. Two gestational periods were investigated to show how the cerebellum develops. All slices were 5µm thick. A) GD90 and B) GD112, scale bar = 10µm. C) GD90 and D) GD112, scale bar = 5µm. EGL – external granule layer, ML – molecular layer, PCL – Purkinje cell layer, IGL – internal granule layer.

### 3.2.3.3.2 Exploring optimal tissue thicknesses for Nissl staining studies

Previous Nissl stain studies have used methodology that cuts tissue sections at a thickness of 50 $\mu$ m using a vibratome (Pilati *et al.*, 2008). We did not have access to a vibratome, so a microtome was utilised, which is more suited to cutting at a lesser thickness. Therefore we cut cerebellar tissue sections of varying thicknesses to determine the optimal thickness (Figure 3.11). Our conclusion was that 5 $\mu$ m tissue sections were the most suitable, due to the fact we could see the cytoarchitecture of the developing cerebellum more clearly than the thicker sections.

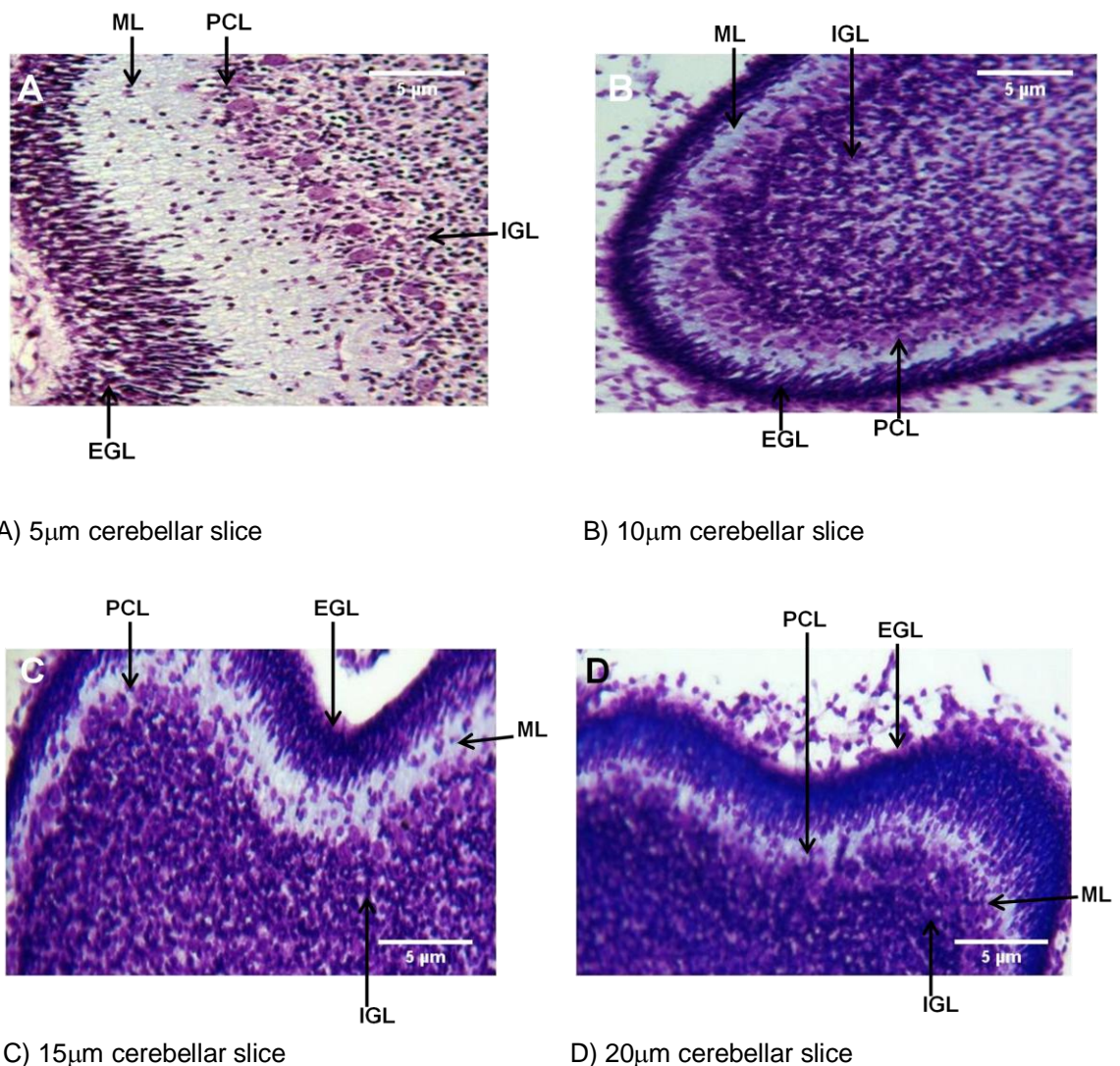


Figure 3.11: Nissl Staining of cerebellar tissue of control male sheep at GD90, scale bar = 5 $\mu$ m. Nissl staining is used to stain neurons and also to understand the cytoarchitecture of different brain areas. Traditionally, the tissue sections are cut thick (approximately 20-50 $\mu$ m). However we are unable to distinguish the different layers and neurons with this thickness, therefore we optimised Nissl staining to identify a suitable different thickness. A) 5 $\mu$ m slice. B) 10 $\mu$ m slice. C) 15 $\mu$ m slice. D) 20 $\mu$ m slice.

### 3.2.3.3.3 Primary antibody titration studies

Titration studies were performed to determine the optimal antibody dilution for immunohistochemical analyses. As an example of the titration experiments, PR is illustrated in Figure 3.12. The more concentrated primary antibody dilution of 1:100 showed the cells expressing PR being masked by non-specific background staining. This would make cell counting more difficult and more prone to errors. Therefore the PR antibody dilution of 1:1000 was more suitable for analysis of protein expression (Figure 3.12).

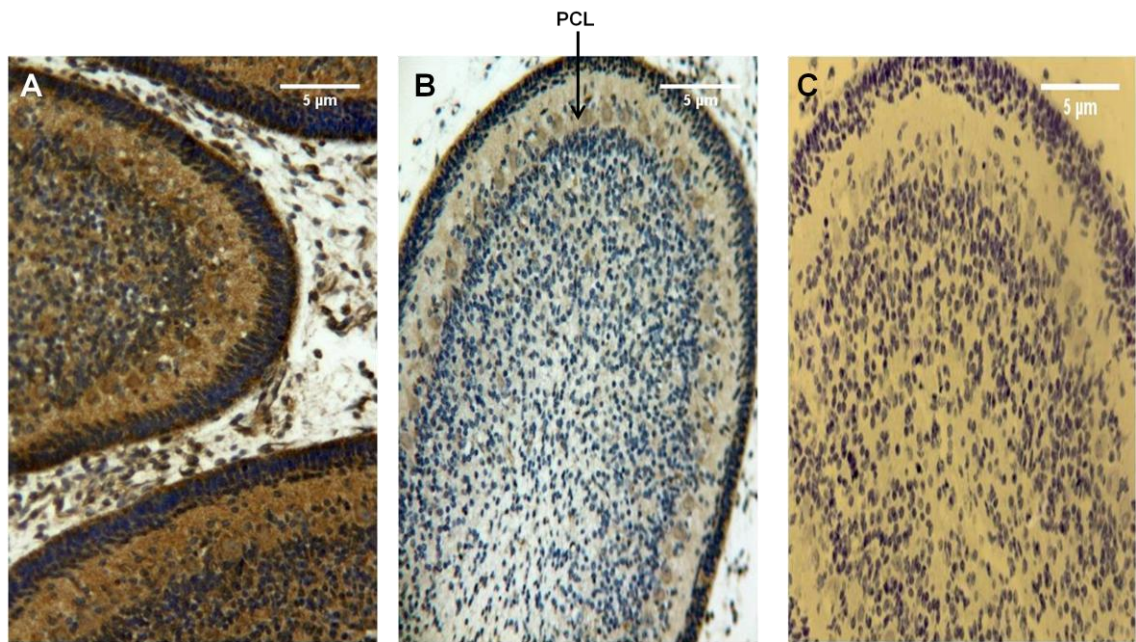


Figure 3.12: An example of dilution titers for anti-PR in GD90 cerebellar ovine tissue, scale bar = 5µm. A) 1:100 dilution. B) 1:1000 dilution. C) Negative control (Rabbit IgG).

#### 3.2.3.3.4 Localisation of AR in the developing cerebellum

As we have treated our samples with an androgen (TP), we decided to examine the location of AR in the cerebellum of GD90 male sheep. We have illustrated, through immunohistochemistry, that AR are located within the nuclei of the Purkinje cells of the cerebellum in GD90 male sheep, as there is strong positive staining (brown) within the nuclei (Figure 3.13, positive cells were stained brown).

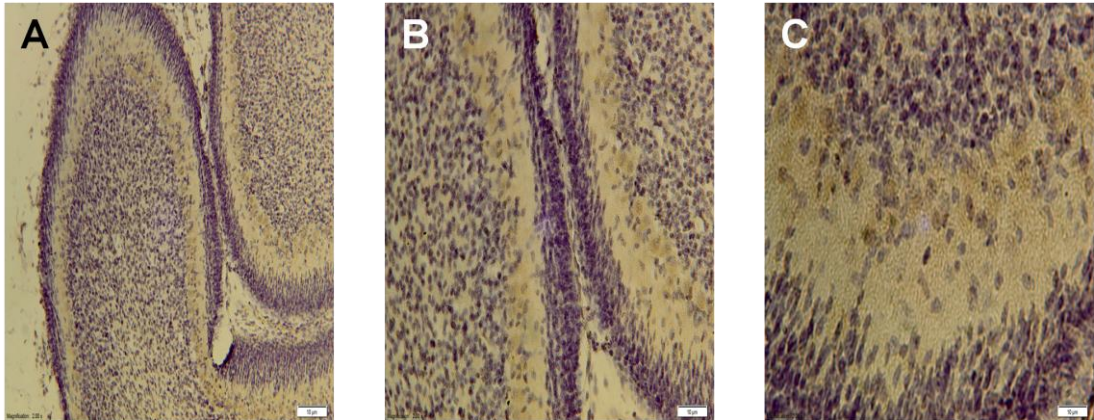


Figure 3.13: Immunohistochemical studies show localisation of AR (dilution 1:500) in the nuclei of Purkinje cells in the cerebellum of GD90 male sheep. A) X10 magnification. B) X20 magnification. C) X40 magnification.

### 3.2.3.3.5 Colocalisation of AR and calbindin in the cerebellum

We investigated the colocalisation of AR and calbindin in the developing male cerebellum. We chose these proteins as we have previously shown that AR is located in the nuclei of Purkinje cells and calbindin is a known Purkinje cell marker (Dougherty *et al.*, 2013).

#### 3.2.3.3.5.1 Examining colocalisation by means of double immunofluorescence

Calbindin is a good neuronal cell marker and Figure 3.14 confirms that the positive calbindin staining is exclusive in the Purkinje cells of the cerebellum. It is difficult to say whether there is any positive nuclear AR staining (in red) or if our results show only background staining. There does appear to be signs of more intense nuclear staining within the PCL which corresponds with the positive cytoplasmic calbindin staining. The top and bottom left panel shows the two images merged.

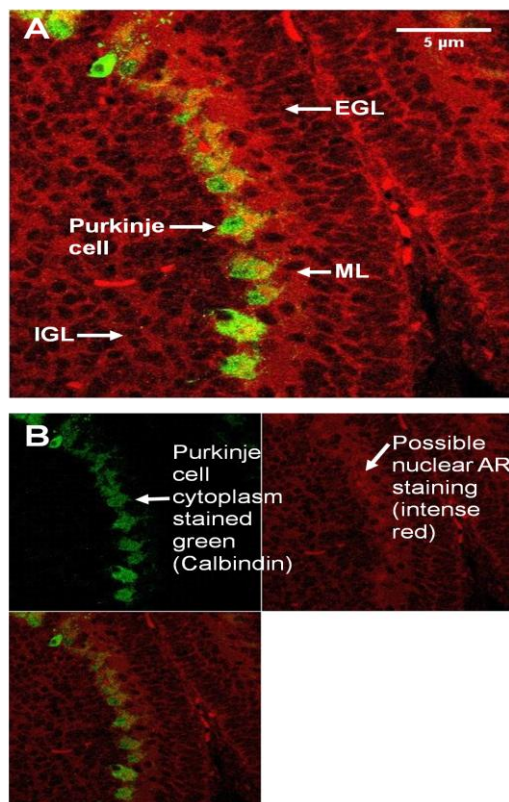


Figure 3.14: Colocalisation of AR and Calbindin in Purkinje cells of the cerebellum in GD90 male sheep, scale bar = 5µm. A) a merged image of AR and calbindin Immunofluorescence. The green fluorescence indicates positive calbindin staining (1:1000 dilution) in the cytoplasm. B) the two fluorescent colours separately. Here we see green positive staining for calbindin (top left panel) and AR positive/background staining in red (right panel).

### 3.2.3.3.5.2 Determining colocalisation of AR and calbindin by using sequential immunohistochemistry

As simultaneous immunofluorescence studies proved to be ambiguous in the determination of colocalisation of AR and calbindin in Purkinje cells of the developing male cerebellum, we chose another method which proved to be more suitable for our study (Figure 3.15).

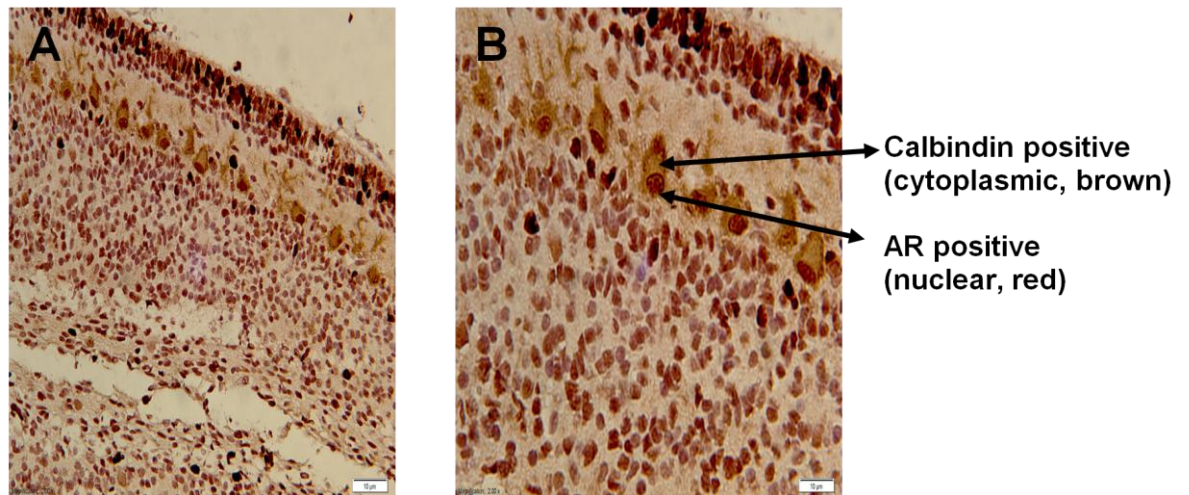


Figure 3.15: Colocalisation studies to show the location of AR and Calbindin (a Purkinje cell marker) in GD90 male sheep. (A and B) Cerebellar tissue was treated with NovaRED (Vector Labs) substrate after the first primary antibody incubation and then DAB after the first primary antibody incubation. A) X20 magnification. B) X40 magnification. Positive staining for DAB is brown and positive staining for NovaRED is red. AR positive cells had nuclear staining whereas calbindin positive cells had cytoplasmic staining.

### 3.2.3.3.6 Protein expression studies

#### 3.2.3.3.6.1 StAR protein expression

StAR protein is located in the mitochondrion of Purkinje cells in the cerebellum of adult rats (Furukawa *et al.*, 1998). Figures 3.16 A and B illustrates that in the developing male cerebellum, StAR is located in the cytoplasm of Purkinje cells and some granule cells within the EGL and IGL, as there is positive staining within this regions (stained brown) and the nuclei are stained blue (the counterstain). Due to limitations of magnification, this study could not identify mitochondria specifically. This study has illustrated that there are more positive Purkinje cells in the developing cerebellum that express StAR protein, as a result of excess androgen exposure ( $p = 0.0002$ , Figure 3.16), compared to the control group.

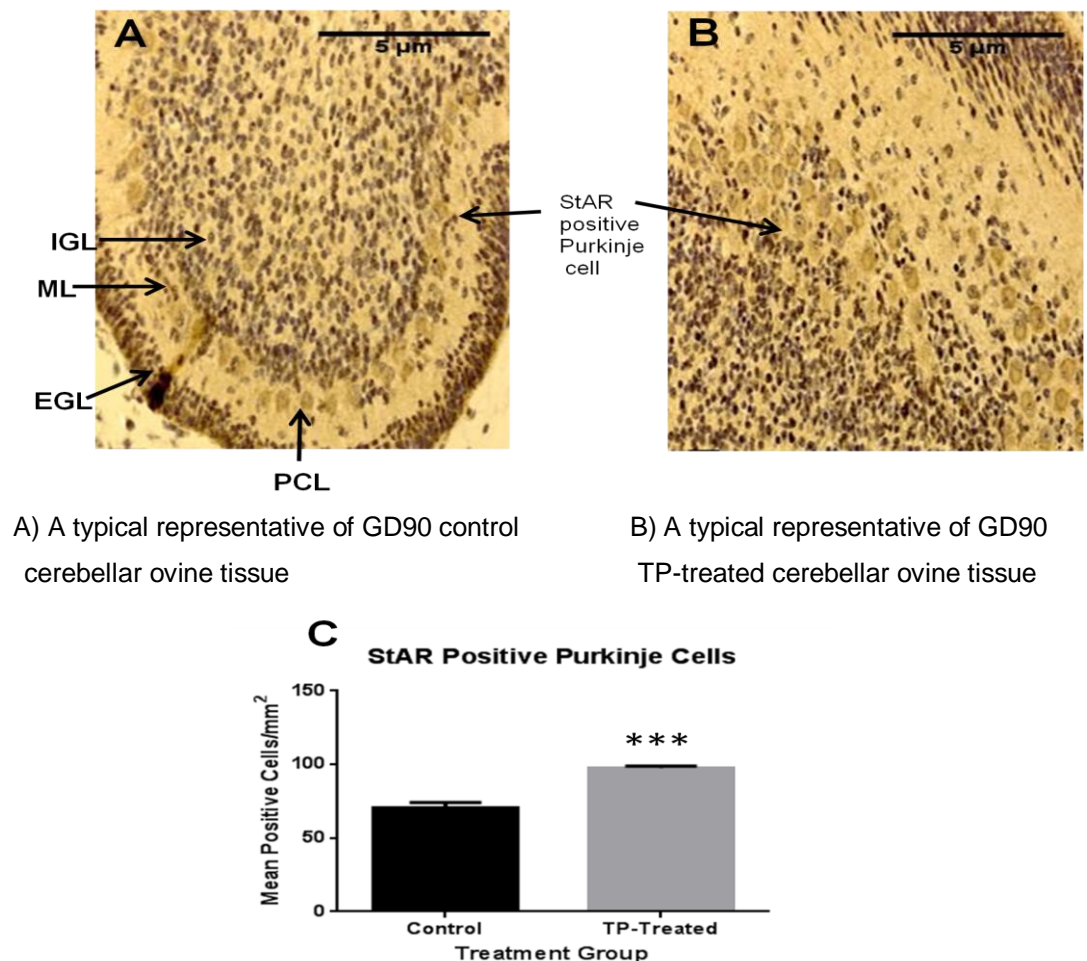
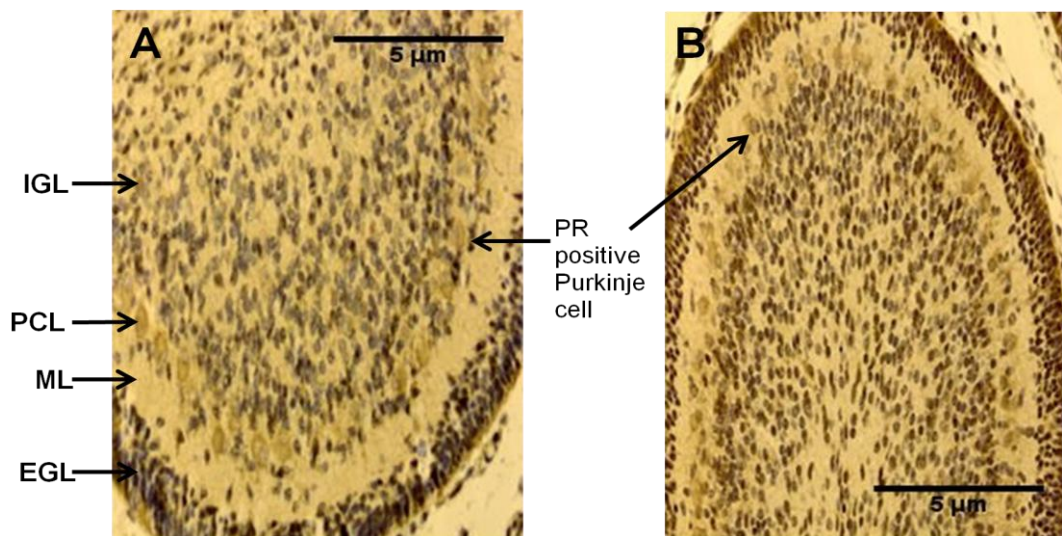


Figure 3.16: An increase in positive Purkinje cells/mm<sup>2</sup> ( $p = 0.0002$ ) expressing StAR in the TP-treated group ( $n=4$ ). A) a typical image of a GD90 control male sample at X20 magnification. StAR positive cells are stained brown (cytoplasmic). Scale bar = 5 $\mu$ m B) a typical image of a GD90 TP-treated male samples at X20 magnification. StAR positive cells are stained brown (cytoplasmic). Scale bar = 5 $\mu$ m C) Graphical representation of data.



### 3.2.3.3.6.2 PR protein expression

Sakamoto *et al.* (2001) demonstrated that the nuclei of Purkinje cells of neonatal rats express PR, using immunohistochemical analysis. The current study shows that PR is detected at the nuclei or perinuclear region of Purkinje cells and some granule cells within the IGL in GD 90 male ovine cerebellar tissue (stained brown, Figures 3.17 A and B). This study has determined that as a result of androgen exposure, there is an increase in the number of Purkinje cells that express PR protein ( $p = 0.0201$ , Figure 3.17), compared to the control group.



A) A typical representative of GD90 control cerebellar ovine tissue

B) A typical representative of GD90 TP-treated cerebellar ovine tissue

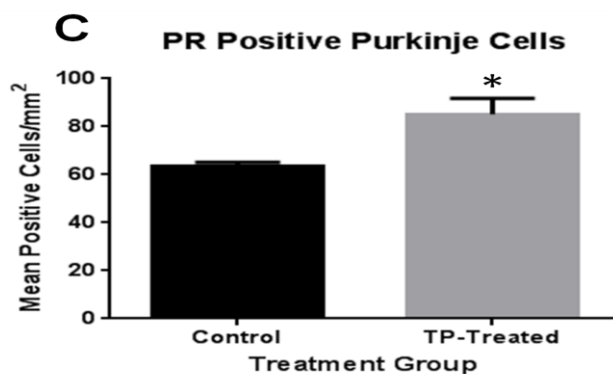
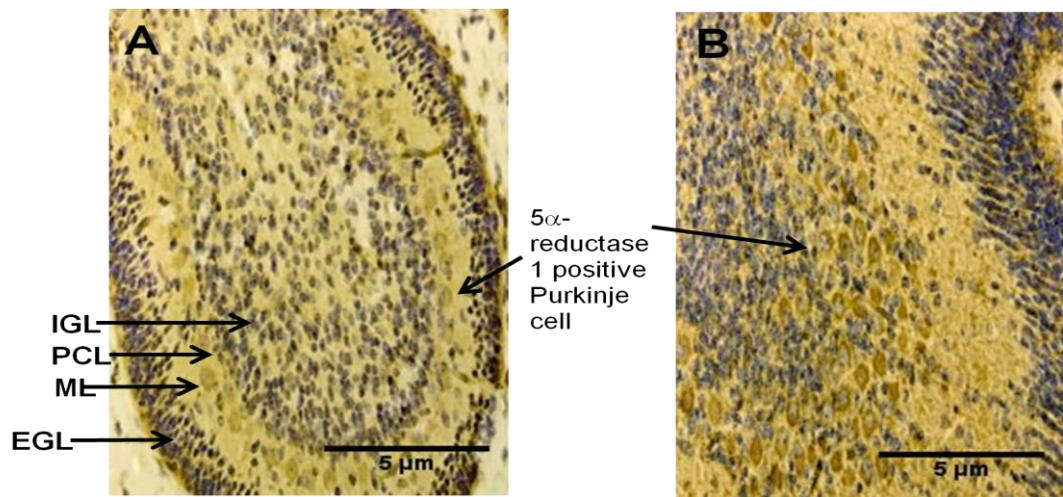


Figure 3.17: Analysis of anti-PR suggests in the TP-treated group, there is an increase in positive Purkinje cells/mm<sup>2</sup> that express PR ( $p = 0.0201$ ,  $n=4$ ). A) a representative image of a GD90 control male sample at X20 magnification. PR positive cells are stained brown (nuclear/perinuclear). Scale bar = 5µm B) a representative image of a GD90 TP-treated male samples at X20 magnification. PR positive cells are stained brown (cytoplasmic). Scale bar = 5µm C) Graphical representation of data.

### 3.2.3.3.6.3 5 $\alpha$ -reductase 1 protein expression

Agís-Balboa *et al.*, (2006) showed that there was 5 $\alpha$ -reductase 1 protein expression within the somata (specifically, the microsomal membrane) of Purkinje cells of adult male mice. This current study has established that 5 $\alpha$ -reductase 1 expression is located in the soma of Purkinje cells and some granule cells within the EGL in fetal male sheep as there is positive staining within this regions (stained brown) and the nuclei are stained blue (the counterstain, Figures 3.18 A and B). This investigation has established that there is an increase in positive Purkinje cells that express 5 $\alpha$ -reductase 1 protein, in the developing cerebellum, as a result of androgen exposure ( $p = 0.0001$ , Figure 3.18).



A) A typical representative of GD90 control cerebellar ovine tissue

B) A typical representative of GD90 TP-treated cerebellar ovine tissue

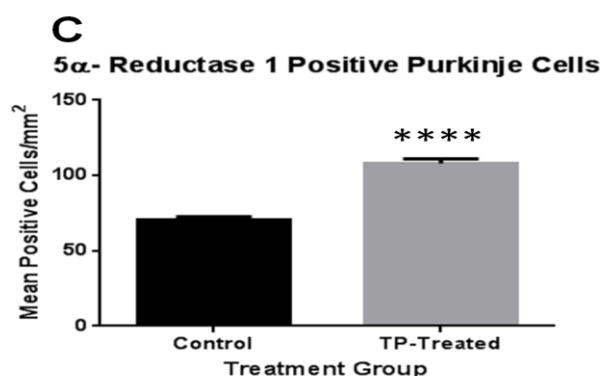
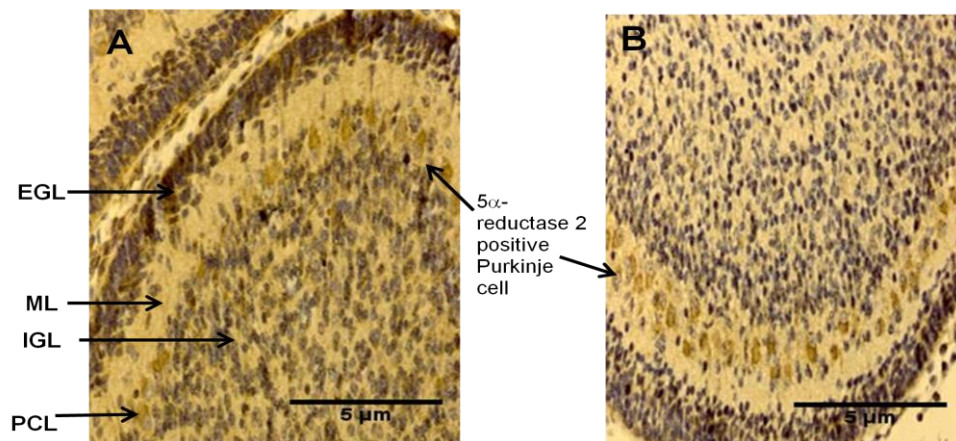


Figure 3.18: This study determined that there was an increase of positive Purkinje cells/mm<sup>2</sup> that express 5 $\alpha$ -reductase 1 in the TP-treated group ( $p = 0.0001$ ,  $n=4$ ). A) a typical image of a GD90 control male sample at X20 magnification. 5 $\alpha$ -reductase 1 positive cells are stained brown (cytoplasmic). Scale bar = 5 $\mu$ m B) GD90 TP-treated male samples at X20 magnification. 5 $\alpha$ -reductase 1 positive cells are stained brown (cytoplasmic). Scale bar = 5 $\mu$ m C) Graphical representation of data.

### 3.2.3.3.6.4 5 $\alpha$ -reductase 2 protein expression

5 $\alpha$ -reductase 2 protein expression is found in the cytoplasm (specifically, the microsomal membrane) of Purkinje cell and some cells in the IGL of the cerebellum in fetal sheep (Petratos *et al.*, 2000). Figures 3.19 A and B also demonstrates that 5 $\alpha$ -reductase 2 expression is detected in the cytoplasm of Purkinje cells and some granule cells within the IGL in developing male sheep as there was positive staining within these regions (stained brown) and the nuclei are stained blue (the counterstain). This study has ascertained that there is an increase in positive Purkinje cells that express 5 $\alpha$ -reductase 2 protein, in the developing cerebellum, as a result of excess androgen exposure ( $p = 0.0001$ , Figure 3.19).



A) A typical representative of GD90 control cerebellar ovine tissue

B) A typical representative of GD90 TP-treated cerebellar ovine tissue

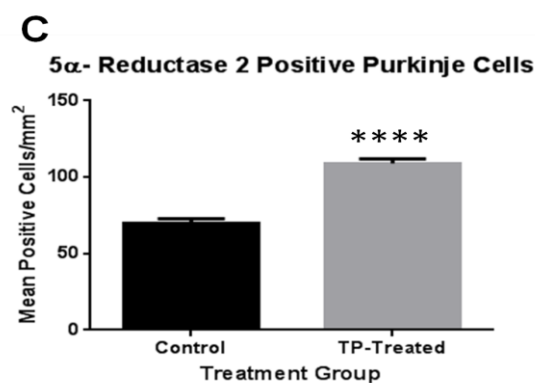


Figure 3.19: An investigation of anti- SRD5a2 revealed an increase in positive Purkinje cells/mm<sup>2</sup> ( $p = 0.0001$ ) in the TP-treated group ( $p = 0.0001$ ,  $n=4$ ). A) a representative image of a GD90 control male sample at X20 magnification. 5 $\alpha$ -reductase 2 positive cells are stained brown (cytoplasmic). Scale bar = 5 $\mu$ m B) a representative image of a GD90 TP-treated male sample at X20 magnification. 5 $\alpha$ -reductase 2 positive cells are stained brown (cytoplasmic). Scale bar = 5 $\mu$ m C) Graphical representation of data.

### 3.3 Discussion

#### 3.3.1 Determining RNA integrity using $A_{260}/A_{280}$ ratio

RNA concentration was determined by measuring the optical density (OD) of each sample. UV spectroscopy is a widely used method to quantify RNA. Contaminants such as residual proteins and phenol can interfere with absorbance readings, thus care was taken during the RNA isolation procedure to remove such things. This method does not discriminate between RNA and DNA; therefore all of our RNA samples were treated with RNase-free DNase to remove contaminating DNA prior to determining concentration. The absorbance of a diluted RNA sample was measured at 260 and 280 nm. The nucleic acid concentration was calculated using the Beer-Lambert law, which predicts a linear change in absorbance with concentration (Fuwa and Vallee, 1966). The  $A_{260}/A_{280}$  ratio was also used to assess RNA purity, therefore in combination with RNA integrity analyses, ensured robust and valid data was generated by PCR methodology.

There are other approaches to investigate RNA integrity. The 2100 Bioanalyzer instrument (Agilent, U.K.) uses a combination of capillary electrophoresis and fluorescent detection to evaluate both RNA concentration and integrity, the latter in an analogous way to that presented here via denaturing gel electrophoresis. The advantages of such an instrument, in addition to low sample input requirements, is that such instruments provide an analysis algorithm to assess the integrity of the RNA sample (the RNA Integrity Number, or RIN) with a maximum value of 10. Significant decreases in the RIN are indicative of degraded total RNA (Lightfoot, 2002). The RIN number was developed in order to standardise RNA integrity interpretation and therefore remove individual interpretation in RNA quality control (Mueller, *et al.*, 2004).

We did not have access to the 2100 Bioanalyser during this part of the study. Although the methods used in this study are more laborious and require larger input amounts of sample than, for example, the 2100 Bioanalyser mentioned above, they were critical in determining that the RNA isolated was of sufficient quality for downstream analysis, providing confidence in the fidelity of the PCR data generated.

### **3.3.2 Fetal ovine cerebellar gene expression and alterations in response to excess androgen exposure in utero**

This study has shown that at GD 90, the male ovine cerebellum expresses genes involved in the steroid synthesis pathway and therefore illustrates that the fetal ovine cerebellum is capable of neurosteroidogenesis. Using assay to measure cholesterol and pregnenolone, two steroidal precursors involved in the rate limiting steps of steroidogenesis, would further establish this theory. All genes of interest have been shown to be expressed in the cerebella of other animal models (e.g. rodent) and so this current study not only confirms this cerebellar expression pattern but also provides novel information on the fetal ovine brain the time point at which these genes are expressed (Table 3.2). Only two of the genes of interest, *SRD5a2* and *CYP11A1*, have been found to be expressed in the developing male sheep cerebellum in previous literature (Petratos, *et al.*, 2000).

The first aim of this study was to identify changes in the cerebellar expression of genetic markers of steroid metabolism that result from exposure to testosterone propionate (TP) during development in male sheep, by utilising qRT-PCR. Using statistical analysis, this study has revealed which genes are altered as a result of prenatal excess testosterone exposure.

#### **3.3.2.1 Steroidogenic transporter, enzymes and steroid hormone signalling**

The majority of the genes encoding steroidogenic enzymes and steroidal signalling investigated in this study have shown no significant alterations in mRNA expression after TP administration, however, there were clear alterations in gene expression of *STAR* and *PR*.

### 3.3.2.2 *STAR*

StAR is the transport molecule for cholesterol and therefore involved in the rate-limiting step of steroidogenesis (Stocco and Clark, 1996). There is a significant decrease in *STAR* mRNA expression in the TP group (n=4, p= 0.0264, Figure 3.4) compared to the control group. The decrease in *STAR* mRNA expression suggests that prenatal excess androgen exposure may reduce local steroid synthesis, as it is the rate-limiting transporter of this pathway, implying the existence of a negative feedback loop. Houk *et al.*, (2004) examined the inhibition of StAR expression by androgens in cultured Leydig cells and suggested that this occurs at the transcriptional level. They proposed that an androgen-dependent feedback loop inhibits cAMP-stimulated StAR expression as this gene is regulated by cAMP-mediated signalling. In order to determine whether this is the mechanism that regulates StAR expression in our model, further experiments should be carried out. Utilising primary cell cultured cerebellar Purkinje cells from control and TP-treated samples would help establish whether levels of cAMP are altered (measured using a cAMP assay) at GD 90 and evaluating the effects of a direct adenylate cyclase inhibitor, would help assess if this is the mechanism involved in our results. Examining whether the inhibition of androgen receptor activity by flutamide, an AR antagonist, or whether DHT, a non-aromatizable androgen, affects *STAR* mRNA expression would allow us to determine whether it is indeed a direct effect of TP that has altered *STAR* mRNA expression in the current study. This could be achieved by treating cultured primary Purkinje cells with TP or DHT, in the presence and absence of flutamide and finally measuring StAR expression by means of qRT-PCR.

Houk *et al.*, (2004) demonstrated that testosterone directly inhibits *STAR* expression in Leydig cells, which suggests an autocrine mechanism through which steroid hormones can regulate their own production at the rate-limiting step of cholesterol transfer to the inner mitochondrial membrane. Investigating the interaction between the activation of the StAR promoter and its feedback inhibition of steroidogenesis would provide more insight into this in the current study's model. A way to be able to determine this would be to indirectly measure *StAR* gene transcription by determining gene promoter activity using reporter genes, where a reporter, such as luciferase or green fluorescent protein

(GFP), is placed next to and controlled by the promoter region of *StAR*. This could be achieved by treating cultured primary ovine Purkinje cells with TP and the addition of a reporter construct. The accumulation of the reporter gene mRNA is measured as an indicator of the promoter activity.

Furthermore, Caron *et al.*, (1997) concurred that cAMP induces *StAR* expression but that unlike many tissue specific and hormonally responsive genes, neither a canonical TATA box nor a consensus cAMP-responsive element (CRE) could be identified within *StAR*'s promoter region. They further suggested that steroidogenic factor 1 (SF-1) was implicated in the regulation of *StAR* expression in fetal mice. Manna, *et al.* (2003) also reported that although SF-1 is necessary, it is not sufficient for complete cAMP responsiveness. Using reporter gene assays, they determined that a functional cooperation between CRE binding protein (CREB), CRE modulator (CREM) and SF-1 was observed in mediating mouse *StAR* gene expression. Therefore, the TP-mediated *StAR* downregulation observed in the present study may be due to alterations in the mechanism of *StAR* gene regulation. To examine whether SF-1, CREM and/or CREB are involved in the regulation of TP-mediated *StAR* regulation, primary cell cultured cerebellar Purkinje cells from control and TP-treated samples could be used for promoter studies involving SF-1, CREM and CREB and utilised for luciferase activity studies. This would have allow us to determine if either of these regions on *StAR*'s promoter are also altered due to androgen over-exposure.

Pregnenolone is the main precursor of various steroid hormones metabolised from cholesterol by the action of P450<sub>scc</sub> (Lieberman *et al.*, 1984). Cholesterol is transported into the inner mitochondria by *StAR* as this is the site of steroidogenesis. To test whether the formation of pregnenolone is altered as a consequence of *StAR* downregulation, cholesterol and pregnenolone levels should be measured in GD 90 male ovine cerebella by utilising radioimmunoassay and comparing the levels of the control and TP-treated groups. This could be achieved by using frozen or fresh tissue samples however, due to limited samples in the present study, this investigation was not performed.

### 3.3.2.3 PR

*PR* mRNA expression was increased in fetal sheep cerebellum from TP-treated fetuses as compared to controls ( $p = 0.0341$ , Figure 3.11). The link between an increase in *PR* expression and an increase in testosterone and estrogen, but not DHT levels has been reported by others (Godwin *et al.*, 2000; Milgrom *et al.*, 1973). This suggests that testosterone's action is mediated by its aromatisation into estrogen and not into the reduction of the potent androgen, DHT. However it does appear that the level of *PR* mRNA expression and the activation/inactivation of this PR vary under different hormonal stimuli. For example, estradiol stimulates the synthesis of *PR*, and therefore *increases* its expression (Milgrom *et al.*, 1973), whereas *PR* also shows sensitivity to progestins, and evidence has illustrated that progesterone *decreases* *PR* expression in most target tissues (Leavitt *et al.*, 1977). Therefore, it could be that the high level of androgen administered to the samples is metabolised, via the aromatase pathway, into high levels of estradiol. This in turn may activate/increase *PR* mRNA expression. However, the current study has evaluated whether the increase observed for PR at the mRNA level was due to an androgenic and estrogenic effect. It was determined that testosterone is acting directly on PR expression, rather than indirectly through the actions of estrogen, as when compared to the control group, the TP-treated group results were significantly different whereas there was no change between the control and DES-treated groups results. On the other hand, the current study's results could imply that *PR* expression changes in the androgen over-exposed group may have been partially caused by a decrease in progesterone levels, which could have been initiated by a decrease in local steroidogenesis due to the downregulation of StAR. It would have been useful to measure the progesterone and DHP levels in the cerebellum, by utilising radioimmunoassay or luminescence immunoassay, to determine whether excess androgen exposure and the increase in *PR* gene expression is due to an alteration in PR ligand levels. Due to limited tissue availability, this was beyond the scope of our study. The current study has evaluated PR mRNA expression by using primers that were designed to specifically amplify an exon boundary that is located in both *PR-A* and *PR-B* gene products. Therefore it would be interesting to examine each isoform separately, by means of designing primer sets unique to each isoforms and utilising these sets in qRT-PCR, in order to determine



whether prenatal androgen over-exposure affects the transcriptional activation or the inhibition of progesterone responsive genes (PR-B is involved in the activation; PR-A is involved in the inhibition, (Tsutsui *et al.*, 2000). It would be expected that the inhibition and therefore PR-A would be altered as this isoform has been established to repress PR-B-mediated transcriptional activity (Merlino *et al.*, 2007).

The increase in *PR* mRNA expression that we see as a result of androgen over-exposure, may result in dysregulated cerebellar development. Progesterone, but not its metabolite allopregnenolone, is thought to act directly via PR on Purkinje cells through intranuclear receptor-mediated mechanisms to promote dendritic growth, spinogenesis and synaptogenesis in neonatal rats (Sakamoto *et al.*, 2003). Therefore, the examination of fetal ovine Purkinje cell morphology was performed in order to observe whether any structural alteration to the cerebellum after prenatal androgen over-exposure was apparent.

#### **3.3.2.3.1 *PR* regulation by estrogen**

Estradiol activates the receptor ER $\alpha$ , which drives the transcription of the PR gene directly by interaction with estrogen response elements (EREs) that exist within the promoter regions of the PR gene (Kraus *et al.*, 1997) . Previous studies have demonstrated that in several brain regions, within different species and age groups (medial preoptic nucleus and ventromedial nucleus in neonatal rats, Quadros and Wagner, 2008; periventricular preoptic area in male lizards, Godwin *et al.*, 2000; cerebellum in adult male rats, Guerra-Araiza *et al.*, 2002). show this regulation of PR gene expression Therefore, it could be assumed that this is the mechanism of PR regulation observed in GD 90 male ovine cerebella, which were over-exposed to androgens. However, the results suggest that this alteration is due to a direct androgen effect.

It is interesting that the present study does not confirm previous literature findings in that *PR* expression is regulated by estrogen however, this cannot be ruled out. Evaluating the inhibition the ERE site by activated ER in the *PR* gene in primary cell cultured cerebellar Purkinje cells from control and TP-treated samples would establish whether the ERE site within the PR promoter region is altered in some way that would affect *PR* gene regulation.

Investigating the activation of a PR promoter, such as ERE, would provide more insight into the mechanisms of gene expression regulation. A way to be able to determine this would be to indirectly measure *PR* gene transcription by determining gene promoter activity using reporter genes, where a reporter, such as luciferase or green fluorescent protein (GFP), is placed next to, and controlled by, the promoter regions of *StAR* and *PR* and the accumulation of the reporter gene mRNA is measured as an indicator of the promoter activity.

In the absence of hormone, steroid hormone receptors exist as inactive oligomeric complexes with a number of other proteins including chaperone proteins, namely the heat shock proteins Hsp90 and Hsp70, and cyclophilin-40 and p23 (Smith and Toft, 1993). The role of Hsp90 and other chaperones may be to maintain the receptors folded in an appropriate conformation to respond rapidly to hormonal signals. Therefore, examining Hsp90, Hsp70, cyclophilin-40 and p23 at the mRNA level, by utilising qRT-pCR, would allow us to determine whether these genes are altered due to excess prenatal androgen exposure. This would enable the identification of altered regulation of gene expression.

#### **3.3.2.4 Is the “TP effect” mediated by androgen or estrogen?**

There were no significant differences in gene expression, measured by qRT-PCR, for the GOIs examined between the control and DES-treated group (Figure 3.13). These experiments aimed to determine whether the observed alterations in mRNA expression are due to an androgenic or estrogenic effect. This was achieved by utilising fetally injected DES-treated samples and comparing the mRNA levels to that of the control samples. The data indicates that the mRNA expression alterations seen for *STAR*, *PR*, *SRD5a1* and *SRD5a2* in the TP-treated group, are due to an androgen effect rather than an estrogen one (Table 3.4). One important point to consider is that RNA extraction DES-treated cerebellar samples were performed by a novice Honour's student. Therefore it may be due to inconsistencies during RNA isolation that have lead to such hypervariance between mRNA levels amongst these particular samples.

### 3.3.2.5 Trends in gene expression

Trends towards alterations in *SRD5a1*, *SRD5a2* and also *ER $\alpha$*  mRNA expression were observed between the control and TP-treated groups. There was a trend towards an increase in *SRD5a1* expression in the TP-treated group ( $p=0.0770$ , Figure 3.5 A), when compared to the control group, whereas there appeared to be a decrease in *SRD5a2* expression ( $p=0.0986$ , Figure 3.5 B). There was also a strong trend towards decreased *ER $\alpha$*  mRNA expression in the developing male cerebellum in the TP-treatment group ( $p=0.0721$ , Figure 3.12 A). The trend observed in the current study may suggest that *ER $\alpha$*  levels are downregulated due to an increase in ligand levels, namely  $17\beta$ -estradiol, which is synthesised by the aromatisation of testosterone. This could be evaluated by measuring  $17\beta$ -estradiol concentrations by means of radioimmunoassay, but was not performed in the current study due to time constraints. However, *ER $\alpha$*  expression is unaltered in DES-treated GD 90 male ovine cerebella ( $p=0.8307$ ) which suggests that ligand levels are not the reason for the trend observed for *ER $\alpha$*  mRNA expression in TP-treated cerebella.

In general, it is acknowledged that sample numbers are low in the current study, which may lead to under powering of the analyses, but unfortunately such matters were out with the control of the researcher, since these were samples which were a by-product from a larger study carried out and funded by collaborators at The University of Edinburgh.

### 3.3.2.6 No change in gene expression

The other steroidogenic enzymes and hormone receptors examined in this study were unaltered in the TP-treated group, when compared to the control group. There was no significant difference in *CYP11A1* gene expression between the control and TP-treated groups ( $p=0.1194$ , Figure 3.6). This suggests that during this specific stage of development, *CYP11A1* mRNA expression is unaffected by excess testosterone levels. There were also no alterations at the mRNA level for the *CYP17*, *CYP19* and *HSD $\beta$ 3* ( $p=0.1102$ , Figure 3.7,  $p=0.1772$ , Figure 3.8 and  $p=0.5111$ , Figure 3.9, respectively), and AR and *ER $\beta$*  ( $p=0.2604$ , Figure 3.10 and  $p=0.3435$ , Figure 3.12 B, respectively). However, previous literature examining a different developmental time point, animal model, brain region or tissue has suggested that testosterone can alter gene expression for these GOIs.

### 3.3.2.6.1 Time point

Ovine mid-gestation is a period of neuronal proliferation, migration, and axonal and dendritic growth and is therefore a vulnerable time for developmental damage. In particular, the cerebellum has a growth spurt at GD 90 (Rees, *et al.*, 1997), which is coincident with the current study. Indeed, McIntosh *et al.*, (1979) suggested that the main growth period for the ovine brain is between GD 95 – 130, which is just after sample collection for the current study (GD 90). The literature has illustrated that androgen can regulate certain genes (*CYP11A1*, *AR*, *ER $\alpha$*  and *ER $\beta$* ) and at various time points from the early post natal period through to adulthood (Sanchez *et al.*, 2006; Luo *et al.*, 2004; Karolczak *et al.*, 1998; Burgess and Handa, 1993; Wu and Gore, 2010; Sharma and Thakur, 2006). Therefore, animals studied in the previous literature are more developed than the time point chosen for the current study (GD 90 in sheep), which is just when a major cerebellar growth period occurs. Investigating these GOIs towards the end of the main growth period suggested by McIntosh *et al.*, (1979, *i.e.* GD 130) in development may have allowed us to determine whether gene expression remains sensitive to prenatal excess androgen exposure later in gestation and allow the results to be comparable to those in the previous literature. Unfortunately, all samples that were collected at a later gestational period were maternally injected with vehicle or TP, as opposed to the fetally injected samples which were used for the entire study.

### **3.3.2.6.2 Determining animal model species or anatomical region differences**

The data from this study suggests that the developing cerebellum has a different steroidal regulatory system compared to the hypothalamus and pituitary gland. Previous literature has shown that testosterone treatment can affect mRNA levels of the genes investigated in this chapter, but in brain regions (and Leydig cells) which are known to play more pivotal roles in the regulation of peripheral steroid synthesis (Karolczak *et al.*, 1998).

(Sanchez *et al.*, 2006; Luo *et al.*, 2004; Karolczak *et al.*, 1998; Burgess and Handa, 1993; Wu and Gore, 2010; Sharma and Thakur, 2006) performed their studies on rodents whereas the current study was examining sheep. These studies also examined different brain areas from the cerebellum, such as the hypothalamus, pituitary, cortex, medial preoptic nucleus and anteroventral periventricular nucleus.

Examining mRNA levels of the hypothalamus and pituitary, which are more heavily involved in peripheral steroid hormone regulation of GD 90 male sheep would allow us to determine whether it is a species or anatomical variation. The data from the current study implies that the level of these steroidogenic enzymes developing male ovine cerebellum is not altered when testosterone levels are elevated.

### 3.3.3 Western blotting

Western blotting of 50µg total protein isolated from TRIsure fractions left over from total RNA extraction was utilised to attempt to measure the relative protein abundance for gene products of interest in each control and TP-treated sample. The method of protein extraction was necessary due to a small sample size. The positive control, β-actin, which is highly abundant in the cerebellum, showed strong protein bands on each immunoblot. This study has demonstrated that StAR and 5α-reductase 2 protein is expressed in the developing male ovine cerebellum. However, further into the study it transpired that poor protein quality in fetal cerebellar tissue and limited sample volume prevented reliable Western blotting detection of StAR, 5α-reductase and PR.

#### 3.3.3.1 Troubleshooting Western blot difficulties

Hummon *et al.*, (2007) demonstrated that the phenol/ethanol supernatant from Trizol RNA isolation could be stored at -20°C for upto three years and successful protein dialysis, and subsequent Western blotting, could be achieved *i.e.* the protein would not be degraded. In this current study, the first phenol/ethanol supernatants were dialysed approximately three months after RNA isolation and storage of the supernatants. The protein did not appear degraded three months after RNA extraction. However after twelve months of storage at -20°C, the protein sample appeared to be smeared and have very low molecular weights. This shows that the protocols in the Hummon, *et al.* (2007) study were incompatible with this study.

Another confounding factor is that there is low expression of these target proteins in the developing cerebellum. We know that these genes are expressed in the fetal cerebellum at the mRNA level from qRT-PCR analysis (Table 3.2) and the immunohistochemistry data show that they are also expressed at the protein level (Figures 3.25-28), therefore we cannot suggest the proteins probed for in Western blotting are not expressed in the developing cerebellum. This was attempted to be rectified by taking half of the protein from each sample and concentrating the protein using centrifugal in concentrator tubes. However, this resulted in faint, ambiguous bands or none at all.

A positive tissue control, the lamb adrenal gland with known high levels of steroid protein expression (Nussey and Whitehead, 2001), was used to attempt

to determine what factor had prevented the quantifying of the proteins of interest. Protein was extracted from the adrenal gland by using the method described in Chapter 2 and then Western blotting was performed. Further investigation of the antibodies, by using lamb adrenal gland and utilising immunohistochemistry on cerebellar tissue demonstrated that these antibodies do work for ovine tissue and that the antibodies had not degraded over twelve months. In conclusion, the transfer of protein of SDS-PAGE gel to nitrocellulose membrane and the antibodies used were successful during this part of the study. Therefore it can be deduced that the problems with Western blotting described in this chapter, were due to low abundance in the fetal ovine cerebellum and protein degradation caused by the protein extraction method and/or the storage of protein at  $-20^{\circ}\text{C}$ .

### **3.3.4 Immunohistochemistry**

#### **3.3.4.1 Optimisation studies**

##### **3.3.4.1.1 Investigation of cell migration in the developing cerebellum**

The haematoxylin and eosin (H&E) stain helps to determine various tissue types as it reveals a broad range of cytoplasmic, nuclear and extracellular matrix characteristics (Fischer *et al.*, 2008). Haematoxylin has a deep blue-purple color and stains nucleic acids whereas eosin is pink and stains proteins nonspecifically. In a typical tissue, such as the cerebellum, nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining. Nucleoli stain with eosin. If abundant polyribosomes are present, the cytoplasm will have a distinct blue cast. The Golgi zone can be tentatively identified by the absence of staining in a region next to the nucleus. Therefore this stain reveals abundant structural information (Fischer *et al.*, 2008) and can help identify the different layers of the cerebellum.

The main cell types, such as the Purkinje cells, of the cerebellum appear at different times during development and at different locations. Purkinje cells were identified through their large nuclei and cytoplasm in comparison with the other cells in the cerebellum (Salouci *et al.*, 2012). Figures 3.19 A and C show H&E staining on GD 90 cerebellar tissue, at different magnifications (A, X10 magnification and C, X20 magnification). It is observed that there are a number of granule cells (stained purple) in the molecular layer (ML). It is known that granules cells migrate from the external granule layer (EGL) to the internal granule layer (IGL) during development (Zhao *et al.*, 2007). The pink cells stained in the Purkinje cell layer (PCL) appear to be Purkinje cells due to their large soma appearance. Using a Purkinje cell marker, such as calbindin, we can confirm that these are indeed Purkinje cells. It is likely that at this developmental time point, the PCL is in the initial stages of establishment. This is supported by the findings of Rees and Harding (1988), who demonstrated that immature Purkinje cells begin to appear in the PCL at approximately GD 80 of fetal sheep. In contrast, Salouci *et al.*, (2012) saw no sign of Purkinje cell development until GD 105 in fetal ovine cerebellar tissue where the cells were small, arranged in several rows and had an irregular shape. However these fetuses had been spontaneously aborted and although no malformations to the brains were found, the examination of the brains was not discussed extensively



nor compared to that of a typical brain. This suggests that the gross anatomy of the brain was unaffected by abortion. The fetal ovine samples in our study were all subjected to the same method of sacrifice (barbiturate injection), therefore a uniform manner of death for every sample in our study, and were tightly monitored up until then. The differences in the way in which the samples were obtained (spontaneous abortion in Salouci *et al.*, (2012) and controlled sacrifice via barbiturate injection in our study), is possibly why we are seeing differences in Purkinje cell migration and development.

To consolidate the theory that the cerebellum is still developing at GD90, cerebellar tissue from GD 112 samples were stained. Figures 3.19 B and D show H&E staining on GD 112 ovine cerebellar tissue, at different magnifications (B, X10 magnification, D, X20 magnification). In these figures, there are fewer granule cells in the ML and the Purkinje cells are more apparent and in the PCL. This gestation timing *does* reflect the findings from Salouci, *et al.* (2012), who showed similar ovine Purkinje cell development at GD 112. Therefore our study confirms that the cerebellum does develop over a long time, which was also demonstrated by Petratos *et al.*, (2000).

There has not been extensive research on ovine cerebellar development. We do know that at GD 90, the fetal ovine cerebellum is immature, which is similar in other species in which neurogenesis and neuronal growth occurs later than in other brain regions (Jacobson, 1997). Neurogenesis in the external granular layer is yet to reach its peak, as judged by the thickness of the layer in sheep (Rees and Harding, 1988). However, a cerebellar growth spurt begins after GD 90 where neuronal proliferation, migration and axonal and dendritic growth (Rees and Harding, 1988) all occur and therefore is a vulnerable period for developmental damage. This is relevant to our study we are investigating whether there are structural alterations in the cerebellum at this gestational period in fetal tissue that has been exposed to TP.

#### **3.3.4.1.2 Exploring optimal tissue thicknesses for Nissl staining studies**

Nissl staining is used to investigate neurons and also to understand the cytoarchitecture of different brain areas. The Nissl-staining method is based on the interaction of a basic dye, such as cresyl violet, with the nucleic acid content of cells (Pilati *et al.*, 2008). These dyes can bind to the DNA content of the cell nuclei, but also to the RNA that is highly concentrated in rough endoplasmic reticulum and ribosomes in the cytoplasm. Neurons are very active protein synthesizing cells and so the cytoplasm of these cells contains a high concentration of rough endoplasmic reticulum (Kosik and Krichevsky, 2002). Due to this special characteristic of neurons, the Nissl staining can specifically stain the cytoplasm of neurons. Nissl staining was subsequently used to measure and calculate EGL, ML and IGL widths and the results from the control and TP- treated group were compared, to determine if there is a difference in the migration of granule cells as a result of androgen exposure.

Some papers suggest that the thickness of each section should be increased up to 50µm for Nissl staining (Pilati *et al.*, 2008). Therefore we investigated what thickness was best for this study. Figure 3.20 A shows ovine cerebellar tissue at GD 90 at a section thickness of 15µm. At this thickness, it was difficult to see single cells and so it would be near impossible to determine cell number by counting. Figure 3.20 B shows ovine cerebellar tissue at GD 112 at a thickness of 5µm, which is the thickness used for H&E staining and immunohistochemistry. Here we can observe clear cellular morphology, determine the different cerebellar cell types, and counting is more plausible. The difference in results between our study and that of Pilati *et al.*, (2008) may be due to differences in methodology e.g. we used a microtome to prepare our sections, whereas Pilati *et al.*, (2008) utilised a vibratome. As a result of this, for subsequent experiments we used tissue sections of 5µm for all histological stains.

The current study has evaluated H&E and Nissl staining in fetal ovine cerebellar tissue. Although Nissl staining is a special stain specifically for identifying neurons, H&E allowed for a clearer histological method to distinguish all cells that comprise the cerebellar structure.

### **3.3.4.1.3 Colocalisation of AR and calbindin in the cerebellum**

#### **3.3.4.1.3.1 Examining colocalisation by means of simultaneous immunofluorescence**

We examined the colocalisation of AR and calbindin in the developing male cerebellum. We chose these proteins as we have previously shown that AR is located in the nuclei of Purkinje cells and calbindin is a known Purkinje cell marker (Dougherty *et al.*, 2013). By using sequential immunohistochemistry, we have shown that AR and calbindin colocalise in Purkinje cells of the developing cerebellum. The red nuclear staining show AR protein expression while the brown cytoplasmic staining demonstrates calbindin protein expression.

Calbindin is a good neuronal cell marker and Figure 3.23 confirms that the positive calbindin staining is exclusive in the Purkinje cells of the cerebellum (green denotes positive staining). It is difficult to say whether there is any positive nuclear AR staining (Figure 3.23, in red) or if our results show only background (*i.e.* non-specific) staining. There does appear to be signs of more intense nuclear staining within the PCL which corresponds with the positive cytoplasmic calbindin staining. However, we cannot assume there *is* colocalisation of calbindin and AR from the simultaneous immunofluorescence method.

This technique has been noted to be time-consuming and prone to background staining (Mason *et al.*, 2000). Furthermore, simultaneous immunofluorescence can be unsuitable in detecting two antigens present at the same site, such as two markers at the cell surface, since one label tends to obscure the other (Valnes and Brandtzaeg, 1984). As a consequence of this, simultaneous immunofluorescence for the study of paraffin-embedded tissues has been largely restricted to the detection of pairs of antigens found either at different sites within a single cell (*e.g.* nucleus and cell surface) or in different cell populations (Mason *et al.*, 2000). We chose this method to examine the colocalisation of calbindin and AR in the developing cerebellum due to the different compartments these protein are expressed in (calbindin is localised in the cytoplasm; AR is localised in the nuclei). The two antibodies we had access to were from different host species and therefore, in theory, this technique should have allowed us to visualise the colocalisation as the antibodies should

not cross react (calbindin was a monoclonal antibody; AR was a polyclonal antibody raised in rabbit).

Reliable double immunofluorescence labeling for confocal microscopy requires that the fluorochromes used exhibit good spectral separation (Kumar *et al.*, 1999). In our study, we have used fluorescently-labelled StreptAvidin 594 (Vector Laboratories, U.K.) and Alexafluor goat anti-mouse 488 (Invitrogen, U.K.) which excites the red and green wavelengths, respectively, and therefore we should not have any ambiguity in regards to what protein is fluorescing at a particular wavelength. Alexa 488 has been shown to be more photostable than fluorescein for conventional immunofluorescence (Kumar *et al.*, 1999) and our results demonstrated that Alexa 488 is a suitable secondary antibody to use within our study.

Streptavidin-based amplification is widely used in fluorescent imaging to improve detection sensitivity. Fluorescent conjugates of streptavidin are used to detect biotinylated biomolecules such as primary and secondary antibodies. Streptavidin exhibits low levels of nonspecific binding. Mammalian cells and tissues contain biotin-dependent carboxylases, which are required for a variety of metabolic functions (Holmberg *et al.*, 2005). These enzymes often produce substantial background signals when biotin–streptavidin or biotin–avidin detection systems are used to identify cellular targets. Endogenous biotin is particularly prevalent in mitochondria and in brain tissues therefore we used a biotin block to quench all endogenous biotin within a tissue section.

In our optimisation studies for simultaneous immunofluorescence, we observed that the antibody dilutions that were most suitable were of a higher concentration than what was required for standard immunohistochemistry, which implies that immunofluorescence is less sensitive. Therefore this may be why we are unable to definitively say whether we are seeing AR nuclear localisation, by means of immunofluorescence, in our study.

#### **3.3.4.1.3.2 Determining colocalisation of AR and calbindin by using sequential immunohistochemistry**

The current study has observed that AR and calbindin colocalise in Purkinje cells of the developing cerebellum (Figure 3.24) by utilising sequential immunohistochemistry. The red nuclear staining show AR protein expression while the brown cytoplasmic staining demonstrates calbindin protein expression.

(Van der Loos, 1999) suggested that this red-brown colour combination was only useful in demonstrating two different cell populations or constituents without any overlap and that colocalisation studies was not possible due to a lack of the visual colour distinctions. If the colours chosen for sequential immunohistochemistry, can be unambiguously differentiated, a technique known as spectral imaging can be applied (Van der Loos, 2008). Spectral imaging allows images of the single stains to be scanned and by using specialised software algorithms the colours are unmixed, displaying the distribution and abundance of the individual chromogens *i.e.* DAB's brown staining and NovaRED's red appearance. However we had no access to the software needed to do this.

As per manufacturer's instructions (A Guide to Multiple Antigen Labelling Handbook, Vector Laboratories, 2005, page 7), the order of the labelling of the primary antibodies is crucial and may significantly affect the quality of labelling. They suggest that if NovaRED is applied to the first primary antibody and DAB applied to the second, then the distinct red and brown colours should be visible after the labelling reactions are completed, using an optimised multiple labeling protocol. We confirm that this method of labelling was successful on fetal ovine cerebellar tissue (Figure 3.24). During our optimisation studies, we examined the result of applying DAB substrate to the first primary antibody and NovaRED to the second, which should not show as distinct colour as the method discussed above. We can also confirm that we were unable to pinpoint the localisation of the brown and red labelling due to the quality and ultimately labelling pattern (data not shown).

#### **3.3.4.2 Cerebellar cells that express the proteins of interest**

The cerebellum has a clear developmental pathway and an established cytoarchitecture that has enabled the identification of the effects of TP treatment on the development of this area of the brain.

Although this study has identified that the proteins of interests are located in Purkinje cells, it was difficult to determine the exact subcellular location of StAR and 5 $\alpha$ -reductase 1 and 2. Due to the limitations of low magnification, this study could not identify mitochondria (the location for StAR protein expression) or the microsomal membrane (the location for 5 $\alpha$ -reductase 1 and 2 protein expression) specifically. This could have been rectified by using a double staining technique, where a mitochondrion, cell or microsomal membrane marker was used as well as the antibody for the particular protein of interest. This would help determine if the protein of interest is indeed located within the mitochondria or microsomal membrane. Electron microscopy would also have given a higher resolution to the precise location of the protein of interest. This study could identify that the subcellular location for PR protein was in the nuclei or perinuclei of Purkinje cells.

Purkinje cells of the cerebellum are the major site for local steroidogenesis and express StAR, 5 $\alpha$ -reductase 1, 5 $\alpha$ -reductase 2 and PR protein (Petratos *et al.*, 2000; Sakomoto *et al.*, 2001). By means of immunohistochemical analysis, this study has determined that the number of Purkinje cells that express these proteins of interest has significantly increased as a result of prenatal excess androgen exposure in the developing male cerebellum.

*SRD5a1* and *SRD5a2* mRNA levels were not significantly altered in the TP-treated group but each gene showed a trend towards an increase, or a decrease (*SRD5a1* and *SRD5a2*, respectively). However, there is an increase in the number of Purkinje cells that express these proteins. A possible explanation for this is that each Purkinje cell is expressing an altered amount of protein in the TP-treated samples, when compared to the control samples. Furthermore, qRT-PCR determined the mRNA levels of one hemisphere of the developing male cerebellum, and therefore all the cells that comprise this tissue, whereas the immunohistochemical analysis examined only the Purkinje cell count in part of the other hemisphere. Therefore, it is possible that mRNA levels

in the whole tissue decline where protein in one cell type increases and vice versa. To determine whether this was case, single cell PCR could be performed. Cerebellar Purkinje cells, from the control and TP-treated groups, could be isolated using laser capture and then DNA extraction could be performed using cell lysis. The appropriate primers sets for each GOI could be designed and then used for qRT-PCR, to measure expression levels.

This increase in the number of Purkinje cells expressing all the proteins of interest could be due to an increase in overall Purkinje cell number as a consequence of androgen exposure during the developmental phase. Therefore the effect of treatment may be directly on Purkinje cell number. Whether these cells are producing more or less of each protein remains unclear and further investigation, such as utilising laser capture microdissection to obtain Purkinje cells and then measuring gene expression levels using qRT-PCR, would have to take place for a conclusive answer. Due to time constraints, this could not be examined in this study. A more detailed examination of Purkinje cell number and size would assess if prenatal androgen over-exposure has altered the cytoarchitecture of the cerebellum.

### 3.4 Conclusions

This study has demonstrated that the number of positive Purkinje cells expressing the proteins of interest had greatly increased as a result of androgen exposure. It has been observed that there is decreased mRNA expression of *STAR* (Figure 3.4,  $p = 0.0264$ ) and increased mRNA expression of *PR* (Figure 3.11,  $p = 0.0341$ ) in the TP-treated group. Immunohistochemical analysis of *StAR*, *PR*,  $5\alpha$ -reductase 1 and  $5\alpha$ -reductase 2 revealed increases in the number of Purkinje cells that express these proteins (Figures 3.25-28,  $p = 0.0002$ ,  $0.0201$ ,  $0.0001$  and  $0.0001$ , respectively) in the TP-treated group. Western blot analysis should have allowed for an examination of whether the amount of protein expression had been altered due to excess androgen exposure, and would have given a better understanding of the relationship between mRNA and protein expression from the same sample, and ultimately cerebellar hemisphere. It was determined that the problems that were encountered in the current study could be very much due to sub-optimal protein quality in the developing male cerebellum protein samples. Little is currently understood regarding the relationship between gene expression as determined by measuring mRNA levels and the corresponding abundance of the protein products. So in conclusion, this study has ascertained possible structural effects, by means of immunohistochemical analysis, as well as alterations in gene expression for *StAR* and *PR*, by means of qRT-PCR, of developmental hormone treatment on the male cerebellum.



## **CHAPTER 4**

### **Alterations in the developing ovine cerebellar cytoarchitecture after prenatal androgen exposure**

## **4. Alterations in the developing ovine cerebellar cytoarchitecture after prenatal androgen exposure**

### **4.1 Aims and Objectives**

#### **4.1.1 Aim 1 and objectives**

To identify whether a panel of genes associated with cell markers and neuronal activity are expressed in GD 90 male sheep cerebella and whether mRNA expression levels are altered due to androgen over-exposure.

Numerous genes, and therefore the functional proteins they encode, are key to the development of the cerebellum but the role of androgen in cerebellar development are unknown. The hypothesis is that the cell marker and neuronal activity-associated genes, specifically chosen for this study, are expressed in the cerebellum of male fetal sheep at GD 90. It was further hypothesised that prenatal excess androgen ( 2x20mg depot injections at GD 62 and GD 82) alters cerebellar steroid-associated gene expression in GD 90 male sheep as measured by qRT-PCR.

#### **4.1.2 Aim 2 and objectives**

To detect any alterations in cerebellar granule cell migration, after prenatal androgen exposure in GD 90 male sheep.

This study wanted to confirm if androgen exposure has any effect on granule cell migration in the cerebellum. Nissl staining was utilised to determine if there is a difference in the width of several cerebellar layers (EGL, ML, IGL) between the control and TP-treated groups. This would establish whether androgen exposure has any effect on granule cell migration in the cerebellum.

#### **4.1.3 Aim 3 and objectives**

To ascertain whether Purkinje cell morphology and distribution is altered in GD 90 male sheep after TP-treatment.

The observation made by immunohistochemical analysis in Chapter 3 was that the Purkinje cell layer appeared different in our TP-treated group samples compared to the control group. Therefore the investigation was expanded to examine specific cell markers and genes involved in neuronal differentiation,

using qRT-PCR and histology. This study further explored the developing male cerebellar cytoarchitecture by means of immunohistochemistry.

#### **4.1.3.1 Purkinje cell number**

In order to determine whether any observed changes in Purkinje cell number were due to an androgenic or estrogenic effect, DES-treated samples were used for immunohistochemical purposes and compared to the control treated samples, using calbindin. The examination of whether there was a difference in the number of Purkinje cells expressing calbindin (a Purkinje cell marker) between the control and TP-treated groups was evaluated using immunohistochemistry.

#### **4.1.3.2 Purkinje cell morphology**

The examination of whether there was a difference in the number, shape, formation and arrangement of Purkinje cells expressing calbindin (a Purkinje cell marker) between the control and TP-treated groups was also undertaken by utilising immunohistochemistry. The Purkinje cell soma were measured to determine if there was a difference in size between the control and TP-treated groups. We also investigated the PCL arrangement and compared between the control and TP-treated groups, to examine if there are any alterations in PCL width of the developing cerebellum. The hypothesis is that Purkinje cell development is altered after excess androgen exposure.

#### **4.1.3.3 Apoptosis and proliferation**

We examined whether there was any changes to apoptosis and proliferation between the control and TP-treated groups, using cleaved caspase 3 as an apoptosis marker and Ki-67 as a proliferation marker. The current study aims to provide insight into whether either of these two cell regulation mechanisms in the cerebellum is affected after prenatal androgen over-exposure.

#### **4.1.3.4 Procedure for measuring Purkinje cell morphology**

Each slide was examined under a light microscope (Leitz Laborlux S, Germany) using the X10, X20 and X40 objectives. The number of positive cells was counted for each slide using a grid eyepiece in five random fields per slide. The sections were digitally captured, using CellSens 1.9 software (Olympus, U.S.A.). Using Image J software (N.I.H., U.S.A.), the images were then able to be measured and in each of the five random areas, five random sections were chosen and the unit width of the PCL was measured. The Purkinje cell

diameters were normalised to the corresponding unit width. The mean of the five measurements was calculated. Five Purkinje cell soma were also randomly chosen and measured in each field view.

The mean of the five grids was calculated and then multiplied by 3.57 to convert the mean into positive cells/mm<sup>2</sup>. The value of each grid of positive cells was 0.28mm<sup>2</sup> and by multiplying each grid by 3.57 converts the mean into mm<sup>2</sup>. This analysis determined whether androgen exposure has altered the number of Purkinje cells, and therefore the cytoarchitecture of the developing cerebellum.

## 4.2 Results

### 4.2.1 Gene analysis

The level of gene expression for each GOI was evaluated using qRT-PCR analysis via the  $\Delta\Delta C_T$  method. The fold difference relative to a single reference (control) sample was then calculated by  $2^{\Delta\Delta C_T*(-1)}$ . Relative gene expression from the male control group and the male TP-treated group were compared to determine differences in gene expression, if any, using a two tailed, unpaired t test (GraphPad Prism software, U.S.A.).

The GOIs were chosen because they have already been established as either neuronal activity or cell markers (Table 4.1).

Gene Name	Classical Name	Reason for being GOI	Reference
<i>BDNF</i>	Brain-derived neurotrophic factor	Modulated by steroids, associated with disease	Numakawa (2010)
<i>FOS</i>	Immediate early gene	Marker for neuronal activity in neuroendocrine systems	Hoffman <i>et al.</i> , (1993)
<i>GABRA6</i>	Gamma-aminobutyric acid A receptor, alpha 6	Altered in autism, target for schizophrenia treatment and granule cell marker	Dean <i>et al.</i> , (2005); Fatemi <i>et al.</i> , (2010)
<i>GAD1</i>	Glutamate decarboxylase 1	Enzyme required for GABA synthesis. GABAergic Purkinje neuron marker.	Westmoreland <i>et al.</i> , (2001)
<i>GFAP</i>	Glial fibrillary acidic protein	Microglial marker	Eng <i>et al.</i> , (2000)

Table 4.1: A list of the genes of interest that were examined. The primary focus of this study was to identify changes in the cerebellar expression of neuronal activity and cell markers that result from exposure to testosterone propionate (TP) during development in sheep.

All cell and neuronal activity genes examined were found to be expressed in GD 90 male cerebellum (Table 4.2).

Gene Name	Previous Evidence For Cerebellar Expression	Reference	Cerebellar Expression In This Study's Sheep Model
<i>BDNF</i>	Fetal sheep	Duncan <i>et al.</i> , (2004)	Yes
<i>FOS</i>	Adult rat	Chen <i>et al.</i> , (2005)	Yes
<i>GABRA6</i>	Neonatal rat	Bovolin <i>et al.</i> , (1995)	Yes
<i>GAD1</i>	Neonatal rat	Chan-Palay <i>et al.</i> , (1981)	Yes
<i>GFAP</i>	Fetal sheep	Mallard <i>et al.</i> , (1998)	Yes

Table 4.2: This table depicts whether mRNA expression for each GOI has been identified in the literature. In the majority of literature, cerebellar gene expression studies have been carried out on neonatal or adult rats. Here, it was ascertained that all GOI are expressed in the male fetal sheep cerebellum.

#### **4.2.1.1 The effect of excess fetal androgen on the mRNA expression of genes associated with cell and neuronal activity in the male cerebellum**

The data indicates that there was no change at the mRNA level for the genes of interest that are cell markers or involved in neuronal activity.

*BDNF* is a critical neurotrophin that regulates cell differentiation, cell survival, neurotransmission, and synaptic plasticity in the CNS (Numakawa, 2010). The data from this present study indicates that there was no change in *BDNF* expression in the cerebellum of TP-treated samples when compared to the controls ( $p=0.3475$ , Figure 4.1 A).

*FOS* is a marker of neuronal activation in the cerebellum (Chen *et al.*, 2005). It was hypothesised that *FOS* mRNA expression in the TP-treated group would be altered. However, there was no change in expression between the control and TP-treated group ( $p=0.2603$ , Figure 4.1 B).

Bovolin *et al.*, (1992) demonstrated that *GABRA6* was abundant in granule cells of neonatal rats. The current study determined that there was no significant change in *GABRA6* expression between the two groups ( $p=0.8058$ , Figure 4.1 C).

*GAD1* is expressed abundantly in Purkinje cells of the cerebellum (Chan-Palay *et al.*, 1981). There was no change in *GAD1* mRNA expression in this study, as a result of TP treatment ( $p=0.3315$ , Figure 4.1 D).

*GFAP* is a known glial marker (Westmoreland *et al.*, 2001). The results from this current study suggest a non-significant trend towards an increase of *GFAP* expression in the TP-treated males in the developing cerebellum ( $p=0.0641$ , Figure 4.1 E).

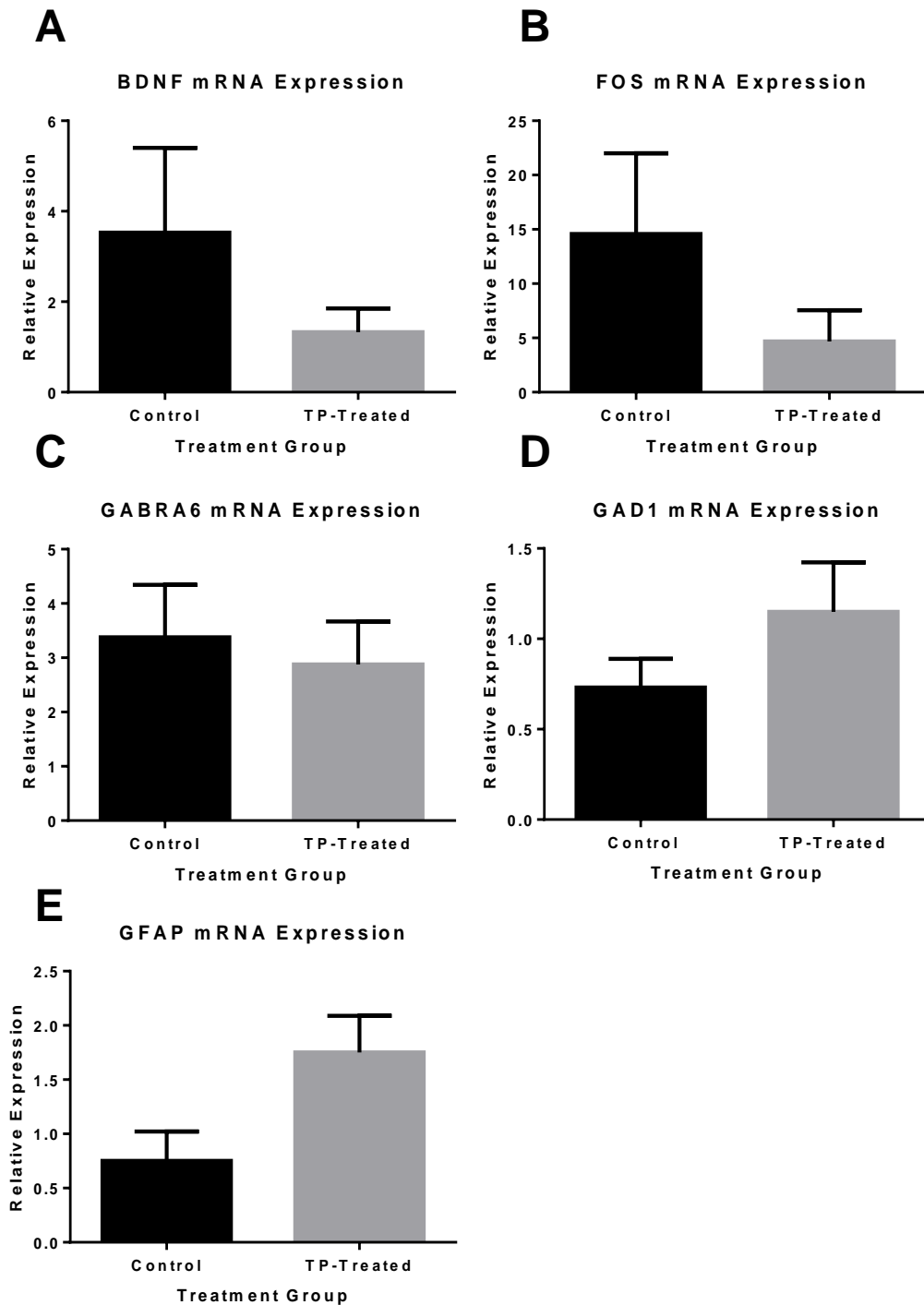


Figure 4.1: qRT-PCR analysis has shown that there are no differences at the mRNA expression level of cell and neuronal activity marker in cerebellar tissue of GD90 male sheep,  $n=4$ . A) BDNF mRNA expression for control and TP-treated samples,  $p = 0.3475$ , a 2.66-fold reduction in the TP-treated group was calculated B) Fos mRNA expression for control and TP-treated samples,  $p = 0.2603$ , a 3.06-fold reduction in the TP-treated group was calculated C) GABRA6 mRNA expression for control and TP-treated samples,  $p = 0.8058$ , a 3.96-fold reduction in the TP-treated group was calculated D) GAD1 mRNA expression for control and TP-treated samples,  $p = 0.3315$  E) GFAP mRNA expression for control and TP-treated samples,  $p = 0.0641$ .



#### 4.2.2 Examination of the developing cerebellar layer widths after androgen exposure

The EGL, ML and IGL cerebellar layers were identified by comparing the slides from the current study with data from Kirsch *et al.* (2012), Pilati *et al.*, (2008) and the Allen Brain Atlas. The widths of the cerebellar layers in GD90 male sheep that were either exposed or not to excess androgen (TP-treated and control group, respectively) were measured and compared to determine whether androgen exposure affected layer width. Figure 4.2 shows that there was no change in EGL, ML or IGL layer width between the control and TP-treated groups ( $p = 0.4042, 0.9293$  and  $0.3727$ , respectively)

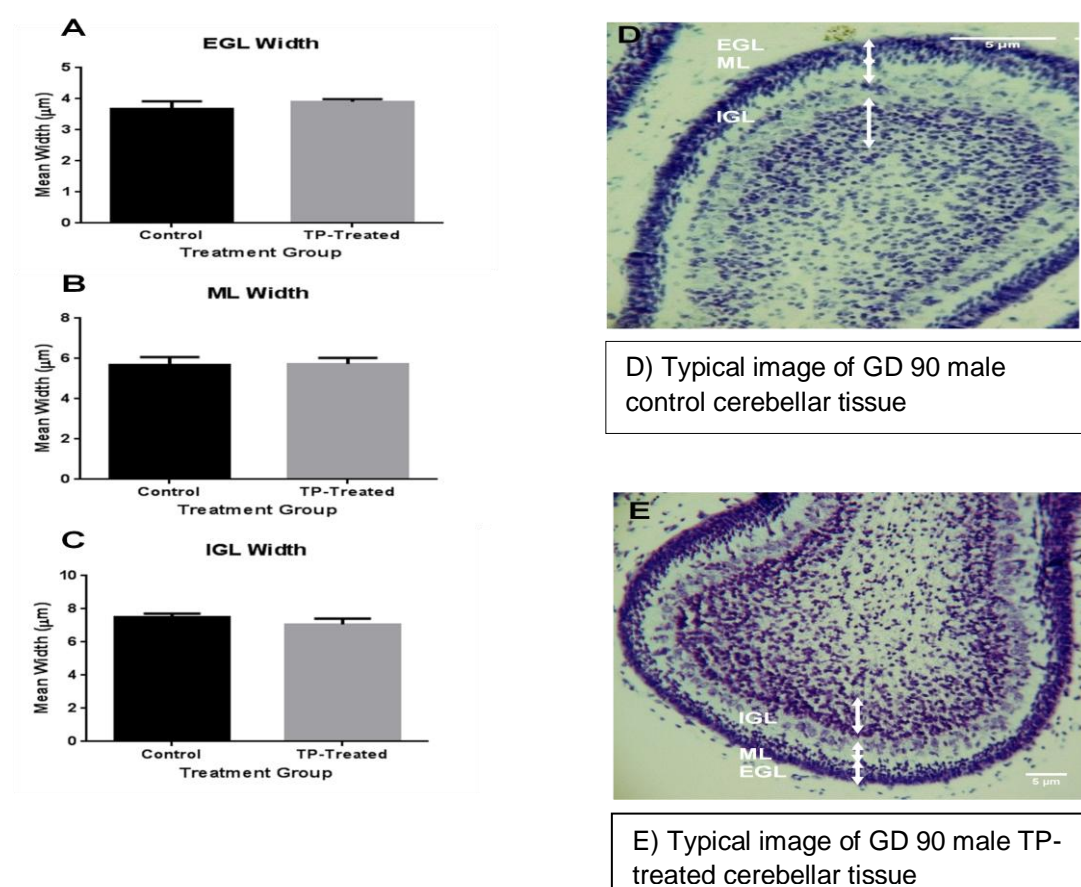
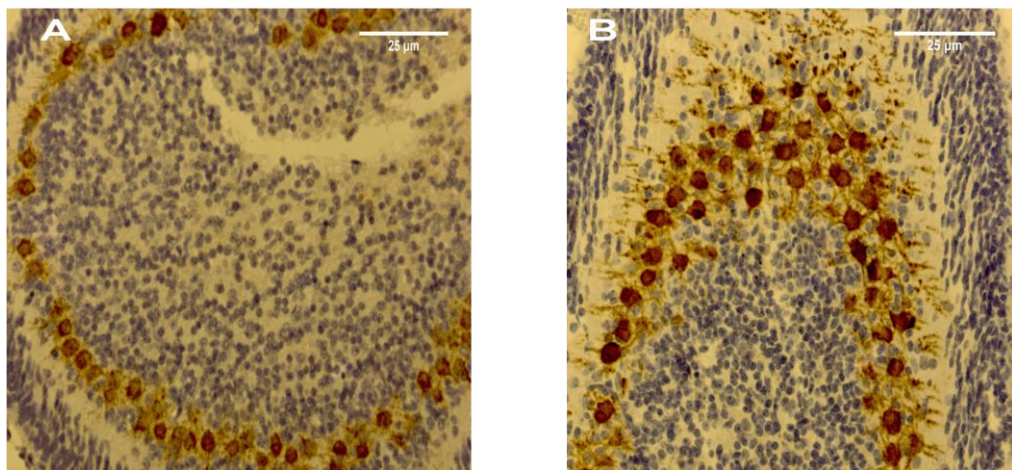


Figure 4.2: Using Nissl staining, the width of each cerebellar layer of GD90 male sheep were measured using Image J software. Using an unpaired t-test, these measurements were used to investigate if there were any differences in layer width between the control and TP-treated groups ( $n=4$ ). A) mean width of EGL for the control and TP-treated groups,  $p = 0.4042$  B) mean width of ML for the control and TP-treated groups,  $p = 0.9293$  C) mean width of IGL for the control and TP-treated groups,  $p = 0.3727$  D) a representative image of a Nissl stained section GD90 control sample at X20 magnification E) a typical image of a GD90 TP-treated sample of a Nissl stained section. The arrows on panels D and E represent how each cerebellar layer was measured.

## 4.2.3 Observation of Purkinje cells, using immunohistochemistry

### 4.2.3.1 The number of Purkinje cells increases after androgen over-exposure

Immunohistochemistry was performed to investigate any Purkinje cell alterations that arose from androgen over-exposure, using calbindin as a Purkinje cell marker. The number of calbindin positive cells were counted using a grid eyepiece at five random areas of each cerebellar sections (GD90 male sheep). The mean was calculated and then converted into positive cells/mm<sup>2</sup>. Figures 4.3 A and B show typical images for the immunohistochemical analysis, using anti-calbindin, for the control and TP-treated groups. This Purkinje cell marker showed an increase in positive Purkinje cells/mm<sup>2</sup> ( $p = 0.0008$ , Figure 4.3) in the TP-treated group.



A) A typical representative of GD90 control cerebellar ovine tissue

B) A typical representative of GD90 TP-treated cerebellar ovine tissue

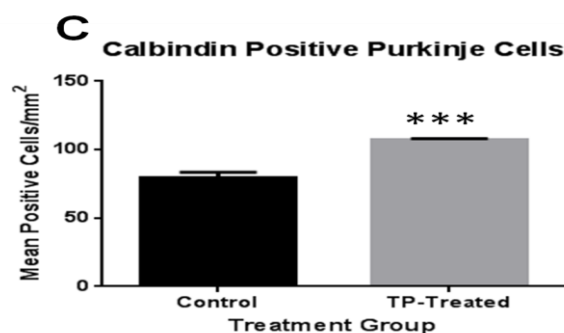


Figure 4.3: Immunohistochemistry was performed to investigate any Purkinje cell alterations as a result of androgen over-exposure, using calbindin as a Purkinje cell marker. The data indicates an increase in positive Purkinje cells/mm<sup>2</sup> ( $p = 0.0008$ ,  $n=4$ ) in the TP-treated group. A) GD90 control male sample at X20 magnification. B) GD90 TP-treated male samples at X20 magnification. C) Graphical representation of data.

#### 4.2.3.2 Is the “TP effect” on Purkinje cell number mediated by androgen or estrogen?

This study also investigated whether the observed alterations in Purkinje cell are due to an androgenic or estrogenic effect. There is potential for the placental aromatisation of testosterone into estrogenic metabolites. Therefore, to define the effects of both sex hormones, fetal male sheep were directly injected with either TP or the potent estradiol receptor agonist, diethylstilbestrol (DES). There was a no significant change between the control and DES-treated groups when comparing Purkinje cell number ( $p = 0.3522$ , Figure 4.4).

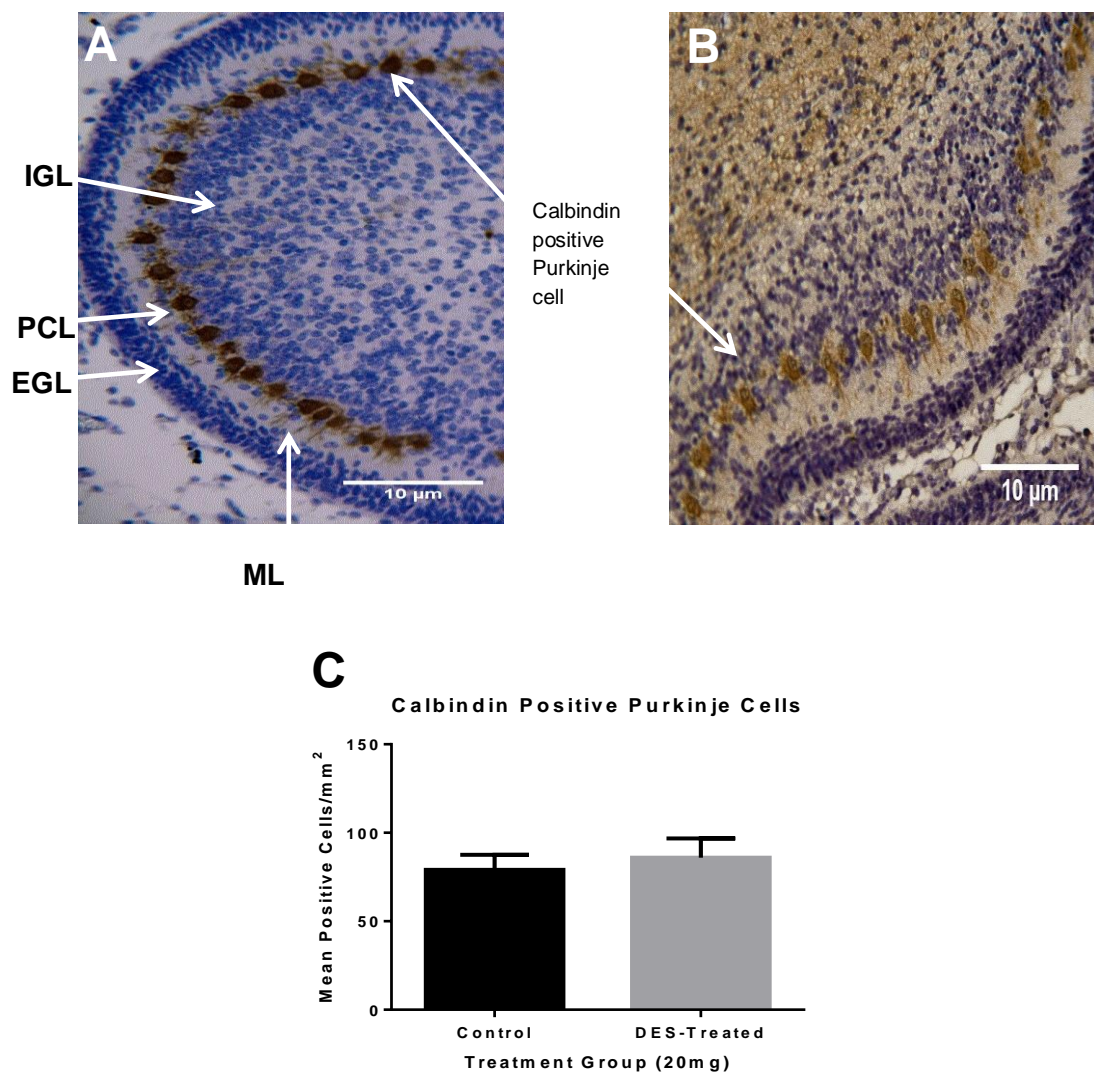
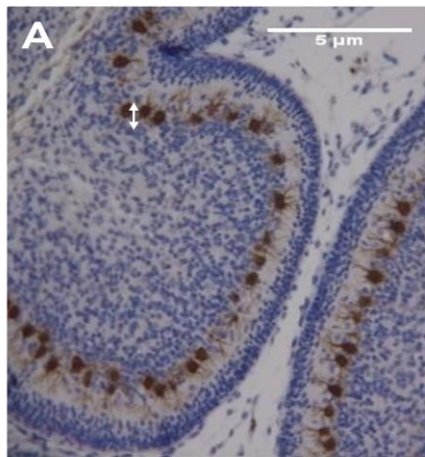


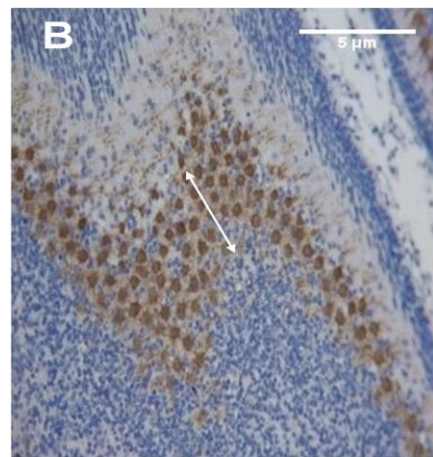
Figure 4.4: Immunohistochemistry was performed to investigate any changes in Purkinje cell number as a result of androgen over-exposure, using calbindin as a Purkinje cell marker. The data indicates no change in positive Purkinje cells/mm<sup>2</sup> ( $p = 0.3522$ ,  $n=4$ ) in the DES-treated group. A) GD90 control male sample at X20 magnification. B) GD90 TP-treated male samples at X20 magnification. C) Graphical representation of data.

### 4.2.3.3 Perturbed Purkinje cell development after prenatal excess androgen exposure

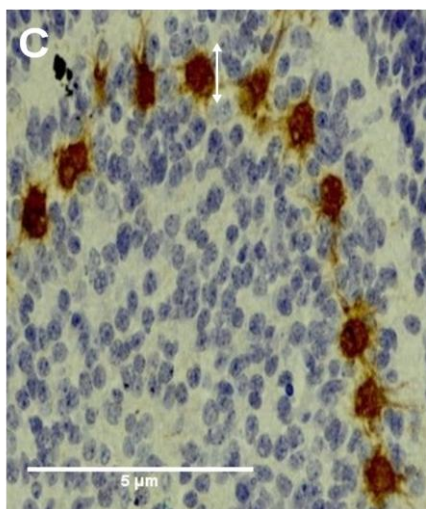
Typically, the PCL is arranged into a monolayer, observed in the majority of the control samples. However, using calbindin as a Purkinje cell marker, we observed an increase in the number of Purkinje cells within the PCL in the TP-treated samples, compared to the control samples (Figure 4.5 E,  $p = < 0.0001$ ). The arrows indicate how this was measured *i.e.* Figures 4.5 A and C) 1 Purkinje cell and Figures 4.5 B and D) 4 Purkinje cells. The Purkinje soma were also reduced in size in the TP-treated samples (Figure 4.5 F,  $p = 0.0328$ ). The PCL width was measured and had increased in the TP-treated samples (Figure 4.5 G,  $p = 0.0002$ ).



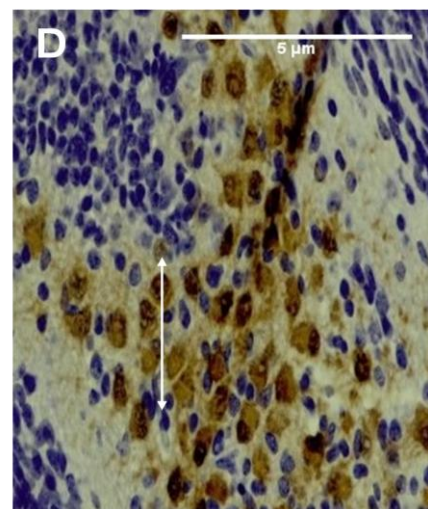
A) GD90 control cerebellar ovine tissue at X20 magnification



B) GD90 TP-treated cerebellar ovine tissue at X20 magnification



C) GD90 control cerebellar ovine tissue at X40 magnification



D) GD90 TP-treated cerebellar ovine tissue at X40 magnification

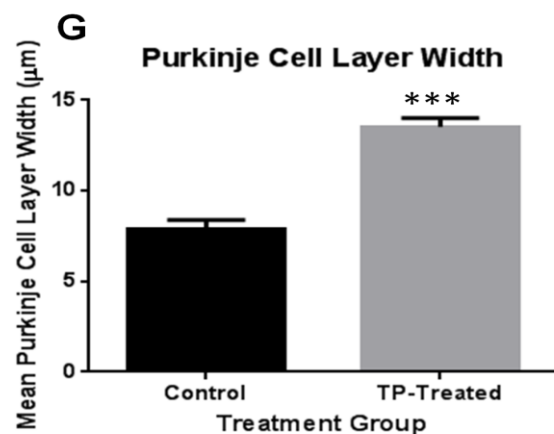
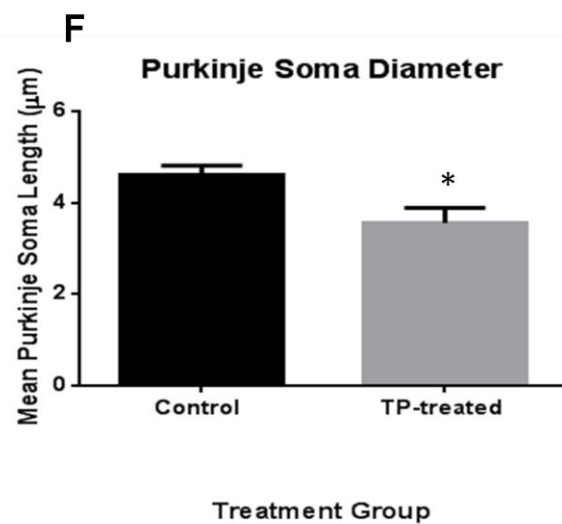
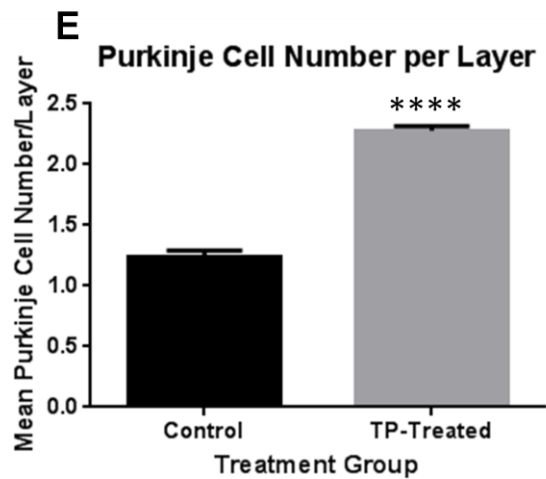


Figure 4.5: In the TP-treated samples, there was an increase in the number of Purkinje cells within the PCL (E,  $p = < 0.0001$ ,  $n=4$ ). The Purkinje soma were also reduced in size in the TP-treated samples (F,  $p = 0.0328$ ,  $n=4$ ). The PCL width was measured and had increased in the TP-treated samples (G,  $p = 0.0002$ ,  $n=4$ ). (A and C) GD90 male control samples. (B and D) GD90 male TP-treated samples. (A and B X20 magnification. (C and D) X40 magnification. Panels A-D are representative images for the control and TP-treated GD90 ovine groups.

This study has demonstrated that there are more, smaller Purkinje cells but with smaller soma diameter in the cerebellum of androgen exposed GD 90 male sheep. The PCL arrangement was also examined. The unit width of the PCL was measured and the Purkinje cell diameters were normalised to the corresponding unit width. This confirmed that there are more Purkinje cells per layer as a consequence of androgen administration (Figure 4.6 E,  $p = < 0.0001$ ). Although an increase of Purkinje cell number and PCL width as a result of androgen exposure was observed, the total area that the Purkinje cells occupy was reduced in the PCL (Figure 4.6,  $p = 0.0078$ ). In the TP-treated samples, there were more gaps, which were also included in the total PCL width measurement, between the Purkinje cells, when compared to the controls.

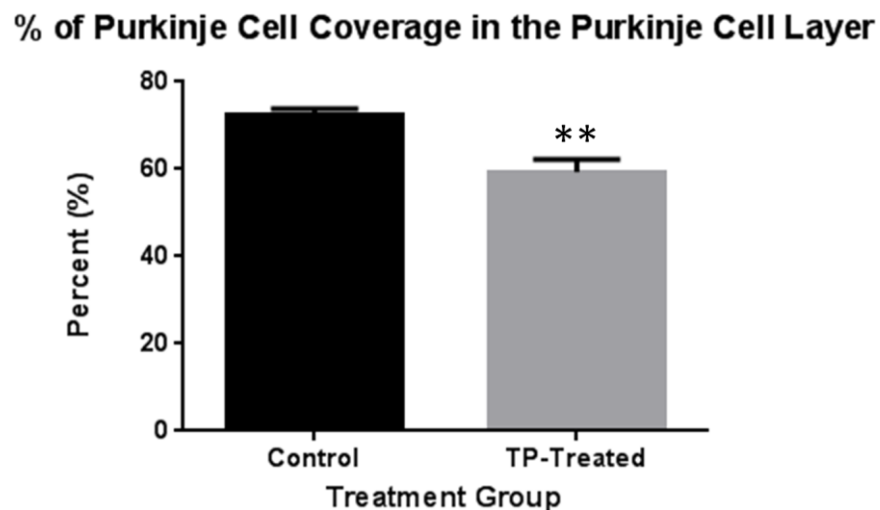
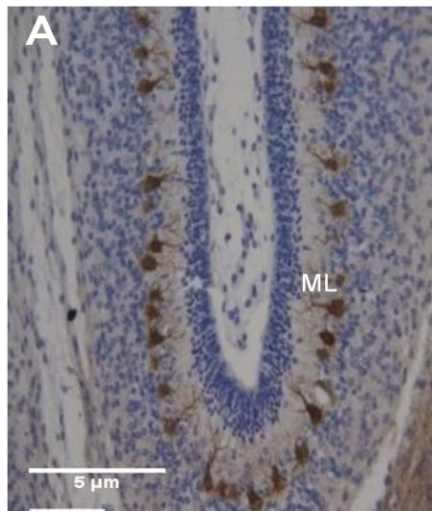


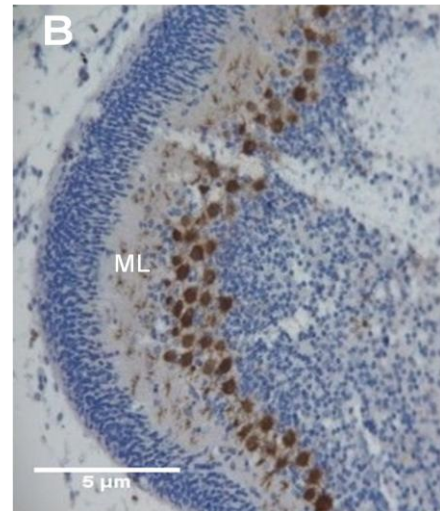
Figure 4.6: The percentage of Purkinje cells that cover the PCL was reduced as a response to prenatal excess androgen exposure ( $p = 0.0078$ ,  $n=4$ ).

#### 4.2.3.4 Purkinje cell observations

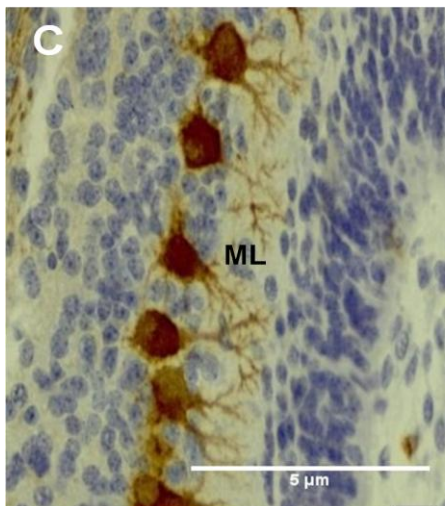
Using calbindin as a Purkinje cell marker, it was possible to observe a more highly developed dendritic network in the ML in the control group (Figures 4.7 A and C) whereas the TP-treated samples appear to have little, or no, dendritic network (Figures 4.7 B and D). Figure 4.7 A and C also illustrates how the Purkinje cells have taken on their typical unipolar shape whereas Figure 4.7 B and D shows that the Purkinje cells have not matured to that extent.



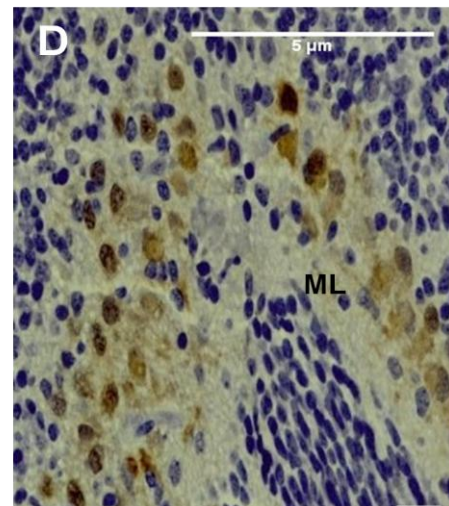
A) GD90 control cerebellar ovine tissue at X20 magnification



B) GD90 TP-treated cerebellar ovine tissue at X20 magnification



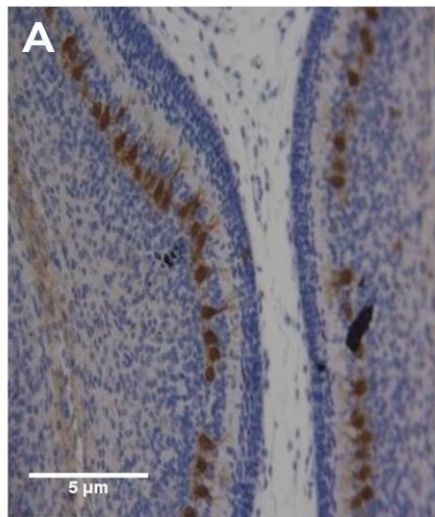
C) GD90 control cerebellar ovine tissue at X40 magnification



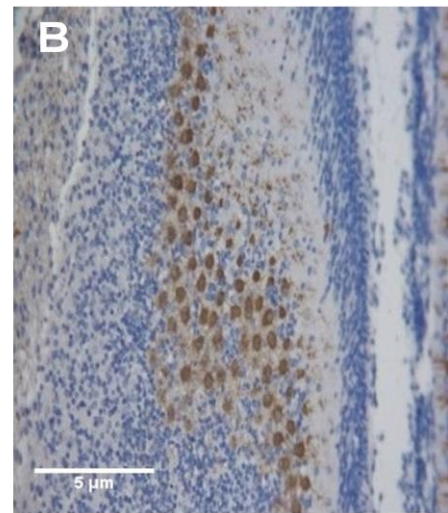
D) GD90 TP-treated cerebellar ovine tissue at X40 magnification

Figure 4.7: A and C) GD90 male control samples. B and D) GD90 male TP-treated samples. A and B) X20 magnification. C and D) X40 magnification. Panels A-D are representative images for the control and TP-treated groups.

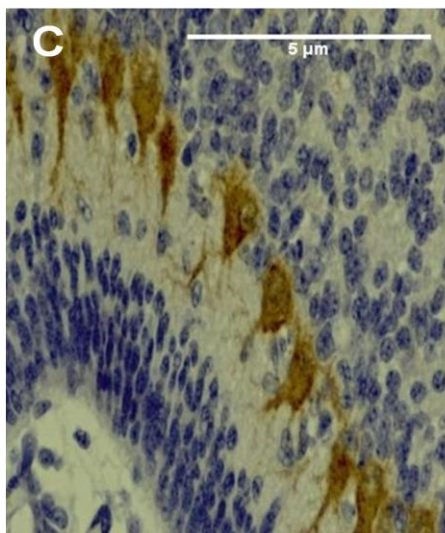
In the control group, Purkinje cells display a typical large, triangular shape (Figure 4.8 A and C) whereas the TP-treated samples appear to exhibit smaller, round Purkinje cells (Figure 4.8 B and D). This may suggest that the Purkinje cells examined in the TP-treated group are immature and not at the same developmental point as that of the control group.



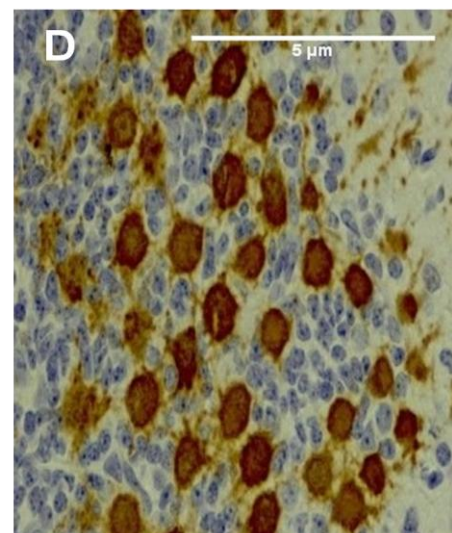
A) GD90 control cerebellar ovine tissue at X20 magnification



B) GD90 TP-treated cerebellar ovine tissue at X20 magnification



C) GD90 control cerebellar ovine tissue at X40 magnification



D) GD90 TP-treated cerebellar ovine tissue at X40 magnification

Figure 4.8: A and C) GD90 male control samples B and D) GD90 male TP-treated samples. A and B) X20 magnification. C and D) X40 magnification. Panels A-D are representative images for the control and TP-treated groups.



## 4.2.4 The apoptosis and proliferation cycle is modified in Purkinje cells due to excess androgen exposure

### 4.2.4.1 Apoptosis

Cleaved Caspase 3 was used as a marker for cell apoptosis in the PCL. Tissue from control and TP-treated (GD 90 male sheep, n=4) was sectioned, stained for cleaved caspase 3 and positive Purkinje cells (stained brown) were counted (4 sections per sample) and converted into a percentage. Figure 4.9 demonstrates an increase in cleaved caspase 3 positive Purkinje cells with TP-treatment ( $p < 0.0001$ ).

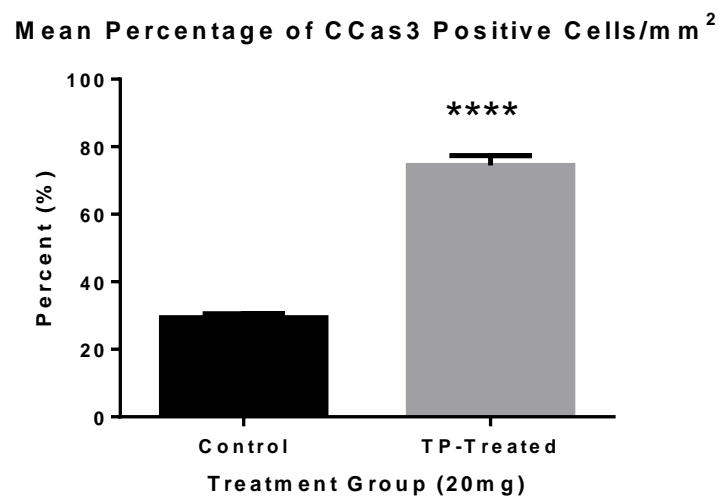
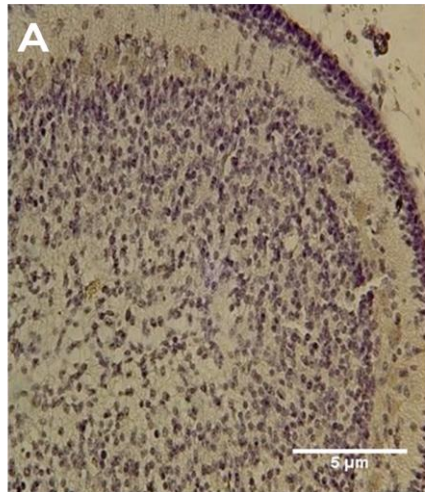
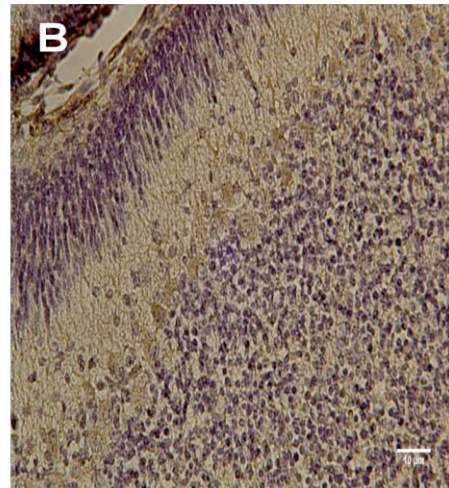


Figure 4.9: an increase in cleaved caspase 3 positive Purkinje cells with TP-treatment ( $p < 0.0001$ , n=4).

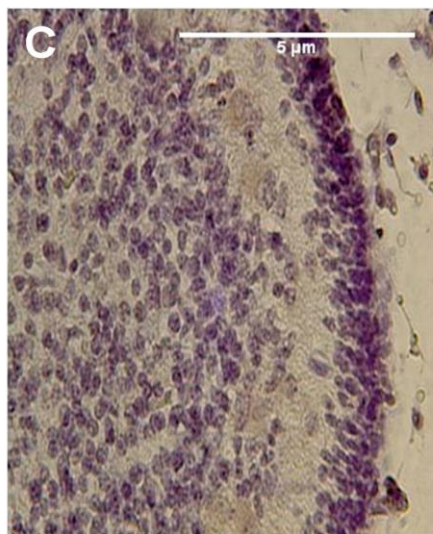
Cleaved caspase 3 was used as an apoptosis marker. Positive cleaved caspase 3 staining revealed which cells were undergoing apoptosis. The data suggests that there is an increase of cleaved caspase 3-positive cells in the TP-treated group (Figure 4.10,  $p = 0.0064$ ).



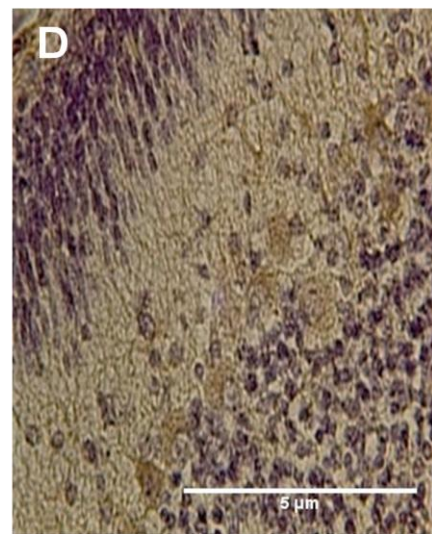
A) GD90 control cerebellar ovine tissue at X20 magnification



B) GD90 TP-treated cerebellar ovine tissue at X20 magnification



C) GD90 control cerebellar ovine tissue at X40 magnification



D) GD90 TP-treated cerebellar ovine tissue at X40 magnification

Figure 4.10: A and C) GD90 male control samples. B and D) GD90 male TP-treated samples. A and B) X20 magnification. (C and D) X40 magnification. Panels A-D are representative images for the control and TP-treated groups.

#### 4.2.4.2 Proliferation

Ki-67 was used as a marker of cell proliferation in the PCL. Tissue from control and TP-treated groups was sectioned, stained for Ki-67 and analysed in terms of positive staining in GD90 male sheep (n=4, 4 sections per sample) and converted into a percentage. Figure 4.11 demonstrates an increase in Ki-67 positive Purkinje cells with TP-treatment (p = 0.0406).

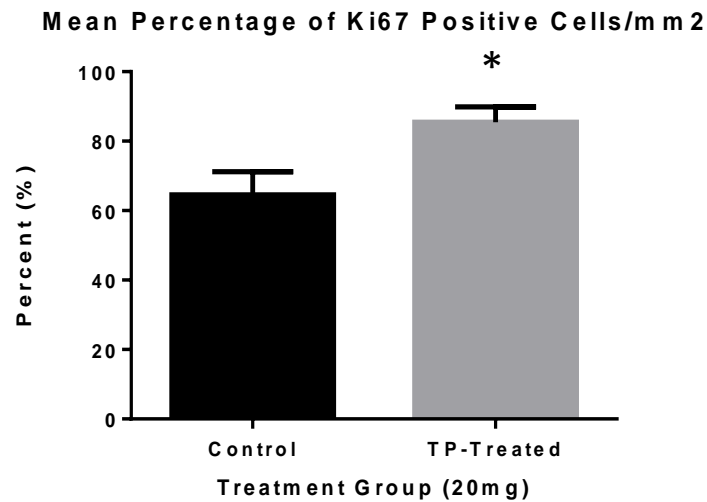
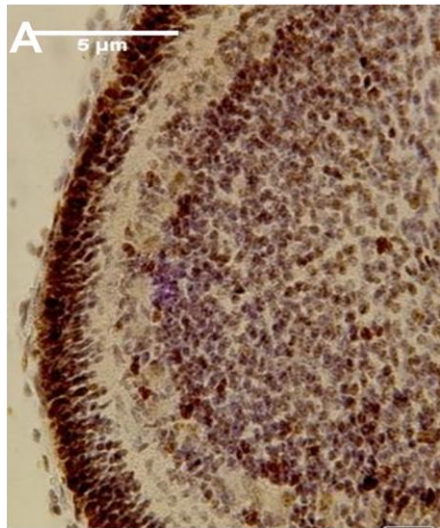
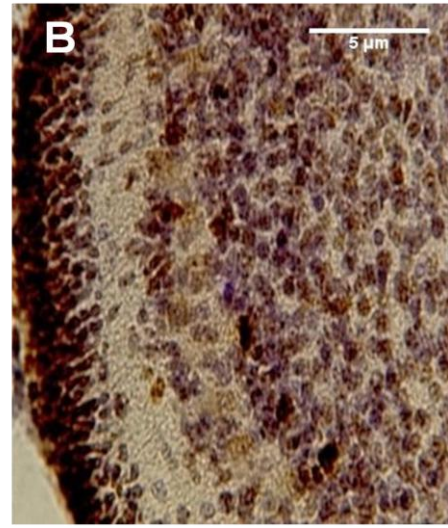


Figure 4.11: an increase in Ki-67 positive Purkinje cells with TP-treatment (p = 0.0406, n=4).

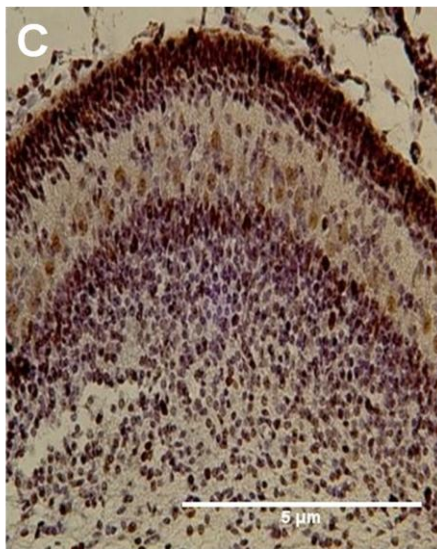
Ki-67 was used as a proliferation marker. The data suggests that there is an increase of Ki-67-positive cells the TP-treated group ( $p = 0.0064$ , Figure 4.12).



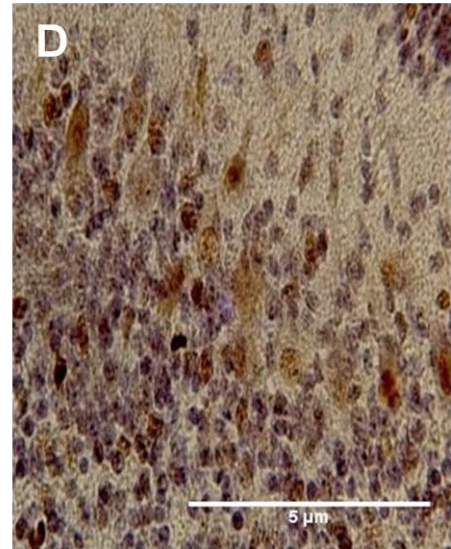
A) GD90 control cerebellar ovine tissue at X20 magnification



B) GD90 TP-treated cerebellar ovine tissue at X20 magnification



C) GD90 control cerebellar ovine tissue at X40 magnification



D) GD90 TP-treated cerebellar ovine tissue at X40 magnification

Figure 4.12: A and C) GD90 male control samples. B and D) GD90 male TP-treated samples. A and B) X20 magnification. C and D) X40 magnification. Panels A-D are representative images for the control and TP-treated groups.

## 4.3 Discussion

### 4.3.1 Fetal ovine cerebellar expression is unaltered in cell and neuronal activity markers

This study has shown that at GD 90, the male ovine cerebellum expresses the cell and neuronal activity markers that were examined in the present study (*BDNF*, *FOS*, *GABRA6*, *GAD1* and *GFAP*). All genes of interest have been shown to be expressed in the cerebella of the rat model so this current study not only confirms this cerebellar expression pattern but also provides novel information on the fetal ovine brain (Table 4.2). Only two of the genes of interest, *BDNF* and *GFAP*, have been found to be expressed in the male ovine cerebellum in previous literature (Duncan *et al.*, 2004; Mallard *et al.*, 1998).

The cell and neuronal activity markers examined in this study were unaltered in the TP-treated group, when compared to the control group. The data implies that there was no change in *BDNF* expression in the cerebellum of TP-treated samples when compared to the controls ( $p=0.3475$ , Figure 4.1 A). However, *BDNF* expression has been found to be up-regulated by increasing testosterone levels in the frontal cortex of pre-pubertal male mice (Hill *et al.*, 2012). There was no observed change in *FOS* mRNA expression as a result of prenatal excess androgen exposure ( $p=0.2603$ , Figure 4.1 B). Previous literature has demonstrated that *FOS* mRNA expression is regulated by testosterone, in the amygdala of male green anole lizards (Neal and Wade, 2007). The results imply that there is no change in cell differentiation, measured via *BDNF* expression, and in neuronal activation, measured via *FOS* expression in the cerebellum of GD 90 male sheep that were over-exposed to androgens and could be attributed to the immaturity of cerebellar neurons.

The current study determined that there was no significant change in *GABRA6* mRNA expression between the two groups ( $p=0.8058$ , Figure 4.1 C). This suggests that granule cell number was not affected by testosterone administration, assuming steady levels of *GABRA6* in the treated and untreated conditions. Using immunohistochemistry, we would expect to observe *GABRA6* positive granule cells within the IGL (Laurie *et al.*, 1992), however using Nissl staining, we detected no difference within the granular layers. There was also no change observed in *GAD1* mRNA expression, as a result of TP treatment

( $p=0.3315$ , Figure 4.1 D). *GAD1* is a key enzyme in GABA synthesis that occurs within cerebellar and cortical interneurons (Benes and Berretta, 2001), therefore the data implies that at this stage of cerebellar development, GABAergic neurons, *i.e.* Purkinje cells, are not altered by prenatal androgen excess. However, the structural effects of excess androgens have been evaluated in the current study and have demonstrated that there are in fact some marked alterations in the Purkinje cell layer development (Figures 4.3-11). It could be that the more, smaller Purkinje cells have the same level of expression as fewer, larger cells. To be able to determine whether this was a possible cause for no significant change in *GAD1* mRNA levels, immunohistochemistry would have allowed the visualisation of Purkinje cells expressing *GAD1* protein and Western blotting would have enabled the quantification of *GAD1* protein. Due to lack of suitable antibody that reacts in ovine tissue and an optimised protein extraction protocol, these investigations were unable to be performed.

There was only one GOI, *GFAP*, where a non-significant trend towards an increase of expression in the TP-treated males in the developing cerebellum ( $p=0.0641$ , Figure 4.1 E). Testosterone has been previously shown to down-regulate *GFAP* mRNA expression in facial nuclei of adult male hamsters (Jones, *et al.*, 1997). Glial cells are crucial in the development of the nervous system and are involved processes such as synaptic plasticity and synaptogenesis (Ullian *et al.*, 2004). Therefore the present study suggests that *GFAP* shows an upward trend as a result of prenatal excess androgen exposure. This would imply that the number of glia cells and the synaptic plasticity and/or synaptogenesis is altered in the TP-treated cerebella, which is indicative of immaturity of the glial cells and ultimately, delayed cerebellar development. Due to limitations in sample size, it was impossible to increase the sample numbers in order to determine if an alteration in *GFAP* mRNA expression would show any significance with a larger population of samples. It would be difficult to identify and determine, if any, differences in glial cell number and morphology by utilising *GFAP* protein, by means of immunohistochemistry due to the small size of glial cells ( $0.37\mu\text{m}^3$  in soma volume and  $7\mu\text{m}$  in stalk length, Grosche *et al.*, 2002).

### **4.3.2 No change in developing cerebellar layer widths after androgen exposure**

Using Nissl staining, the mean widths of the EGL, ML and IGL were measured and calculated in the control and TP-treated male groups and then compared. The study determined that there were no significant differences in the EGL, ML or IGL ( $p = 0.4042, 0.9293$  and  $0.3727$ , respectively, Figure 4.2) between the two groups. This implies that granule cell migration was not affected by androgen exposure.

The processes of granule cell differentiation and migration depend on many extracellular factors, such as BDNF, which is expressed in cerebellar granule cells in neonatal rats (Rocamora *et al.*, 1993). Rocamora *et al.*, (1993) demonstrated that BDNF mRNA expression increases during development in rats. Furthermore, BDNF mRNA expression is up-regulated by increasing testosterone levels in the frontal cortex of pre-pubertal male mice (Hill *et al.*, 2012). Therefore, the current study had hypothesised that there would be some change in granule cell migration, as a result of androgen exposure. However, no alterations were observed for BDNF expression, as previously discussed ( $p = 0.3475$ , Figure 4.1 A), therefore the results indicate that cerebellar granule cells of the TP-treated samples, develop in a typical granule cell migration pathway (Borghesani, *et al.*, 2002). Hill *et al.*, (2012) examined pre-pubertal mice and the granule cell migratory pattern was not established. In conclusion, androgen exposure does not seem to affect the widths of each of the cerebellar layers, and ultimately granule cell migration, during ovine gestation. This, taken together with the altered Purkinje cell development observed in the TP-treated samples; suggest that only Purkinje cells are particularly vulnerable to androgens.

### **4.3.3 Purkinje cell morphology is altered due to androgen over-exposure**

Calbindin was used as a specific Purkinje cell marker (Dougherty *et al.*, 2013) as it is abundantly expressed in these cells and is a Purkinje cell-specific protein in the cerebellum (Bastianelli, 2003). The present study has evaluated whether there is a difference in Purkinje cell numbers between the control and TP-treated samples. The data also shows multiple differences in Purkinje cell development between the control and TP-treated groups.

#### **4.3.3.1 Is the “TP effect” on Purkinje cell number mediated by androgen or estrogen?**

The data indicates that the number of cerebellar Purkinje cells in GD 90 male sheep have increased as a result of prenatal androgen over-exposure (Figure 4.3). The current study has established that the observed change is due to a direct androgen effect on these cells as there was no significant change in the number of Purkinje cells in the DES-treated group, when compared to the control group (Figure 4.4). There is very little literature that examines the effect of androgens on Purkinje cell development. Wright and Smolen (1983) have demonstrated that TP administration in neonatal male rats resulted in a 40% increase in the number of superior cervical ganglion neurons compared to the control group.

On the other hand, estrogen, progesterone and their metabolites have been shown to promote the development and survival of Purkinje cells in non-castrated rats (Sakamoto *et al.*, 2003). A previous study has shown that indeed androgens do affect Purkinje cell number but rather in the opposite manner to the current study. Zhang *et al.*, (2000) demonstrated testosterone administration decreased undifferentiated and differentiated cell number in cultured cerebellar neurons from GD 14 rats (The equivalent to GD 18-22 in sheep); whereas estrogen increased neuronal cell number. However the current study's results indicate that this is not the reason in our cohort of samples. This could be further investigated by evaluating the effects of DHT, a non-aromatizable androgen, has on the developing ovine cerebellar cytoarchitecture. If the results were similar as the ones observed for the present study then it could be deduced that it is indeed an androgen-driven mechanism.



#### **4.3.3.2 Purkinje cell number alterations in neurodevelopmental disorders**

Bailey *et al.*, (1998) observed that again adult male humans with autism had a lower number of Purkinje cells, however when a four year old male was examined, there was no change in Purkinje cell number. This may suggest that the loss of Purkinje cells occurs during adult life, a time point not examined in the current study. There was also no report of Purkinje cell abnormalities in sixteen year old autistic females (Guerin *et al.*, 1996) which suggests that Purkinje cell dysfunction is more likely in autistic males and somehow females are “protected” from structural cerebellar abnormalities by high estrogen levels. Why this occurs remains unclear. Examining the cytoarchitecture of GD 90 female ovine cerebellar samples that have been treated with TP, would allow us to determine whether females demonstrate the same abnormal development as males of the same gestational age. However due to time constraints, this was not investigated.

#### **4.3.3.3 Purkinje soma diameter**

The results from the current study indicate that there was a reduction in Purkinje cell soma diameter in the TP-treated group (Figure 4.5 F). Androgen regulation of cerebellar development has not been investigated in depth in previous literature but various different brain regions have. For example, Wright and Smolen (1983) demonstrated that TP administration in neonatal male rats does not affect Purkinje soma diameter. The current study and Wright and Smolen (1983) demonstrate that TP alters neuronal development; however as different brain regions have been investigated; they cannot be compared to one another. These findings, from both the present study and previous literature, give more insight into how androgen exposure can affect different regions in a variety of ways.

#### **4.3.3.4 PCL arrangement**

The PCL width had increased in the TP-treated samples (Figure 4.5 G,  $p = 0.0002$ ). Although an increase of Purkinje cell number and PCL width as a result of androgen exposure was observed, the total area that the Purkinje cells cover was reduced in the PCL (Figure 4.6,  $p = 0.0078$ ). In the TP-treated samples, there were more gaps, which were also included in the total PCL width measurement, between the Purkinje cells, when compared to the controls.

Rees and Harding (1988) observed that Purkinje cells were generally arranged in a monolayer, although multilayered regions were found in GD 90-92 sheep, which is what was observed in the current study's control samples. The current study also observed that in the TP-treated group, multilayered regions of Purkinje cells were predominant over the typical monolayer arrangement, which is usually observed at an earlier gestational time point (Rees and Harding, 1988). This suggests that excess prenatal androgen exposure delays the progression of Purkinje cell development. In the rat, Purkinje cell clusters begin to disperse after birth (Chung *et al.*, 2009), the equivalent of GD 25-35 in ovine gestation. Therefore, it is possible that the increase in Purkinje cell number and PCL width, demonstrated in the TP-treated group of the current study, may be due to delayed development. Cerebellar samples from a later gestational time point, the neonatal and pubertal periods and adulthood would help to determine whether this developmental delay ever catches up to typical development by comparing the morphology from control and TP-treated samples. However, this was beyond the scope of the current study.

#### **4.3.3.5 Observations**

When Purkinje cells were examined using immunohistochemistry in the current study, it became apparent that there were other anatomical features that may be altered by prenatal androgen over-exposure. However these variations were not statistically analysed but are important to point out. Rees *et al.*, (1997) performed Golgi staining on GD 90-92 ovine cerebellar tissue. They observed large, triangular Purkinje soma with several processes radiating mainly from the apical dendrite during typical cerebellar development. However, dendritic arborisation was still immature at this developmental stage and so it was deemed not possible to carry out any morphometric analysis.

The control cerebellar samples displayed typical large, triangular shaped Purkinje cell soma (Figures 4.8 A and C); whereas the TP-treated samples exhibited small and round soma (Figures 4.8 B and D). Salouci *et al.*, (2012) demonstrated that Purkinje soma shape is modified during cerebellar development in fetal sheep. The data suggested that prior to the Purkinje cell displaying large and triangular soma, the soma were small and round; which is what was observed in the TP-treated samples. It was also observed that the TP-treated cerebellar samples displayed an underdeveloped dendritic network

compared to that of the control samples (Figures 4.7 B and D; Figures 4.7 A and C, respectively). This implies that the cerebella over-exposed to androgens, has not matured at same pace as the control tissue. Sotelo (1978) demonstrated that postnatal rats (the approximate equivalent to GD 90 in sheep) possessed extensive dendritic trees from which Purkinje cells extended upward in a single plane into the molecular layer, where they synapsed with parallel fibers of granule cells. Indeed, Purkinje cells of the control samples displayed signs of apical dendrites forming and therefore, an indication that these cells were assuming their typical unipolar organisation (Salouci *et al.*, 2011). However the dendritic network and apical dendrite was not observed in TP-treated group. This further suggests that what is observed in the current study is the delayed development of the cerebella over-exposed to androgens.

In order to be able to investigate and statistically analyse any true soma shape and dendritic morphological differences that have occurred through prenatal androgen over-exposure, the Golgi staining technique could be utilised. This would allow the proper visualisation of the Purkinje cell dendritic network and enable the number of dendritic processes and/or the total length of processes per Purkinje cell to be measured and quantified, to give further insight into the development of the Purkinje cell and its dendritic network. Due to time constraints, this was beyond the scope of the current study.

Sakamoto, *et al.*, (2003) demonstrated that treatment of the neonatal male rat with exogenous estradiol and progesterone promoted the dendritic growth and spine formation of Purkinje cells. The increase in *PR* mRNA expression that is observed as a result of androgen over-exposure (Chapter 3) may result in the dysregulated cerebellar development observed in the current study. The observation of an underdeveloped dendritic network of Purkinje cells, among other Purkinje cell alterations, were detected as a result of androgen over-exposure. Further investigation into measuring Purkinje cell dendritic and spine length, using Golgi staining would allow statistical analysis to be carried out. If the results from this experiment were the same to ones discussed in this thesis *i.e.* increased *PR* gene expression, delayed Purkinje cell development then it could be implied that these alterations are mediated by *PR*. Furthermore, Zhang *et al.*, (2000) demonstrated that testosterone inhibited neuronal differentiation in cultured cerebellar neurons from GD 14 rats. These findings taken together,

suggest that progesterone contribute to the development of Purkinje cells during neonatal life whereas androgens appear to operate in the opposite manner, which is what the current study has observed in the TP-treated cerebellar tissue samples, compared to the controls.

#### **4.3.3.6 Apoptosis and proliferation of altered Purkinje cells**

Within the ovine cerebellum, there was an increase in Purkinje cell apoptosis in the TP-treated samples compared to the control group (Figures 4.9 and 4.10). There is evidence to suggest that neurosteroids can influence the typical physiological rate of apoptosis and in the developing brain. For example, Ahlbom *et al.*, (2001) demonstrated that testosterone administration rendered cultured cerebellar granule cells less susceptible to apoptosis that was induced by oxidative stress. Furthermore, Yawno *et al.* (2007) revealed that the suppression of allopregnanolone via finasteride, a  $5\alpha$ -reductase 2 inhibitor, increased apoptosis in the granular and molecular cerebellar layers, in GD 125 fetal sheep. This suggests that the reduction in  $5\alpha$ -reductase 2 mRNA expression observed in the current study (Chapter 3, Figure 3.5 B), may play some part in the increased apoptosis in the TP-treated samples. In humans, apoptosis involves the normal loss of 50% or more of the neurons within a brain region (Stiles and Jernigan, 2010) therefore, it could be assumed that the level of apoptosis observed in the TP-treated cerebellar samples may have occurred earlier in gestation in the control samples, which is further evidence for the immature Purkinje cell development detected in the androgen exposed tissue. Zhang *et al.*, (2000) determined that these changes did not occur through an alteration in proliferation, *i.e.* more/less neuronal cell growth, but rather through the regulation of apoptosis. This finding is in contrast to the current result as both apoptosis *and* proliferation have significantly increased in the TP-treated group, which again suggests this may be a symptom of immaturity of the TP-treated cerebella.

In the current study, there was also an increase in Purkinje cell proliferation as a result of prenatal androgen over-exposure (Figures 4.11 and 4.12). Zhang *et al.*, (2000) examined whether testosterone or estrogen modulated cell proliferation in cultured cerebellar neurons. The results suggested that testosterone decreased neuronal cell numbers whereas estrogen increased neuronal cell numbers. (Thomaidou *et al.*, 1997) suggested that apoptosis is important in the

proliferating neuroepithelium of the developing cerebellum in rats. Therefore, the increase in apoptosis may buoy the rate of proliferation in developing Purkinje cells that have been prenatally exposed to excess androgens. The high rate of apoptosis and proliferation are also indicative of immaturity of Purkinje cells (Stiles and Jernigan, 2010). As there was no change in Purkinje cell number between the control and DES-treated groups, apoptosis and proliferation were not examined in the DES-treated samples.

#### 4.4 Conclusions

The results of the investigation into gene expression changes in GOI that are cell and neuronal activity markers showed no significant changes between the control and TP-treated groups. GFAP was the only GOI which showed a trend towards increased mRNA expression in the TP-treated group ( $p=0.0641$ , Figure 4.1 E). The results of the current study suggest that the time point used in the current study, GD 90 in ovine gestation may be too early to detect any changes in mRNA levels of these GOIs. Androgens have been shown to regulate these GOIs, by the action of testosterone, in pre-pubertal and adult animal models (Hill *et al.*, 2012; Janmaat *et al.*, 2011; Neal and Wade, 2007; Jones *et al.*, 1997). Therefore, the age of the animal models in previous literature are more developed whereas the specific time point chosen for the current study was GD 90 in sheep, which is just when a major cerebellar growth period occurs. Examining male ovine cerebellar samples from a later point in gestation or even into adulthood would allow the results to be comparable to those in the previous literature. Another factor to take into consideration is that the expression of these genes does not reflect the conclusions of previous studies (Hill, *et al.*, 2012; Janmaat, *et al.*, 2011; Neal and Wade, 2007) on other brain regions. One possible explanation for this is that we are investigating a brain area that has a different functional role (the cerebellum plays a role in motor function) than the other well-characterised areas of the brain (such as the hippocampus which is involved in memory and spatial capacity).

The gestational age for the current study (GD 90) was chosen as it is within the period of neuronal proliferation, migration and axonal and dendritic growth (Rees and Harding, 1997) in the brain region of interest in the study (the cerebellum), and thus is likely to represent a vulnerable period for developmental damage. Therefore, cerebellar development is still occurring during the time point that was evaluated in the current study.

The control cerebellar tissue samples demonstrated the typical Purkinje cell monolayer arrangement, with large, triangular soma and signs of a growing dendritic network and apical dendrites, which also demonstrates that these cells are showing signs of becoming unipolar. In contrast to this, we observed Purkinje cell clustering, small and round Purkinje soma, a larger number of Purkinje cells and a very underdeveloped dendritic in network TP-treated

cerebellar tissue. This cytoarchitecture is found in normal ovine cerebellar development at an earlier stage (GD 80, Rees and Harding, 1988). Therefore, the control cerebellar samples likely had this disorganised pattern earlier in gestation but since developed in a typical fashion. The current study illustrates how excess prenatal androgens dysregulate and delays cerebellar Purkinje cell development and begs the question “does delayed development reach the typical cerebellar cytoarchitecture at a later stage?” Examining ovine cerebellar samples from a later gestational period, postnatally and into adulthood would give us insight into this. If the delayed development never reaches a fully matured cerebellum then this may have detrimental consequences on cognitive and/or motor function as the cerebellum plays a vital role in these areas. This could be achieved by a battery of tests that examine executive and cognitive function, such as discrimination and reversal learning, attentional set shifting and intra- and extradimensional set shifting tasks. These tests have already been assessed in sheep and it was concluded that sheep can not only perform these tasks but also display executive and cognitive function (Morton and Avanzo, 2011).

The ovine gestational period examined, GD 90, is a time of neuronal apoptosis and proliferation. Taken together, the increase in apoptosis and proliferation of Purkinje cells in the TP-treated group, may suggest during typical development, most of the immature Purkinje cells undergo apoptosis in order to allow some Purkinje cells to fully develop into maturity. This alteration in the apoptosis/proliferation cycle may be due to direct androgen over-exposure or perhaps is a knock-on effect from another cycle that androgen has disrupted. The fact that estrogen enhances cell survival while testosterone promotes cell death (Zhang *et al.*, 2000) also begs the question of what is driving this dysregulated development in our model and why is there an increase in both apoptosis and proliferation in the androgen over-exposed group. Investigating cerebellar samples that have been over-exposed to estradiol or DHT would confirm which steroid hormone is the driving force behind this. As progesterone promotes dendritic growth (Sakamoto *et al.*, 2003), examining the effects of excess progesterone on ovine cerebellar development would also allow more insight on Purkinje cell dendritic growth.

The *staggerer* mutation, a deletion of the *RORA* gene, causes severe ataxia which is associated with dysgenesis of the cerebellum. A typical feature of this is that Purkinje cells are small, ectopic and possess rudimentary dendritic arbors (Crepel and Mariani, 1975). Sarachana *et al.*, (2011) proposed that *RORA* was a candidate gene for autism and that the gene is regulated by androgens. There is evidence that links testosterone as a contributing factor in the occurrence of autism (Manning *et al.*, 2001). Baron-Cohen (2002) described the “extreme male brain” theory in which the behaviour seen in autism is an exaggeration of typical sex differences and that exposure to high levels of prenatal testosterone may be a risk factor. This, taken together with the present study’s findings, suggests that this altered cerebellar cytoarchitecture which is seen in male ovine tissue that was treated with TP, an androgen, may in some part be involved in the aetiology of autism.

Ju *et al.*, (1998) reported that the growth arrest specific gene (*GAS7*) is expressed preferentially in the brain and particularly in the Purkinje neurons of the cerebellum. Treatment of primary cerebellar cell cultures with antisense oligonucleotides inhibited *GAS7* expression and interfered with neurite formation in Purkinje cells. Conversely, overproduction of *GAS7* in mouse neuroblastoma cells promoted neurite-like outgrowth. Together, these results suggest that *GAS7* may have a role in promoting, and possibly maintaining, maturation and morphological differentiation of these cerebellar neurons. However, in contrast to the morphological consequences of inhibiting *GAS7* expression or overproducing the *GAS7* gene product, at least some biochemical concomitants of neuronal differentiation do not appear to be affected by *GAS7* in Purkinje cells. Examining genes that have been reported to arrest Purkinje cell development, such as *GAS7*, by utilising qRT-PCR may provide more insight into the mechanism behind the delayed Purkinje cell development observed in the current study.



## **CHAPTER 5**

### **Neurodevelopmental disorder candidate genes in the cerebellum**

## 5. Neurodevelopmental disorder candidate genes in the cerebellum

### 5.1 Aims and objectives

To determine whether any candidate genes associated with autism and schizophrenia are altered at the mRNA level in GD 90 male sheep, after TP-treatment.

The current study has shown that there are Purkinje cell abnormalities as a result of prenatal androgen exposure – an increase in cell number, smaller and rounder cells, an underdeveloped dendritic network and clustering of Purkinje cells (*i.e.* larger PCL width, Chapter 4). Previous literature has suggested a *reduction* in eventual Purkinje cell number and size in neurodevelopmental disorders. It remains to be seen whether this dysregulated structural developmental pattern of the cerebellum observed in the TP-treated sheep is a relevant model for neurodevelopmental disorders.

There is mounting evidence that gender is a risk factor and may contribute to neurodevelopmental disorders such as autism and schizophrenia (Baron-Cohen, 2002; Rees and Harding, 2004). A link between fetal testosterone levels, structural abnormalities and neurodevelopmental disorders has already been investigated in the literature. The *RELN* and *RORA* genes are candidate genes for autism (Bonora *et al.*, 2003; Nguyen *et al.*, 2010) and *DISC1* and *NPAS3* are associated with schizophrenia (Nguyen *et al.*, 2010; Thomson, *et al.*, 2005). Alterations in some candidate genes (*RELN* and *RORA*) for neurodevelopmental disorders have been described in the cerebellum, however we do not know the effects that prenatal androgen exposure would have on the gene expression of these candidate genes in the cerebellum. Within the current study, four candidate genes were examined, to determine whether prenatal androgen exposure alters the mRNA levels of these genes. The hypothesis is that the full complement of candidate genes (*DISC1*, *NPAS3*, *RELN* and *RORA*) examined are expressed in the cerebellum of male fetal sheep at GD 90. It is further hypothesised that prenatal excess androgen (2x20mg depot injections at GD 62 and GD 82) alters cerebellar candidate gene expression in GD 90 male sheep as measured by qRT-PCR, and ultimately suggest that androgen over-exposure may be a contributing factor of some neurodevelopmental disorders.

## 5.2 Results

### 5.2.1 Gene expression analysis

The level of gene expression for each GOI was evaluated using qRT-PCR analysis via the  $\Delta\Delta C_T$  method. The fold difference relative to a single reference (control) sample was then calculated by  $2^{\Delta\Delta C_T*(-1)}$ . Relative gene expression from the male control group and the male TP-treated group were compared to determine differences in gene expression, if any, using a two tailed, unpaired t test (GraphPad Prism software, U.S.A.).

The genes of interest (GOIs) were chosen because they are suggested to be candidate genes for autism or schizophrenia (Table 5.1).

Gene Name	Classical Name	Reason for being GOI	Reference
<i>DISC1</i>	Disrupted in schizophrenia 1	Altered in schizophrenia	Millar <i>et al.</i> , (2000)
<i>NPAS3</i>	Neuronal PAS domain protein 3	Altered in schizophrenia and regulates glucose metabolism	Pickard <i>et al.</i> , (2005)
<i>RELN</i>	Reelin	Autism marker	Serajee <i>et al.</i> , (2006)
<i>RORA</i>	Retinoid-related orphan receptor alpha	Autism marker modulated by steroids	Sarachana <i>et al.</i> , (2011)

Table 5.1: A list of the genes of interest that were examined. The primary focus of this study was to identify changes in the cerebellar expression of candidate genes for neurodevelopmental disorders that result from exposure to testosterone propionate (TP) during development in sheep.

All candidate genes examined were found to be expressed in GD 90 male cerebellum (Table 2). With the exception of *RELN*, this is the first time that the other GOIs have been found to be expressed in the fetal male sheep cerebellum.

Gene Name	Previous Evidence For Cerebellar Expression	Reference	Cerebellar Expression In This Study
<i>DISC1</i>	Fetal and adult mice	Schurov <i>et al.</i> , (2004)	Yes
<i>NPAS3</i>	Fetal mice	Brunskill <i>et al.</i> , (2005)	Yes
<i>RELN</i>	Neonatal lambs	Suarez-Vega <i>et al.</i> , (2013)	Yes
<i>RORA</i>	Neonatal mice	Koibuchi <i>et al.</i> , (2001)	Yes

Table 5.2: This table depicts whether mRNA expression for each GOI has been identified in the literature. In the majority of literature, cerebellar gene expression studies have been carried out on neonatal or adult rats.

### 5.2.2 Neurodevelopmental disorder candidate genes mRNA expression data

*DISC1* has been identified as a susceptibility gene for schizophrenia due to the link between psychiatric disease and a large Scottish family (Millar *et al.*, 2000). This current study wanted to examine whether androgen over-exposure altered mRNA levels of *DISC1* however, there was no change in *DISC1* expression in the TP-treated group, when compared to the control group ( $p=0.2289$ , Figure 5.1 A).

Pickard *et al.*, (2005) demonstrated that *NPAS3* expression is altered in schizophrenia. No significant changes in *NPAS3* mRNA expression in the developing male ovine cerebellum were observed between the control and TP-treated groups in this current study ( $p=0.2755$ , Figure 5.1 B).

Low reelin expression in the cerebellum of adult humans has been found to be associated with autism (Fatemi *et al.*, 2005). This study has demonstrated that no significant changes were observed in *RELN* mRNA expression between the two groups ( $p=0.5673$ , Figure 5.1 C) at the GD90 stage of cerebellar ovine development.

*RORA* has been proposed as a novel candidate gene in autism (Sarachana *et al.*, 2011). No significant changes in cerebellar *RORA* mRNA expression were observed between the control and TP-treated groups ( $p=0.3041$ , Figure 5.1 D).

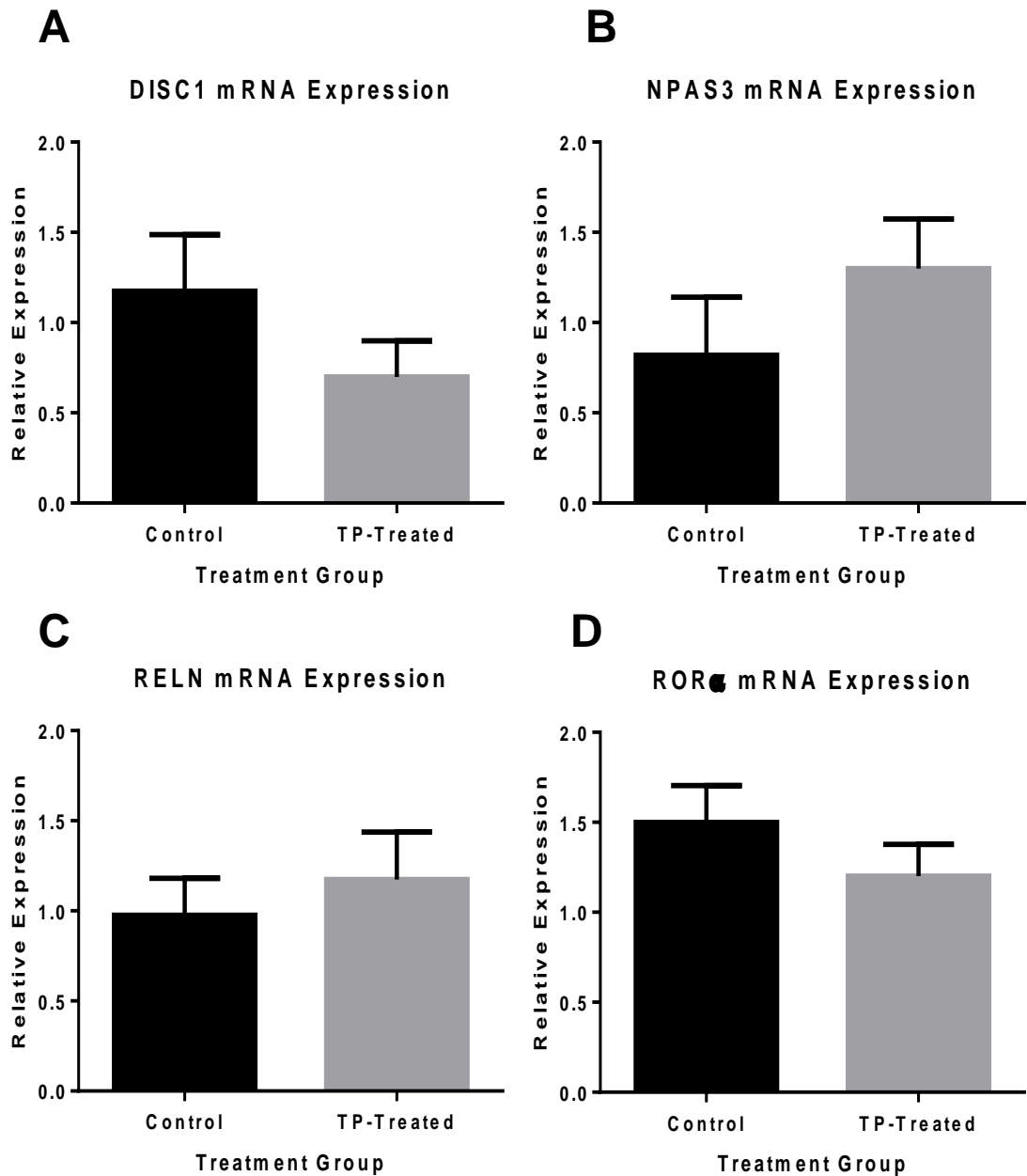


Figure 5.1: qRT-PCR analysis has shown that there are no differences at the mRNA expression level of neurodevelopmental disorder candidate gene marker in cerebellar tissue of GD90 male sheep, n=4. A) DISC1 mRNA expression for control and TP-treated samples,  $p = 0.2289$  B) NPAS3 mRNA expression for control and TP-treated samples,  $p = 0.2755$  C) RELN mRNA expression for control and TP-treated samples,  $p = 0.5673$  D) ROR $\alpha$  mRNA expression for control and TP-treated samples,  $p = 0.3041$ .

## 5.3 Discussion

### 5.3.1 Fetal ovine cerebellar expression of neurodevelopmental disorder candidate genes

This study has shown that at GD 90, the male ovine cerebellum expresses candidate genes of autism and schizophrenia (*DISC1*, *NPAS3*, *RELN* and *RORA*) and therefore illustrates that the fetal ovine cerebellum is capable of being vulnerable, to contributing to the aetiology of neurodevelopmental disorders. All genes of interest have been shown to be expressed in the cerebella of other animal models and so this current study not only confirms this cerebellar expression pattern but also provides novel information on the fetal ovine brain (Table 5.2). Only one of the genes of interest, *RELN*, has been found to be expressed in the male ovine cerebellum from the neonatal period and into adulthood, in previous literature (Suarez-Vega *et al.*, 2013) and is therefore after the gestational period examined in the current study (GD 90).

### 5.3.2 No change in gene expression of candidate genes as a response to prenatal androgen over-exposure

#### 5.3.2.1 Schizophrenia

There is no literature that documents whether *DISC1* and *NPAS3* cerebellar mRNA expression is regulated by androgens or whether over-exposure to androgens during gestation alters their gene expression. Therefore, this current study can provide insight in that these genes are not regulated or influenced by androgens in the developing ovine cerebellum. Further experiments on GD 90 male cortex tissue would determine whether these genes are altered as a result of over-exposure to androgen *in utero*.

The prefrontal cortex region is strongly implicated in schizophrenia and it has been previously reported that the density of *DISC1* positive neurons was significantly reduced in certain layers of the cortex of schizophrenics (Millar *et al.*, 2000). Schurov *et al.*, (2004) demonstrated that *DISC1* expression is developmentally regulated with two major peaks at embryonic day (ED) 13 and post-natal day (PND) 35 in the cortex and hypothalamus of mice whereas the current study is examining the cerebellum. This is the equivalent of GD 20 and approximately 1 postnatal month, respectively, in the sheep. These time periods are critically important in the development of rodents and correspond to the period of neurogenesis in the developing rodent brain (Nadri *et al.*, 2003) but

not in the ovine cerebellum. Therefore this current study avoids disturbing the critical period of as the ovine fetuses were injected at GD 62 and 82, assuming the same *DISC1* expression pattern occurs in the cerebellum. Pieper *et al.*, (2005), reported reduced neurogenesis in the hippocampus of *NPAS3 knockout* mice and suggested the possibility that impaired hippocampal neurogenesis may be relevant to some aspects of hippocampal pathology, that are associated with schizophrenia. Schizophrenia candidate genes have been extensively examined in the prefrontal cortex and hypothalamus and shown to have altered expression patterns in people with this neurodevelopmental disorder (Millar *et al.*, 2000; Schurov *et al.*, 2004; Pieper *et al.*, 2005).

### **5.3.2.2 Autism**

One theory concerning the aetiology of autism is the “extreme male brain theory” which suggests that behaviours seen in autism are an exaggeration of typical sex differences and that exposure to high levels of prenatal testosterone might be a risk factor (Baron-Cohen, 2002). The *RELN* gene encodes for Reelin protein and has a major role in neuronal migration and in prenatal development of neuronal connections (D’Arcangelo *et al.*, 1995). Low reelin expression in the cerebellum of neonatal mice has been found to be associated with autism (Fatemi *et al.*, 2005). There is evidence that testosterone may regulate *RELN* mRNA expression in the hypothalamus and cerebellum of rats aged PND 10 and 2.5 months (Lakatosova *et al.*, 2010). However this current study does not reproduce this finding which could be due to the earlier time point used in our animal model (GD 90). *RORA* activates genes in the cerebellum that stimulate proliferation of granule cell precursors in the external granule layer while also regulating a set of genes in Purkinje cells involved in processing neurotransmitter signalling (Gold *et al.*, 2003). Sarachana *et al.*, (2011) demonstrated that estradiol treatment significantly enhanced *RORA* expression in the human neuroblastoma cell line, SH-SY5Y, whereas dihydrotestosterone (DHT) had the opposite effect and led to reduced *RORA* expression. These findings imply that *RORA* expression is regulated by steroids however the current study did not reflect this. This could be due to the time point chosen for examination in this study (GD 90) whereas the literature has previously focused on postnatal and adulthood gene expression in autism. Re-visiting *RELN* and *RORA* gene expression at a later time point, such as adulthood, by means of



qRT-PCR, would help establish if indeed androgen only regulate these genes post natally.

## 5.4 Conclusions

The results of the investigation into gene expression changes of GOIs involved in neurodevelopmental disorders showed no significant changes or trends between the control and TP-treated groups. This suggests that prenatal androgen over-exposure does not alter the expression of these candidate genes at GD 90 stage of development of the ovine cerebellum. Androgen exposure has been shown in other areas of the brain to be influential in affecting gene expression of these genes (Millar *et al.*, 2001; Fatemi *et al.*, 2005; Sarachana *et al.*, 2011; Baron-Cohen, 2002). It is feasible that the period of cerebellar development and therefore the age of the animals in the current study may be too early to detect any changes in these genes as the literature above evaluates adult tissue of other models. Therefore, evaluating the expression of candidate genes in male ovine cerebellar samples from a later point in gestation, or even into adulthood which is similar to the previous studies mentioned, would allow the results to be comparable to those in the previous literature.

Previous literature has demonstrated that in neurodevelopmental disorders such as autism and schizophrenia, there is abnormal cytoarchitectural development in the cerebellum (Bauman and Kemper, 1985; Bailey *et al.*, 1998; Reyes and Gordon, 1981; Tran *et al.*, 1998). Chapter 4 demonstrated marked Purkinje cell layer dysregulation as a result of prenatal androgen over-exposure, and this abnormal cytoarchitecture, *i.e.* reduced Purkinje cell size, is seen in post mortem adult tissue from people afflicted with autism and schizophrenia. However, the candidate gene expression data implies that excess androgen administration does not alter their expression pattern in the fetal cerebellum of sheep at this stage of development. It is also important to point out that *DISC1*, *NPAS3*, *RELN* and *RORA* also play major roles in neurogenesis, neuronal migration, proliferation of granule cells and act as regulators of neurotransmitter signalling in the brain (Pieper *et al.*, 2005; D'Arcangelo *et al.*, 1995; Gold *et al.*, 2003) and so the changes seen in the cerebellar cytoarchitecture must be due to alterations on development via other pathways.

There has been extensive research on structural abnormalities of the frontal and temporal cortex in schizophrenia. So perhaps investigating the prefrontal

cortex and/or the hippocampus would allow us to illustrate how androgen over-exposure may alter the genetic and structural development of the area of the brain heavily involved in cognitive and social functions. Due to time constraints, the examination the prefrontal cortex cytoarchitecture was beyond the scope of the current study. However, microarray analysis was performed on GD 90 ovine prefrontal cortex tissue to evaluate any change in gene expression in this brain region and is discussed at length in Chapter 6.

## **CHAPTER 6**

### **Microarray analysis of gene expression in the cortex**

## **6. Microarray analysis of gene expression in the cortex**

### **6.1 Aims and objectives**

#### **6.1.1 Aim 1 and objectives**

Examining the cortical cytoarchitecture of GD 90 male sheep and identifying whether pyramidal cell number and distribution are affected by TP-treatment.

This study explored the developing male cortex cytoarchitecture by means of Nissl staining and immunohistochemistry, with the intention of evaluating whether there was a difference in pyramidal cell number, shape, formation and arrangement of pyramidal cells expressing T-box brain 1 (*tbr1*, a pyramidal cell marker, Bulfone *et al.*, 1995) between the control and TP-treated GD 90 ovine samples (2x20mg depot injections at GD 62 and GD 82). The hypothesis is that pyramidal cell development is altered as a consequence of prenatal androgen over-exposure.

#### **6.1.2 Aim 2 and objectives**

To evaluate and compare gene expression levels between the control and TP-treated GD 90 male sheep cortices.

A link between fetal testosterone levels, structural abnormalities and neurodevelopmental disorders has already been investigated in the literature. The *DISC1* and *NPAS3* are associated with schizophrenia (Nguyen, *et al.*, 2010; Thomson, *et al.*, 2005). The role of androgens and their effect on gene expression during the development of the cortex is unknown. In order to evaluate and compare the gene expression between the control and TP-treated cortex samples, microarray analysis was performed. This allowed the measurement of 15 000 genes and their expression levels, in order to gain a broader insight into how prenatal androgen over-exposure can alter various genes in GD 90 ovine male cortex.

#### **6.1.3 Aim 3 and objectives**

To validate the microarray data.

A selection of significantly altered genes, as determined by microarray analysis, was then validated by utilising qRT-PCR. This was performed in order to verify the microarray data.

## 6.2 Results

### 6.2.1 Identifying pyramidal cells by Nissl staining

The current study set out to determine whether there was a change in the number of pyramidal cells in GD90 male sheep, as a result of prenatal excess androgen exposure. This was investigated by the use of Nissl staining, whereby distinct pyramidal cells are usually seen (Santana *et al.*, 2004). However the current study could not differentiate between cell type although the different layers of the cortex were determined using Nissl staining (Figure 6.1).

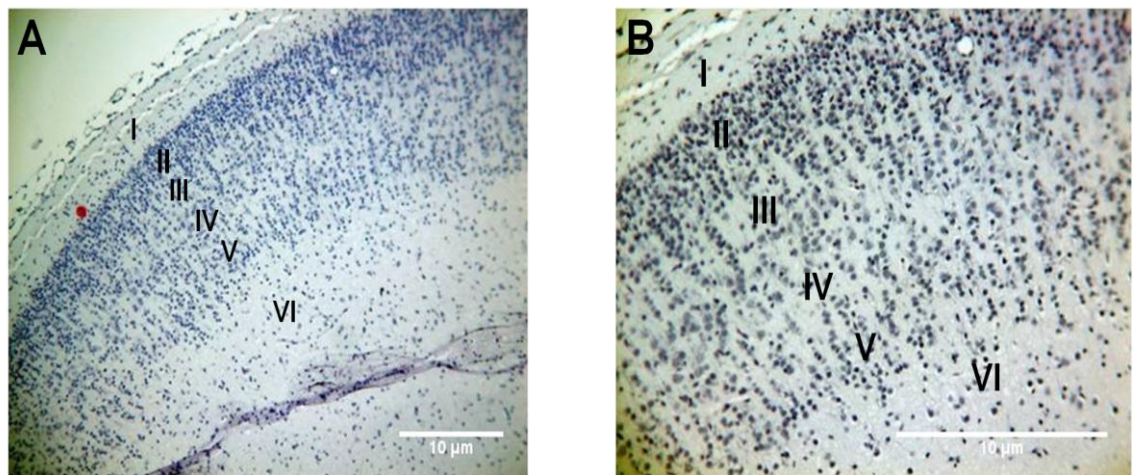


Figure 6.1: Representative images of a Nissl stained section GD90 control cortex tissue sample. A) X10 magnification. B) X20 magnification. The pyramidal cells were undistinguishable to granule cells at this developmental period. Layers I-VI are annotated on A) and B).

## 6.2.2 Examining the cortex using neuronal markers

As Nissl staining was unsuccessful in illustrating pyramidal cells of the developing cortex, neuronal markers were utilised as an alternative method. T-box, brain, 1 (Tbr1) was used as a pyramidal cell marker for the present study, as it is expressed in migratory and postmigratory neurons (Bulfone *et al.*, 1995). However there was no success in observing any positive Tbr1 cells at different dilutions (Figures 6.2 A and B). Calbindin is a Purkinje cell marker (Dougherty, *et al.*, 2013) as it is abundantly expressed in these cells in the cerebellum (Bastianelli, 2003). Due to its specificity to neurons, we elected to try this as a pyramidal cell marker, but once again, there does not appear to be any positive staining (Figure 6.2 C). Both neuronal markers did work in the developing cerebellum (Figures 6.2 D and E).

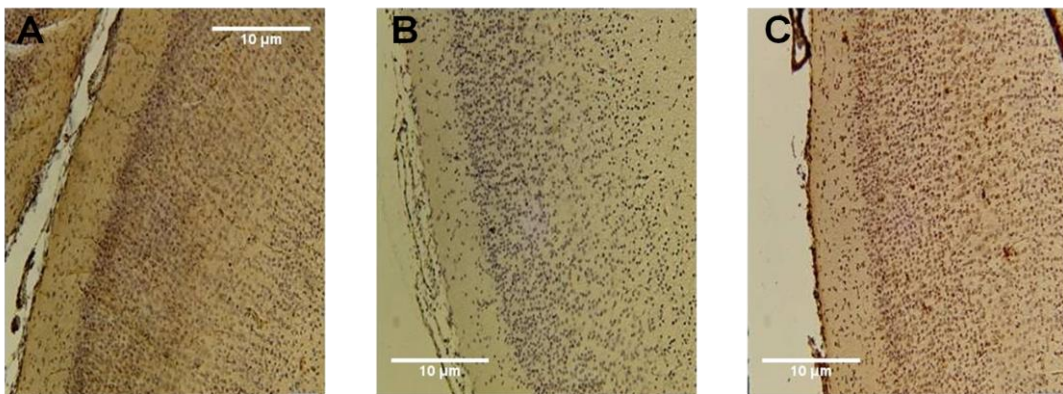


Figure 6.2 A) GD90 cortex tissue. Anti-TBR-1 at 1:50 dilution B) GD90 cortex tissue. Anti-TBR-1 at 1:100 dilution C) GD90 cortex tissue. Calbindin at 1:1000 dilution

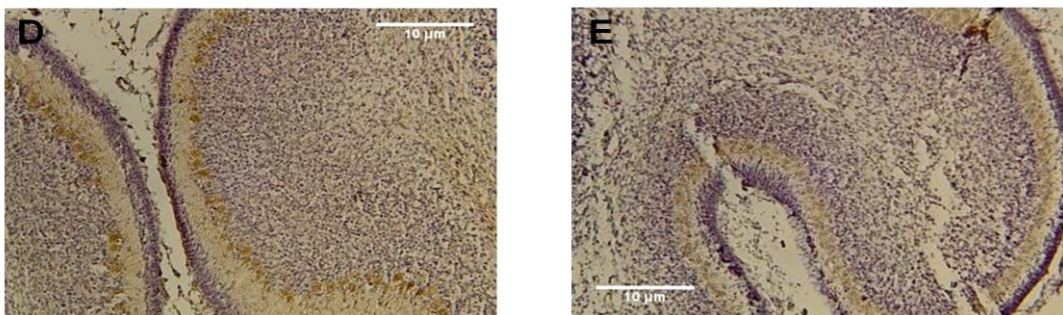


Figure 6.2 D) GD90 cerebellar tissue. Anti-TBR-1 at 1:50 dilution E) GD90 cerebellar tissue. Anti-TBR-1 at 1:400 dilution

Figure 6.2: Anti-TBR1 was used for the detection of Pyramidal cells in the cortex in GD90 male sheep. A) 1:50 dilution. Pyramidal cells were undistinguishable. B) 1:100 dilution. No positive staining for Pyramidal cells were seen and this low dilution. C) Due to its remarkable staining of the Purkinje cells of the cerebellum, calbindin (1:1000 dilution) was used as another neuronal marker. D) To determine whether there was a problem with the anti-TBR1, GD90 male ovine tissue was used as a tissue control. TBR1 (1:50 dilution) shows positive staining for cerebellar Purkinje cells and so does the recommended 1:400 dilution (E). Therefore it was determined that the antibody used was suitable for ovine tissue.

### 6.2.3 Localisation of AR in the developing cortex

AR localisation has been demonstrated in the nuclei of pyramidal cells of the cortex in neonatal rats (Nunez *et al.*, 2003). However, there was no positive staining for AR in GD90 male sheep (Figure 6.3).

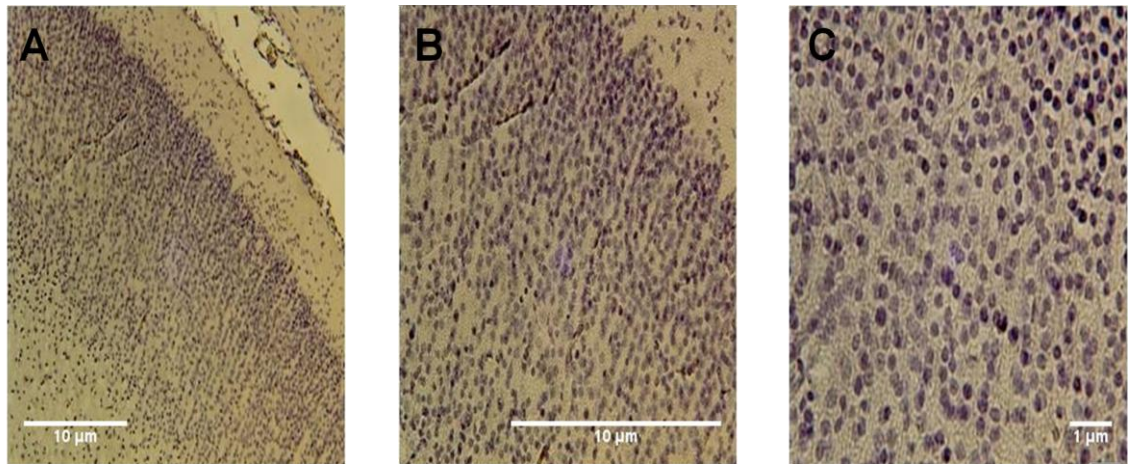


Figure 6.3: Immunohistochemical studies shows no localisation of AR (dilution 1:500) in the nuclei of Pyramidal cells in the cortex of GD90 male sheep. A) X10 magnification. B) X20 magnification. C) X40 magnification.



## **6.2.4 Cortex Gene Expression using Microarray Analysis**

### **6.2.4.1 Differences in gene expression in the cortex, as a result of prenatal androgen over-exposure**

Microarray analysis evaluated 15 000 genes, in order to determine whether prenatal androgen over-exposure alters gene expression in the developing cortex. Using volcano analysis, the genes identified as being significantly altered in their expression in the cortex of GD 90 male sheep, are listed in order of significance in Table 6.1.

Gene Name	Accession number	Description	P value (sig < 0.05)	Fold change
<i>SLIT2</i>	EF627036	Signals through the Robo receptor as a repellent neuronal migration	0.007976612	2.08-fold increase
<i>ERBA BETA1</i>	NM001190391	Thyroid hormone receptor beta1	0.01801177	2.03-fold increase
<i>NPY3R</i> AKA <i>CXCR4</i>	NM_001277168	Neuropeptide Y receptor type AKA Chemokine (C-X-C motif) receptor 4	0.019454515	2.13-fold reduction
<i>SNX19</i>	XM_004019833	Sorting nexin 19	0.019792868	2.29-fold increase
<i>SRRM2</i>	XM_005224631	Serine/arginine repetitive matrix 2	0.01983914	2.28-fold increase
<i>ALG10</i>	XM_004006737	Asparagine- linked glycosylation 10, alpha 1,2 glucosyl transferase	0.02029975	2.01-fold reduction
<i>FBXW7</i>	XM_004017195	F-box & WD repeat domain containing protein 7	0.020762052	2.12-fold reduction
<i>RERE</i>	XM_004014019	Arginine glutamic acid dipeptide (RE) repeats	0.02133858	2.11-fold increase
<i>WDR6</i>	XM_004018477	WD repeat domain 6	0.023501296	2.53-fold increase
<i>COL4A2</i>	XM_004012356	Collagen type IV alpha 2 chain	0.024283078	2.66-fold increase
<i>THBS1</i>	XM_004010435	Thrombospondin-1 precursor	0.02572934	2.1-fold increase
<i>PPARD</i>	XM_004018768	Peroxisome proliferator-activated receptor delta, transcript variant 1	0.027523464	2.08-fold increase
<i>NUCB1</i>	XM_004015361	Nucleobindin 1	0.031315632	2.0-fold increase
<i>NNT</i>	XM_004017007	Nicotinamide nucleotide transhydrogenase	0.033152565	2.05-fold increase
<i>POMC</i>	NM001009266	Proopiomelanocortin	0.037072923	2.0-fold increase
<i>YLPM1</i>	XM_004010791	YLP motif containing 1	0.039331213	2.17-fold increase
<i>EPB41L2</i>	XM_004011315	Erythrocyte membrane protein band 4.1-like 2, transcript variant 1.	0.039527547	2.54-fold increase
<i>APP</i>	XM_004002806	Amyloid beta (A4) precursor protein, transcript variant 8	0.044590697	2.23-fold increase
<i>CARM1</i>	XM_004009301	Coactivator-associated arginine methyltransferase 1	0.047516413	2.13-fold increase

Table 6.1: The genes which are affected in GD 90 male ovine cortex as a consequence to androgen over-exposure (n=6). These results were determined using microarray analysis.

#### **6.2.4.2 Determining whether gene expression changes are due to an androgenic or estrogenic effect**

Microarray analysis evaluated 15 000 genes, and compared expression levels between the TP- and DES- treated GD 90 male ovine cortical tissue. If there is a change in gene expression between the TP- and DES-treated samples, then the expression would have changed in one treatment and not the other. If the same altered gene is reported in the comparison of control and TP-treated samples, then it is the TP administration that has changed the expression of the gene and is therefore deemed to be due to an androgenic effect. The gene expression levels for DES-treated sample wouldn't be altered in this scenario thus, it is similar to comparing the control and TP-treated samples. Using volcano analysis, the genes identified as being significantly altered in their expression in the cortex of GD 90 male sheep of TP- and DES-treated samples, are listed in order of significance in Table 6.2.

Gene Name	Accession number	Description	P value (sig < 0.05)	Fold change
<i>SRRM2</i>	XM_005224631	Serine/arginine repetitive matrix 2	0.01685605	2.13-fold reduction
<i>PPARD</i>	XM_004018768	Peroxisome proliferator-activated receptor delta, transcript variant 1	0.017093947	2.23-fold reduction
<i>COL4A2</i>	XM_004012356	Collagen type IV alpha 2 chain	0.01757203	2.98-fold reduction
<i>RERE</i>	XM_004014019	Arginine glutamic acid dipeptide (RE) repeats	0.018418575	2.63-fold reduction
<i>NPY3R</i> <i>AKA</i> <i>CXCR4</i>	NM_001277168	Neuropeptide Y receptor type AKA Chemokine (C-X-C motif) receptor 4	0.018649546	2.05-fold increase
<i>PC1</i>	AF_063110	prohormone convertase 1	0.021528961	2.05-fold reduction
<i>SNX19</i>	XM_004019833	Sorting nexin 19	0.022351159	2.26-fold reduction
<i>APP</i>	XM_004002806	Amyloid beta (A4) precursor protein, transcript variant 8	0.022832684	2.56-fold reduction
<i>CARM1</i>	XM_004009301	Coactivator-associated arginine methyltransferase 1	0.022832684	2.18-fold reduction
<i>NUCB1</i>	XM_004015361	Nucleobindin 1	0.02313712	2.28-fold reduction
<i>WDR6</i>	XM_004018477	WD repeat domain 6	0.028551904	2.26-fold reduction
<i>ERBA BETA1</i>	NM_001190391	Thyroid hormone receptor beta1	0.02919307	2.16-fold reduction
<i>EPB41L2</i>	XM_004011315	Erythrocyte membrane protein band 4.1-like 2, transcript variant 1.	0.031731732	2.82-fold reduction
<i>YLPM1</i>	XM_004010791	YLP motif containing 1	0.032558337	2.36-fold reduction
<i>NNT</i>	XM_004017007	Nicotinamide nucleotide transhydrogenase	0.039746065	2.01-fold reduction

Table 6.2: The genes which have altered expression in GD 90 TP-treated male ovine cortex, compared to the DES-treated samples (n=6). These results were determined using microarray analysis.

### **6.2.5 qRT-PCR validation of the microarray data**

qRT-PCR analysis was used to validate some of the significant findings of microarray analysis. The genes that were selected for validation were altered in the TP-treated group, and were chosen due to their roles in either cerebellar development, cellular/neuronal migration, steroidogenesis, endocrine disruption and neurodegenerational disease. However, the validation illustrated no significant differences at the mRNA level for five of the six genes of interest: *SLIT2*, *POMC*, *ERBA  $\beta$ 1* and *NPY3R* and *APP* (Figures 6.4 A-E). There was an increase in *COL4A2* mRNA expression in the TP-treated cortex tissue with a 8.95-fold increase in expression, which correlates with the result from microarray analysis for this particular gene (Figure 6.4 F).

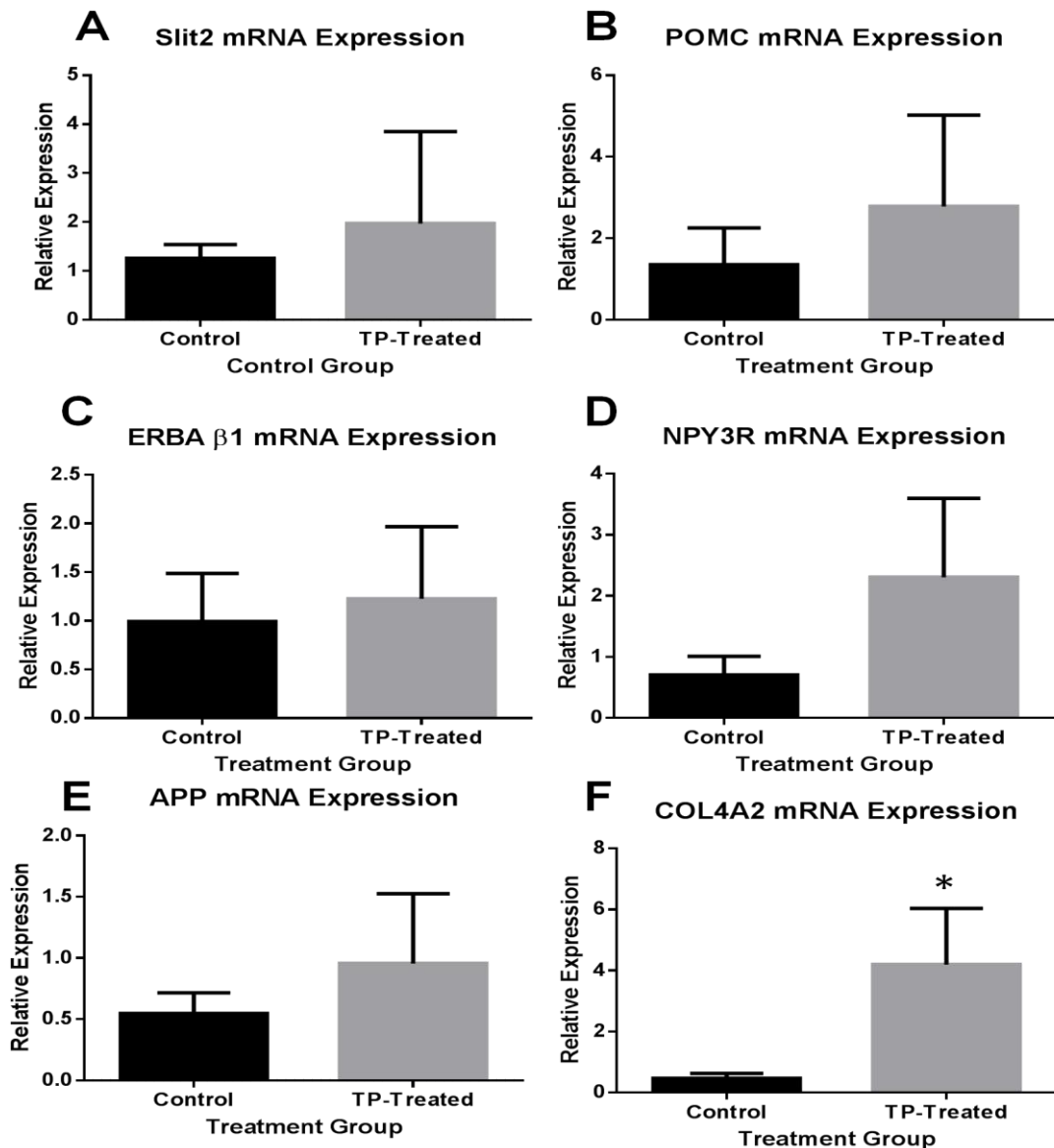


Figure 6.4: qRT-PCR analysis has shown that there were no differences at the mRNA expression level of these gene of interests in cortex tissue of GD90 male sheep (n=6). A) Slit2 mRNA expression for control and TP-treated samples,  $p = 0.4138$  B) POMC mRNA expression for control and TP-treated samples,  $p = 0.516$ , a 2.05-fold increase in the TP-treated group was calculated C) ERBA  $\beta 1$  mRNA expression for control and TP-treated samples,  $p = 0.7907$  D) NPY3R mRNA expression for control and TP-treated samples,  $p = 0.1796$ , a 3.97-fold increase in the TP-treated group was calculated E) APP mRNA expression for control and TP-treated samples,  $p = 0.4538$ . There was an increase in COL4A2 mRNA expression in the TP-treated group and this validates the microarray analysis (F,  $p = 0.0352$ ) for this GOI.

The *SLIT/ROBO* pathway was also investigated due to *SLIT2* being the most altered gene in the TP-treated cortex tissue in the microarray analysis. This pathway has already been investigated in the context of fetal programming (Hogg *et al.*, 2011). *SLIT2* is mediated by members of the *ROBO* receptor family (Andrews *et al.*, 2007); therefore, due to the availability of primer sets, certain *ROBO* receptors were examined to determine whether androgen over-exposure alters their gene expression. There was a significant increase in mRNA expression of *ROBO1* (Figure 6.5 A). This suggests that after TP administration, there was a 3.54-fold increase in *ROBO1* mRNA expression in the developing male cortex. However there was no change in the expression of *ROBO2* and *ROBO4* in GD90 male sheep, which were exposed to androgens (Figures 6.5 B and C).

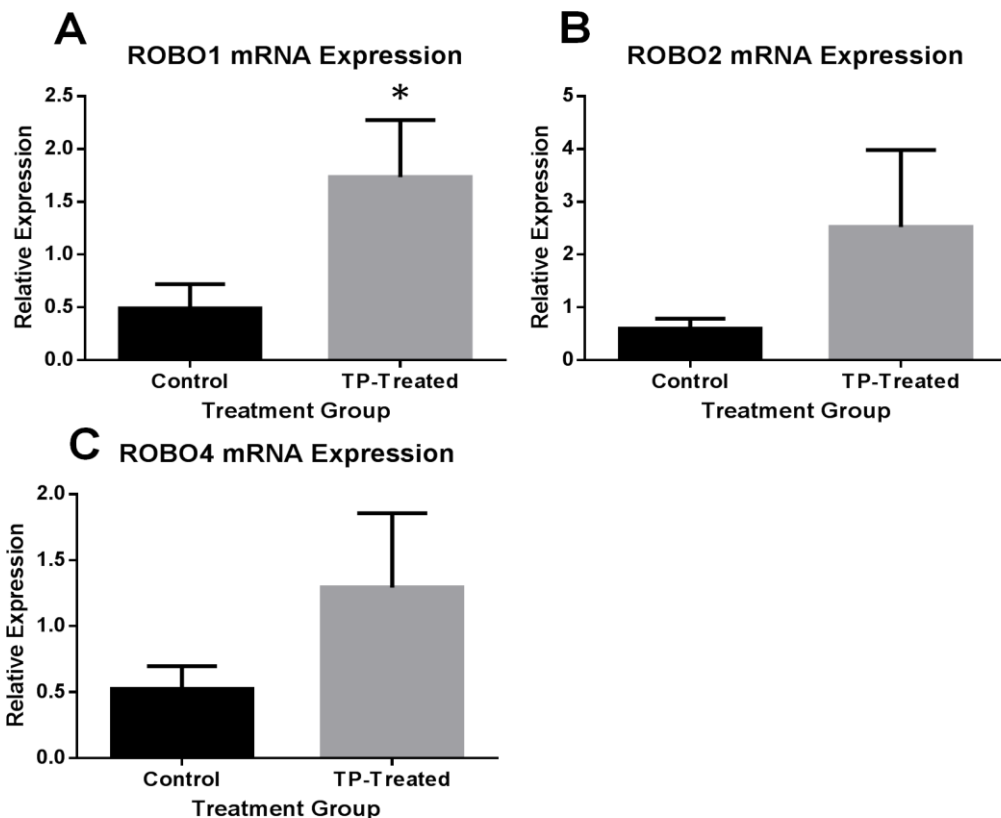


Figure 6.5: qRT-PCR analysis was used to further examine the *SLIT/ROBO* pathway, at the mRNA expression level in cortex tissue of GD90 male sheep (n=6). There was a significant increase in mRNA expression of *ROBO1* in the TP-treated group, when compared to the control group (A,  $p = 0.0422$ ) and no change in the expression of *ROBO2* and *ROBO4* in GD90 male sheep, that were exposed to androgens (B and C,  $p = 0.1406$  and  $0.1584$  respectively). A 4.21-fold increase in *ROBO2* and a 2.47-fold increase in *ROBO4* mRNA expression in the TP-treated group were calculated.

### 6.2.6 Comparison of microarray and validation results

A comparison of a panel of significant microarray results taken (TP-treated versus control-treated group) and their validation by qRT-PCR on GD90 male sheep was performed (Table 6.3). Although the validation showed a trend towards upregulation of the GOIs, only one showed a significant increase in mRNA as a result of androgen exposure (COL4A2,  $p = 0.0352$ ). Interestingly, NPY3R was downregulated in the microarray analysis but in the validation method of qRT-PCR, the mRNA expression showed a trend towards upregulation but this was not significant ( $p = 0.1796$ ).

Gene Name	Fold Change	Compacted P value (sig < 0.05)	Fold Change	P (sig < 0.05)
SLIT2	2.08-fold increase	0.007976612	< 2	0.4138
ERBA BETA1	2.03-fold increase	0.01801177	< 2	0.7907
NPY3R	2.13-fold reduction	0.019454515	3.27-fold <b>increase</b>	0.1796
COL4A2	2.66-fold increase	0.024283078	8.95-fold increase	0.0352
POMC	2.0-fold increase	0.037072923	2.05-fold increase	0.516
APP	2.23-fold increase	0.044590697	< 2	0.4538
ROBO1	N/A	N/A	3.54-fold increase	0.0422
ROBO2	N/A	N/A	4.21-fold increase	0.1406
ROBO4	N/A	N/A	2.47-fold increase	0.1584

Table 6.3: A table to summarise the panel of significant microarray results and their validation by qRT-PCR on the cortex of GD 90 male sheep (n=6).



## 6.3 Discussion

### 6.3.1 No identification of pyramidal cells of the developing cortex

We have observed that androgen over-exposure *in utero* dysregulates Purkinje cell development in the cerebellum. Therefore, we wanted to determine whether similar changes occur in pyramidal cells of the cortex in GD90 male sheep, as a result of androgen exposure. Basic features of the dendritic arbors of pyramidal neurons emerge during gestational weeks 17–25 in humans (Mrzljak *et al.*, 1988), approximately GD 50-80 in sheep. From weeks 26 to 34 (approximately GD 85-120 in sheep), migration should be completed and layer III and V dendrites continue to mature, as spines develop, basal dendritic length increases, and interneurons differentiate in layer IV (Mrzljak *et al.*, 1992). Therefore, the literature suggests that pyramidal cells can be detected in GD 90 ovine cortex tissue. Initially, the use of Nissl staining was utilised, whereby distinct pyramidal cells are usually observed (Ongur, *et al.*, 2003). However, we could not differentiate between cell type using this method but the developing different layers of the cortex could be observed, using Nissl staining (Figure 6.1).

Neuronal markers were used as an alternative method in identifying pyramidal cells. There were no positive Tbr1 cells within the ovine cortex observed when evaluating the optimal primary antibody dilution (Figures 6.2 A and B). Bulfone *et al.*, (1995) examined the protein expression of Tbr1 in the developing cortex and showed that Tbr1 is expressed in migratory and post-migratory pyramidal cells of fetal mice, and is an important factor in early brain development; which was why Tbr1 was chosen as a pyramidal cell marker for the current study. Calbindin is a Purkinje cell marker (Dougherty *et al.*, 2013) as it is abundantly expressed in these cells in the cerebellum (Bastianelli, 2003). Due to its specificity to neurons, we elected to try this as a pyramidal cell marker but there does not appear to be any specific, positive staining; but there was some staining of artefacts (Figure 6.2 C). Both neuronal markers did work in the developing cerebellum and therefore it can be concluded that the antibodies reacted with the ovine species (Figures 6.2 D and E). One explanation for no positive staining of pyramidal cells could be that these neurons are too immature *i.e.* undifferentiated at the gestational time point we are examining (GD90), which conflicts previous findings (Mrzljak *et al.*, 1988). Another option

is that perhaps the use of another pyramidal cell marker may be more suitable. Muller *et al.*, (2006) used calcium/calmodulin-dependent protein kinase II (CaMK) as a pyramidal neuron marker in rats whereas Chan *et al.*, (2001) used EMX1, a homeobox-containing gene, as the preferred pyramidal cell marker for neonatal and adult rats. Due to time constraints, we were unable to investigate whether these markers would be successful in fetal ovine tissue. Therefore the investigation into whether prenatal androgen exposure altered the cytoarchitecture of the developing ovine cortex was abandoned. Pyramidal neurons compose approximately 80% of the neurons of the cortex, and release glutamate as their neurotransmitter, making them the major excitatory component of the cortex (Santana *et al.*, 2004). Santana *et al.*, (2004) used adult male human tissue to show pyramidal cells of the cortex whereas the current study examined the developing cortex of fetal sheep, therefore this suggests that the time point, GD 90 in sheep, was too early to detect cell differentiation. To determine whether androgen over-exposure affects cell migration in the developing ovine cortex, the layer widths could be measured and compared between the control and TP-treated groups.

### **6.3.1.1 No protein expression of AR in the developing cortex**

In the current study, AR protein expression was not observed in the developing cortex of sheep (Figure 6.3). By the end of the first postnatal week of the rat, neuronal migration into the cortex is complete (Bayer *et al.*, 1991). Pyramidal cells need to arrive at the appropriate layer of cortex prior to expression of the androgen receptor and therefore pyramidal cells do not express androgen receptors until they are differentiating (Bayer *et al.*, 1991). This may be the reason that in the present study AR expression was not observed in GD 90 sheep, due to immature pyramidal cells. This theory is supported by the finding that neuronal nuclei (NeuN), a marker for mature neurons, is detected in AR positive cells in the rat neonatal cortex (Nunez *et al.*, 2003). Thus, the increase in the density of AR immunoreactive cells may reflect an increase in neuronal complexity and synaptic connectivity.

Androgen receptors have been documented in the developing rat cerebral cortex, with the density of AR positive cell nuclei varying as a function of age and cortical layer (Nunez *et al.*, 2003). Specifically, Nunez *et al.*, (2003) demonstrated that AR positive staining in pyramidal cells of the cortex was less intense than in the pyramidal cell layer of the hippocampus. At PND 0 (the equivalent to GD 36-42 in ovine gestation), AR positive neurons were only observed in layer V and VI of the developing cortex of male rats. At PND 4 (the equivalent to GD 50-55 in ovine gestation), Nunez *et al.*, (2003) demonstrated that AR immunoreactivity was observed in layers II–VI. On PND 10 (the equivalent to GD 70-75 in ovine gestation), there were robust increases in the density of AR positive cells in layers II–IV and small increases in layers V and VI. This suggests that AR expression increases as the cortex develops. The current study has illustrated that this particular AR antibody was successful in locating AR positive nuclei in the developing male cerebellum (also GD 90) therefore it can be assumed that there was no issue with AR species reactivity. One explanation of why AR protein was not detected in GD 90 ovine cortex tissue include the gestational time point that has been examined in the current study is before neuronal differentiation in sheep and may be why AR positive pyramidal cells were not observed in the present study. Another may simply be that there is low AR protein expression in GD 90 ovine cortex tissue and therefore is below the threshold of detection, using immunohistochemistry.

### **6.3.1.2 Testosterone may be indirectly mediated by the estrogen receptor**

Shughrue *et al.*, (1990) showed that cortical cells migrate while expressing the estrogen receptor (ER), so it could be suggested that testosterone acts indirectly by the aromatisation into estrogen and ultimately mediated through ER. (Nunez *et al.*, 2003) illustrated that at PND 0, ER expression was restricted to pyramidal cells in layers V and VI of the cortex. At later stages of cortical development, ER expression occurred in the more superficial layers and not in the deep layers. Cortical cell migration into layers V and VI of the cortex is completed by PND 0, which is also when Nunez *et al.*, (2003) detected AR expression in pyramidal cells. These findings suggest that during early stages of cortex development, testosterone effects maybe indirectly be mediated through ER, which could be why no AR expression was observed in the current study's cytoarchitectural investigations. However, qRT-PCR analysis demonstrated that, AR, ER $\alpha$  and ER $\beta$  mRNA expression was detected in the cortex, but at low levels ( $C_T$  values for each GOI was approximately 30). This suggests that the reason AR expression could not be detected using immunohistochemistry, is due to the level of expression being below the threshold detection limit for this technique.

### **6.3.1.3 Summary**

Although Nissl staining revealed the different layers that comprise the cortex and neurons in the developing cortex, we could not identify pyramidal cells. This is because pyramidal cells were indistinguishable from granule cells. As a consequence, the investigation of whether the cytoarchitecture of the developing cortex, and in particular the pyramidal cells, was altered as a result of androgen exposure was abandoned. The current study has also demonstrated that there is no detectable AR protein expression at this particular time point in the gestational period of sheep. Although the cortex cannot be examined at the cellular level, an investigation into whether there are alterations at the mRNA level as a result of androgen exposure can still be evaluated.

## 6.3.2 Microarray analysis of GD 90 male ovine cortex tissue

### 6.3.2.1 Microarray validation using qRT-PCR

Microarray analysis revealed nineteen genes had altered expression in TP-treated GD 90 cortex tissue, when compared to the control. Fourteen of these genes were confirmed to be due to an androgenic effect, as they were altered when analyses were performed to compare TP- and DES-treated samples. The remaining five genes were only altered when comparing expression levels of TP-treated samples with the control samples, thus, implying the genes are also due to an androgen-driven mechanism.

To confirm the differential expression of genes identified by microarray analysis, a panel of six upregulated (five genes) and downregulated (one gene) genes was evaluated using qRT-PCR, as a means of microarray validation. The data from the microarray validation (using qRT-PCR) indicates that only one of these genes, *COL4A2* (Figure 6.4 F), was significantly altered (upregulated) at the mRNA level, with a further four genes, *SLIT2*, *POMC*, *ERBA-BETA1* and *APP* (Figure 6.4 A, B, C and E), revealing trends towards increases in the TP-treated cortex samples but no significant differences between the control and TP-treated cortex groups. Interestingly, *COL4A2* has the largest fold change for both microarray and qRT-PCR analysis, compared to the other GOIs. The qRT-PCR data positively corresponds to the microarray data *i.e.* both techniques imply that these five genes are increased, significantly or not, as a response to prenatal androgen over-exposure. However, one gene, *NPY3R* (Figure 6.6 D), indicated a trends towards an increase in mRNA expression in the TP-treated samples using qRT-PCR, which is contradicting.

#### 6.3.2.1.2 Differences between microarray and qRT-PCR approaches

Although both techniques measure mRNA levels of a particular sample, it is important to point out some of the differences between microarray and qRT-PCR. Total RNA used in microarray experiments is subjected to an additional linear amplification step. This is used to amplify nanogram quantities of total RNA, where only small amounts of sample are available for microarray analysis (Jenson *et al.*, 2003) whereas micrograms of total RNA is typically used in qRT-PCR experiments. In addition, data normalisation fundamentally differs between microarray analysis and qRT-PCR, as microarray requires global normalisation, while qRT-PCR generally utilises the expression of one or more reference

genes against which all other genes are standardised to (Morey *et al.*, 2006). Therefore, selection and appropriate application of normalisation criteria may play a major role in the differences found between these two methods. It is also likely that the probe and primer sequences chosen for microarray and qRT-PCR analysis, respectively, are not identical and therefore why we observe inconsistencies in the data from microarray and its validation using qRT-PCR. One approach to eliminate this factor would be to design primer and probe sequences that can be utilised in both microarray and qRT-PCR experiments. This would give confidence that both techniques are detecting the same fraction of the particular gene of interest. Investigating and quantifying protein levels would allow the evaluation of any alterations in protein expression of the aforementioned genes would also be beneficial to evaluate the functional consequence prenatal androgen exposure has on the translation of these genes. Another point to mention is that the two methods may have amplified different splice variants of each gene of interest. Previous literature suggests that all the genes of interest, *SLIT2*, *POMC*, *ERBA BETA1*, *NPY3R*, *APP* and *COL4A2*, have splice variants (Gupta and Pillarisetti, 1999; Karolak *et al.*, 2011; Leder and Silverstein, 2006; Levy *et al.*, 2006; Little *et al.*, 2002; Strait *et al.*, 1991). Therefore, the qRT-PCR data may not reflect the microarray analysis as the may detect different splice variants of each gene.

### **6.3.2.2 Microarray analysis - changes in gene expression**

The microarray data indicates that *SLIT2*, *POMC*, *ERBA-BETA1*, *APP* and *COL4A2* genes are upregulated as a response of prenatal androgen overexposure (Table 6.1) whereas *NPY3R* was downregulated (Table 6.1). Here, each gene and function is briefly described (*SLIT2* is discussed in greater detail below).

#### **6.3.2.2.1 ERBA BETA1**

Thyroid hormones are known to be important for optimal development of the human central nervous system. The thyroid hormone receptor beta1 (*ERBA BETA1*) has the ability to bind triiodothyronine ( $T_3$ ) with high affinity and specificity and has been shown to be expressed in the human developing cortex (Chan *et al.*, 2002). Thyroid hormone deficiency during development results in multiple morphological alterations in the rat brain. These alterations include a decrease in dendritic spine number and reduced number and altered distribution of pyramidal cells of the cortex (Ruiz-Marcos *et al.*, 1983; Schwartz, 1983). These may be possible consequences of the up-regulation of *ERBA BETA1* observed in the microarray data and could be established by examining pyramidal cell morphology by utilising histology, at a later time point, such as adulthood, in male ovine cortex tissue.

#### **6.3.2.2.2 NPY3R**

The neuropeptide Y receptor (NPY) is a 36-amino acid peptide that is widely distributed in the CNS. NPY stimulates food intake in sheep (Adam *et al.*, 1997), modulates cognition in humans (Flood *et al.*, 1987) and inhibits neuronal excitability (Colmers and Bleakman, 1994). In addition, NPY is thought to play a role in the pathophysiology of certain mood disorders and also in the mechanism of action of antidepressant drugs (Heilig *et al.*, 1988; Wang *et al.*, 2013). There are 6 receptor found subtypes (*NPY1R-NPY6R*) and in the current study, *NPY3R* is downregulated in the TP-treated cortex samples. It has been proposed that *NPY3R* possesses a high affinity for NPY in the rat brain (Grundemar *et al.*, 1991) and bovine adrenal glands (Wahlestedt *et al.*, 1992). However, evidence for the existence of this subtype is circumstantial as the clone initially reported as the  $Y_3$  receptor (Rimland *et al.*, 1991) does not actually bind NPY (Herzog *et al.*, 1993). In fact, this clone was later found to

belong to the cytokine receptor family (Feng *et al.*, 1996), and so the gene is also known as CXCR4.

#### **6.3.2.2.3 COL4A2**

Collagen type IV alpha 2 chain (*COL4A2*) is ubiquitously expressed in basement membranes during early developmental stages. (Favor *et al.*, 2007) observed defects in mutant heterozygotes mice that reflect disturbances in the migration of neurons. They established that in the areas of defective migration, pseudocysts arise in the cortical plate and the neurons that normally would populate this area either migrate abnormally around this area, with pathological attachments to the arachnoid or remain trapped basal to the cyst. We have observed an upregulation of *COL4A2* expression, both in microarray and qRT-PCR, in GD 90 male ovine cortex that that been over-exposed to androgens. Therefore, this suggests that pyramidal cell migration is altered in these samples. Examining a later timepoint, such as the neonatal period, and investigating *COL4A2* mRNA expression using qRT-PCR and pyramidal cell number, location and morphology using histology would aid in establishing dysregulation of neuronal migration.

#### **6.3.2.2.4 POMC**

Proopiomelanocortin (*POMC*) encodes a polypeptide hormone precursor that undergoes extensive, tissue-specific, post-translational processing via cleavage by prohormone convertases (PC1 and PC2). The encoded protein is predominantly synthesised in corticotroph cells of the anterior pituitary and expressed in various brain regions. Adrenocorticotrophin (ACTH) is the only *POMC*-derived peptide with a clear action on adrenocortical function (Peytremann *et al.*, 1973). Binding of ACTH induces cAMP production and steroidogenesis which results in the secretion of glucocorticoids, androgenic steroids and mineralocorticoids. The acute effect of ACTH is to increase conversion of cholesterol into pregnenolone, the initial step in cortisol biosynthesis and ultimately steroidogenesis. It has been suggested by two groups that PC1 deficits leads to an increase in *POMC* plasma levels is generated by highly stimulated corticotroph cells responding to the negative feedback loop between cortisol and ACTH the absence of PC1 disturbs *POMC* processing, which leads to striking accumulations of unprocessed *POMC*, and a significant upregulation of *POMC* mRNA in PC1 knockout mice (Jackson *et al.*,



1997). We have observed a similar relationship between PC1 and POMC – POMC gene expression is up-regulated in the TP-treated group, when compared to the control group whereas its precursor, PC1, gene expression is down-regulated in the DES-treated group, when compared to the TP-treated group. In other tissues, including the hypothalamus, placenta, and epithelium, *POMC* is also cleaved, giving rise to peptides with roles in melanocyte stimulation, and immune modulation (Cowley *et al.*, 2001; Heijnen *et al.*, 1987) Mutations in this gene have been associated with early onset obesity, adrenal insufficiency and red hair pigmentation (Krude *et al.*, 1998).

#### **6.3.2.2.5 APP**

Amyloid beta (A4) precursor protein, transcript variant 8 (*APP*) encodes a cell surface receptor and transmembrane precursor protein that is cleaved by secretases to form a number of peptides. Multiple transcript variants encoding several different isoforms have been found for this gene. APP variants are highly expressed in neurons and have been localised to somata, dendrites and axons. APP undergoes fast axonal transport and recently all three APP variants have been identified as constituents of the pre-synaptic active zone of CNS neurons (Marcinkiewicz and Seidah, 2000). APP generates  $\beta$ -amyloid which is central to the pathogenesis of Alzheimer's disease. Therefore, the up-regulation of APP gene expression, observed in the TP-treated group, implies that  $\beta$ -amyloid production is increased and this could be a predisposing factor of Alzheimer's disease. Alzheimer's disease, the most common type of progressive dementia in the elderly, is characterized by neurofibrillary tangles and parenchymal deposits of  $\beta$ -amyloid (Terry and Katzman, 1983). In the United States of America, approximately two thirds of people with Alzheimer's disease are women (Alzheimer's Association, 2014). Furthermore, it has been suggested that APP is an androgen-induced gene that promotes proliferation activity of breast carcinoma cells (Taqagi *et al.*, 2013).

### 6.3.2.3 An evaluation of the SLIT/ROBO pathway

The most significant change in gene expression, in terms of the highest p value, from the microarray data was *SLIT2*, where we observe an increase in gene expression in the TP-group, when compared to the control group (Table 6.1). This gene's signalling mechanism was further investigated by examining the receptors which mediate SLIT2s effects - *ROBO1*, *ROBO2* and *ROBO4*. qRT-PCR was performed and the data indicated that only *ROBO1* was altered at the mRNA level, as a response to prenatal androgen over-exposure (Figure 6.5 A).

The *SLIT/ROBO* system represents an evolutionarily conserved chemorepulsive ligand-receptor system which is involved in axon guidance, axonal branching (Ozdinler and Erzurumlu, 2002; Sang *et al.*, 2002; Wang *et al.*, 1999) and regulation of neural cell migration (Hu, 1999; Yuan *et al.*, 1999). In vertebrates, three Slit homologs are known (*SLIT1*, *SLIT2*, *SLIT3*), The Robo family of transmembrane receptors encompasses four members in vertebrates - *ROBO1*, *ROBO2*, *ROBO3* and *ROBO4* (Mertsch *et al.*, 2008). *SLIT2* is thought to act as molecular guidance cue in cellular migration and its function appears to be mediated by interaction with roundabout homolog receptors (Ghose and Van Vactor, 2002). During neural development, *SLIT2* is involved in axonal navigation at the ventral midline of the neural tube and projection of axons to different regions and further plays a role in the branching and arborization of CNS sensory axons (Dickson and Gilestro, 2006).

Previous literature emphasises that a lack of expression of these two genes influences neurogenesis; but there appears to be no evidence for how an upregulation of *SLIT2* and/or *ROBO1* may shape cortical development. For example, the absence of *ROBO1* leads to premature migration of interneurons to the cortex in *ROBO1* knockout mice (Andrews *et al.*, 2008) and evidence has further suggested that *ROBO1* and *SLIT2* signalling plays a role in the morphological differentiation of neurons of the forebrain (Andrews *et al.*, 2008). In a similar study to that of the current one, Hogg *et al.*, (2011) demonstrated no difference in *SLIT2* mRNA expression between GD 70 ovine control and TP-treated ovary samples, which were over-exposed to TP *in utero*, which implies that *SLIT2* is not regulated by androgens in the ovary. This finding contradicts with our results, however this could be due to the earlier gestational period (GD 70 as opposed to GD 90 in the present study) or simply that the ovine central

nervous system differs in the mechanism for the regulation of *SLIT2*, compared to the ovaries.

## 6.4 Conclusions

The developing ovine cortex is an androgen target tissue, which, if exposed to excess androgens, shows dysregulated gene expression of genes involved in diverse developmental functions such as cellular/neuronal migration, steroidogenesis, endocrine disruption and neurodegenerational disease. The altered genes of the ovine cortex, which were established by microarray analysis, need to be examined at the protein level, by means of protein quantification, in order to determine whether the changes observed at the mRNA level, are reflected at a functional level. *SLIT2*, *ERBA-BETA1* and *COL4A2* are genes that encode for proteins that are implicated in neuronal migration, synaptogenesis and differentiation. The genes, *NPY3R*, *POMC* and *APP* are associated with a variety of functions in the developing brain including appetite, steroidogenesis and neurodegenerative disorders. The altered mRNA levels observed for these genes would imply that these aforementioned processes are affected by androgens. The relationship between the upregulation of *SLIT2* and *ROBO1*, needs to be established, in terms of any structural effects on the developing ovine male cortex. Another ROBO receptor that mediates *SLIT2* signalling is *ROBO3*, whose interactions are also associated with neuronal migration (Barber *et al.*, 2009). It would be interesting to examine *ROBO3* in order to determine whether this ROBO isoform is altered at the mRNA level as a result of prenatal androgen overexposure as this was not investigated in the current study due to primer set availability.

The validation of microarray needs to be reassessed by the production of primer sequences that are identical to the probe sequences used, in order to determine whether the significant changes in gene expression found through microarray analysis are truly altered at the mRNA level. Furthermore, an investigation should be carried to evaluate whether there are any alterations in the cytoarchitecture of the cortex. This could be achieved by utilising Golgi staining, optimising the immunohistochemistry protocols already identified in this chapter and the visualisation of such work by using an electron microscope. It would also be interesting to examine ovine cortex tissue at a later gestational time point through to adulthood, to determine whether the changes observed at the mRNA level at GD 90 remains throughout life. Due to time constraints, these options were not fully explored. It would have been interesting to be able to quantify and compare protein expression between the control and TP-treated

sample, in order to determine if the same result *i.e.* upregulation of these genes, is reflected at the functional protein level.

The prefrontal cortex is known to play a pivotal role in cognitive function, personality expression, decision making, moderating social behavior and executive function (Fuster, 1997). Therefore examining the cognitive function of sheep from lambs into adulthood would give new insight in to whether prenatal androgen over-exposure has an impact on this cognitive ability. This could be achieved by a battery of tests that examine executive and cognitive function, such as discrimination and reversal learning, attentional set shifting and intra- and extradimensional set shifting tasks. These tests have already been assessed in sheep and it was concluded that sheep can not only perform these tasks but also display executive and cognitive function (Mortan and Avanzo, 2011).

## **CHAPTER 7**

### **General discussion**

## 7. General Discussion

### 7.1 Summary

Steroids exert effects on the nervous system and play a major role in the development of the CNS, e.g. in the hypothalamus and pituitary gland. These brain areas are involved in sexual behaviour and have been the focus of most neuroendocrine studies to date. However it is known that other areas of the brain, e.g. the cerebellum and cortex, express those proteins necessary for local steroidogenesis and steroid signalling (Baulieu, 1998) however the impact of steroids on cerebellar and cortex development has yet to be investigated.

#### 7.1.1 Methods

We utilised a fetal programming model whereby ovine fetuses were subjected to an ultrasound-guided intraperitoneal 20mg injection of TP or sham (vegetable oil vehicle) at GD 62 and repeated at GD 82. The fetuses were sacrificed at GD 90 and their brain tissue was dissected. The brain was bisected along the midline and one hemisphere and half of the cerebellum stored in neutral buffered formalin. The other hemisphere cerebellum, hippocampus and prefrontal cortex was frozen on dry ice before being stored at -80°C.

#### 7.1.2 The cerebellum

Total RNA and cDNA was isolated from the frozen fetal ovine cerebellar tissue. The expression of a panel of genes of interest, including genes that are responsible for steroidogenesis and steroid signalling, was measured using quantitative polymerase chain reaction (qRT-PCR). Using the  $\Delta\Delta C_T$  method, a decreased mRNA expression of Steroidogenic acute regulatory (*StAR*, p value = 0.0264) and an increased mRNA expression of progesterone receptor (PR, p = 0.0341) was observed in the TP-treated group. There were also trends towards alterations in mRNA expression for several other genes; *SRD5a1*, *SRD5a2*, *ER $\alpha$*  and *GFAP* observed between the control and TP-treated groups (an increase in *SRD5a1* mRNA expression p= 0.0770; a decrease in *SRD5a2* expression p=0.0986; decreased *ER $\alpha$*  mRNA expression, p = 0.0721 and increased *GFAP* expression, p = 0.0641).

The cerebella that were stored in neutral formalin buffer were used for immunohistochemical analyses, in order to determine whether the number of Purkinje cells that express particular steroid proteins are altered due to prenatal

androgen exposure. Using light microscopy, Purkinje cells were counted (positive cells/mm<sup>2</sup>), data from treated and control groups were compared using statistical analysis. This revealed an increase in the number of cells which express of StAR, PR, SRD5a1 and SRD5a2 ( $p = 0.0002, 0.0201, 0.0001$  and  $0.0001$ , respectively) in the TP-treated group. Using calbindin, a Purkinje cell marker, an increase in positive Purkinje cells/mm<sup>2</sup> ( $p = 0.0008$ ), a reduction in Purkinje soma size ( $p = 0.0328$ ), increased Purkinje cell layer width ( $p = 0.0002$ ) and that the Purkinje cells were arranged in clusters, as opposed to the typical monolayer usually observed ( $p = < 0.0001$ ) were also observed.

### **7.1.3 The cortex**

Total RNA and was isolated from the frozen fetal ovine cortex tissue from both the control and TP-treated groups ( $n=6$ ). The concentration of each RNA sample was measured using a spectrophotometer. These samples were sent to Oxford Gene Technology (Oxford, U.K.) where microarray analysis was performed in order to detect differences in gene expression between the control and TP-treated groups. From the 15 000 genes that were analysed on the Agilent sheep transcriptome wide microarray (AGEOD-10778), 19 genes had altered gene expression in the TP-treated cortex samples. To confirm the differential expression of genes identified by microarray analysis, a panel of six genes (five upregulated and one downregulated) was evaluated using qRT-PCR, as a means of microarray validation. The data from the microarray validation (using qRT-PCR) indicates that only one of these genes, *COL4A2*, was significantly altered (upregulated) at the mRNA level, with a further four genes, *SLIT2*, *POMC*, *ERBA-BETA1* and *APP*, revealing trends towards increases in the TP-treated cortex samples but no significant differences between the control and TP-treated cortex groups. The qRT-PCR data positively corresponds to the microarray data *i.e.* both techniques imply that these five genes are increased, significantly or not, as a response to prenatal androgen over-exposure. However, for one gene, *NPY3R*, we identified a trend towards an increase in mRNA expression in the TP-treated samples using qRT-PCR, whereas the microarray analysis indicated a significant downregulation of this gene and therefore, both data obtained contradict one another.

Nissl staining revealed neurons in the developing cortex, however, pyramidal cells were not identified. The cortex tissue that was stored in neutral formalin



buffer were used for histological analyses, in order to determine whether the number, size and location of Pyramidal cells are altered due to prenatal androgen exposure. The different layers that comprise the cortex were ascertained, however, the migration of the layers seem immature. This finding suggested that the development of the cortex of GD 90 sheep was still at a very early stage. This may be why pyramidal cells were indistinguishable from granule cells. As a consequence, the investigation of whether the cytoarchitecture of the developing cortex was altered as a result of androgen exposure was abandoned. The current study has also demonstrated that there is no detectable AR protein expression at this particular time point (GD 90) in the gestational period of sheep.

#### **7.1.4 Summary of results**

The results observed throughout this thesis provide strong evidence that prenatal androgen over-exposure leads to delayed development of the ovine cerebellum and cortex. Specifically, we have demonstrated dysregulated cerebellar Purkinje cell development in terms of number and morphology which suggests immaturity of the cerebellum. Altered cortical gene expression for genes associated in neuronal migration, synaptogenesis and differentiation was observed, thus indicating that the cortical development is also effected by TP administration. Further investigation into gene expression of these genes in the cerebellum using qRT-PCR; and immunohistochemical analysis in cortical tissue in order to determine pyramidal cell number and morphology would provide additional insight into the delayed brain development and immaturity of neurons already observed.

Furthermore, genes which are associated with steroidogenesis have altered expression levels in the ovine cerebellum. In the cortex, changes in the level gene expression of a variety of functions including appetite, steroidogenesis and neurodegenerative disorders were also observed. The altered mRNA levels detected for these genes would imply that these aforementioned processes are also affected by androgens.

## **7.2 Limitations in methodology**

### **7.2.1 Sample availability**

In general, it is acknowledged that sample numbers (n=4) were low in the current study, which may lead to under powering of the analyses. This was due to limited sample availability. It would have been ideal if more samples were obtained, in order to observe whether the alterations in gene expression reflect the bigger population. More samples would have also allowed for steroid assays, such as pregnenolone and progesterone, to be performed so that levels of these steroids could be determined. This would have provided more insight into the mechanisms of the regulation of *StAR* and *PR* genes.

### **7.2.2 Protein expression**

It is of importance to evaluate whether the changes observed at the mRNA level are also reflected in the functional product *i.e.* protein level. Western blotting would enable the protein product to be quantified and the results from control and TP-treated sample could be compared. Due to limited sample size, protein extraction was attempted on the by-product of RNA isolation from the samples. This method of protein extraction, although published by Hummon *et al.*, (2007), was deemed unsuitable as it resulted in poor quality protein thus, several proteins of interest could not be measured using Western blotting.

The quantification of protein would have helped to identify whether changes at mRNA level are reflected in the functional product. Therefore at present, little is known about the functional consequences of the alterations observed at the mRNA level as a result of prenatal androgen over-exposure. Future studies should try and resolve the fundamental question of whether excess androgens also alter protein expression.

### 7.3 Androgenic or estrogenic effect?

Testosterone can be metabolised into estradiol (by the action of aromatase) *and* into DHT (by the action of 5 $\alpha$ -reductase), a non-aromatisable androgen with a high affinity to AR. Therefore, it should be determined whether the changes observed in the developing male ovine cerebellum and cortex are due to a direct androgen effect or an indirect estrogen effect.

There is potential for the placental aromatisation of testosterone into estrogenic metabolites. Therefore, to define the effects of both sex hormones, fetal male sheep were directly injected with either TP or the potent estradiol receptor agonist, diethylstilbestrol (DES). We did investigate whether the observed alterations in cerebellar mRNA expression were due to an androgenic or estrogenic effect. The present study has examined DES-treated GD 90 male ovine cerebellar samples and compared gene expression levels for *StAR*, *PR*, *SRD5a1* and *SRD5a2* to the control samples. The results confirmed that the mRNA expression alterations seen for *STAR*, *PR*, *SRD5a1* and *SRD5a2* in the cerebellar TP-treated group are due to an androgen effect rather than an estrogen one. Therefore, an androgen-dependent mechanism is driving the changes observed at the mRNA level. The DES-treated samples should be further explored at the gene and protein level by utilising qRT-PCR and Western blotting in order to determine whether any of the observed changes are driven by an estrogenic effect.

The current study has also established that the observed change in Purkinje cell number is due to a direct androgen effect on these cells as there was no significant change in the number of Purkinje cells in the DES-treated group, when compared to the control group. One observation from examining the DES-treated GD 90 male ovine cerebellar tissue was that some of the random field views appeared to have the typical Purkinje cell monolayer, a sign of maturation whereas as others had several rows of Purkinje cells, which indicates immaturity. This implies that the Purkinje cell development seems to fall between the typical development observed in the control group, and the dysregulated development detected in the TP-treated group. Another observation is that the Purkinje cell typical shape, large and triangular soma, is prominently displayed in the DES-treated samples and these cells appear even more mature than the control samples. Estrogen has been shown to promote

the development and survival of Purkinje cells in rats (Sakamoto, *et al.*, 2003), therefore the observed alterations may be due to estrogen over-exposure. The analysis of Purkinje and Pyramidal cell length from both cerebellar and cortical tissue from control and DES-treated samples would give insight into the observed morphological changes in cytoarchitecture.

There appears to be hypervariance at both the mRNA level and Purkinje cell numbers amongst the DES-treated cerebellar samples. As discussed previously, the RNA isolation was performed by a student and therefore, the high variance was deemed to be due to inexperience and inconsistency. However, immunohistochemical analyses was performed by the experienced author and therefore, would suggest that manual error may not be the cause of hypervariance but down to natural causes.

### **7.3.1 Examining androgen receptor activity to confirm whether TP effect is indeed mediated by androgen**

Examining fetal male sheep that were directly exposed to dihydrotestosterone (DHT) would also help identify whether the changes observed in the TP-treated samples were indeed an androgenic effect. If the DHT-treated samples showed similar significant changes, when compared to the control group, as the TP-treated group did, then it could be concluded that androgen is directly generating alterations at the gene and protein level.

Investigating whether the inhibition of androgen receptor activity by flutamide, an AR antagonist, or whether DHT affects *StAR* or *PR* mRNA expression would allow us to confirm whether it is indeed a direct effect of TP that has altered *STAR* and *PR* mRNA expression in the current study. This could be achieved by treating cultured primary Purkinje cells with TP or DHT in the presence and absence of flutamide and measuring downstream *StAR* and *PR* mRNA expression by means of qRT-PCR. If the TP and DHT treated cells both had significant differences in gene expression, compared to the control, then this would confirm that it is indeed an androgen effect. Alternatively, if cells treated with DHT showed no alterations at the mRNA level and the one treated with TP did, then the observed changes are most likely due to the mediating of testosterone into estrogen, thus an indirect effect of androgens. However, there was an issue with the DHT-treated cohort of animals which prevented the use of them and therefore these samples were not investigated. In short, our

collaborators at The University of Edinburgh were unable to verify any DHT treatment in positive control experiments.

### **7.3.2 Lack of androgen receptors in the cortex**

The present study revealed that AR protein expression was not found in GD 90 male ovine cortex tissue using immunohistochemistry. However, we have observed changes in gene expression due to androgen over-exposure. These changes therefore may have been mediated by the aromatisation of androgen into estrogen which then acted on estrogen receptors. By utilising qRT-PCR analysis, AR, ER $\alpha$  and ER $\beta$  mRNA expression was detected in the cortex, but at low levels ( $C_T$  values for each GOI was approximately 30). Conversely, the mRNA expression of these genes in the cerebellum appears to be higher with  $C_T$  values ranging from 27-28 (AR), 19-24 (ER $\alpha$ ) and 25-27 (ER $\beta$ ). This suggests that the reason AR expression could not be detected using immunohistochemistry, is due to the level of expression being below the threshold detection limit for this technique. Therefore, we cannot rule out that the gene expression alterations observed in GD 90 male ovine cortical tissue, by utilising microarray analysis, are driven by an androgen-dependent mechanism.

Future experiments should include exploring estrogen receptor localisation in the cortex by utilising immunohistochemistry to determine whether it is possible for androgens to be mediated by this mechanism. Another possible explanation is that immunohistochemistry analysis was unable to detect any AR protein as the level of expression was below the threshold of detection, as mRNA levels for AR were detected, albeit the levels were low.

## **7.4 The consequences of prenatal androgen over-exposure**

### **7.4.1 The cerebellum**

Cerebellar Purkinje cells are a major site of neurosteroid formation (Tsutsui *et al.*, 2003), implying that these cells may be affected by developmental androgen exposure. The cerebellum has a clear developmental pathway and an established cytoarchitecture that has enabled us to more easily identify effects of TP treatment on the development of the brain. How cerebellar gene expression is affected by androgen exposure during development - and how this subsequently changes the brain's cytoarchitecture and function was investigated.

#### **7.4.1.1 Structural cerebellar alterations**

The current study has illustrated how excess prenatal androgen dysregulates cerebellar Purkinje cell development. How this occurs still remains unclear. As a result of prenatal androgen over-exposure, we have observed that Purkinje cells are smaller, rounder, more numerous, clustered together and appear to display an underdeveloped dendritic network. It appears that these findings may imply delayed Purkinje cell development, as Rees and Harding (1997) demonstrated similar findings in sheep (untreated, control group) of an earlier gestational period than the one used in the current study. The way in which androgen dysregulates development should be examined, by evaluating signalling pathways and Purkinje cell arrester genes, such as *GAS7* (Ju *et al.*, 1998). *COL4A2* gene expression was up-regulated in GD 90 male ovine cortical tissue that was exposed to excess androgens. This gene encodes for a protein that is implicated in neuronal migration (Favor *et al.*, 2007) and should be investigated in the developing cerebellum to determine whether this gene is altered, by utilising qRT-PCR analysis, in the same manner as it is in the cortex. This may aid in establishing a mechanism by which Purkinje cell development is dysregulated due to androgen over-exposure. The migration of Purkinje cells is also regulated by factors secreted from the EGL, such as Reelin (Jensen *et al.*, 2002). The current study has examined *RELN* mRNA levels, using qRT-PCR, and saw no changes in expression between the control and TP-treated groups. This suggests that the Purkinje cell dysregulation that is observed as a result of androgen over-exposure does not involve *RELN*.

There is little literature on the effects of androgens on Purkinje cell development. Zhang *et al.*, (2000) revealed that testosterone administration decreased differentiated cell number in cultured cerebellar neurons from GD 14 rats. The current study confirms this. Previous studies have shown that estrogen and progesterone are implicated in facilitating cerebellar Purkinje cell dendritic growth in neonatal rats, however soma diameter is not influenced by these steroids (Sakamoto *et al.*, 2003; 2001). This suggests that in our study, dendritic growth has been dampened by a reduction of estrogen and/or progesterone. We have observed that *PR* gene expression is upregulated in the TP-treated samples which could be the result of low progesterone levels and therefore would support this theory. The measurement of progesterone levels and the dendritic networks and spines in the control and TP-treated groups and comparing them would establish whether there is a significant difference in the dendritic distribution between the two groups.

## **7.4.2 The cortex**

Grobin *et al.*, (2003) demonstrated that developing rat forebrain neurons are sensitive to perturbation by neuroactive steroids. Pyramidal cells are the output neurons of the cerebrum (Elston, 2003), and so transmit signals to other parts of the CNS. There is substantial literature that illustrates that stress hormones, such as glucocorticoids, are associated with increased risk in illness and changes in cognition. Furthermore, anomalies in the cortex, genetic or structurally, have been implicated in several mental health disorders such as schizophrenia. For instance, stressful life events appear to increase the probability of a psychotic episode in schizophrenics (Ventura *et al.*, 1989). Therefore, we wanted to examine whether androgen, a male sex hormone, would alter gene expression or cause structural affects in GD 90 male ovine cortex tissue. This was investigated by means of microarray analysis and histology.

### **7.4.2.1 Microarray validation**

qRT-PCR was utilised as the preferred microarray validation method in the present study. However, only one gene of interest, *COL4A2*, was validated using this method. This gene had the largest fold change in both the microarray and qRT-PCR analysis and so it is possible that the issue with microarray validation is due to sensitivity and that qRT-PCR as a technique, was only sensitive enough to confirm alterations in gene expression for *COL4A2*. Therefore, as extensively discussed in Chapter 6, further work should be performed in order to re-attempt to validate the microarray results, such as the re-designing of primers. Nineteen genes were revealed to be altered as a result of prenatal androgen excess and from this, a panel of six genes were utilised for the validation. The other thirteen altered genes should also be examined by qRT-PCR in order to validate the microarray data.

### **7.4.2.2 Gene expression alterations**

For the cerebellar samples, genes of interest were specifically chosen and investigated using qRT-PCR, as these genes had either been affected in other tissues after androgen exposure or the genes were vital in androgen metabolism and ultimately the steroid signalling pathway. In contrast, microarray analysis was performed on the cortex samples which allowed 15 000 genes to be evaluated. Although both techniques measure mRNA levels of a



particular sample, it is important to point out some of the differences between microarray and qRT-PCR. It is interesting to note that the observed gene alterations in the cerebellar samples by qRT-PCR were not detected in the cortex tissue samples by microarray analysis. This demonstrates that androgens affect brain development in a region-specific manner. There were also differences in methodology when evaluating the two regions, therefore, selection and appropriate application of normalisation criteria may also play a major role in the differences found between these two methods.

#### **7.4.2.3 Establishing whether cytoarchitectural alterations are observed in the cortex**

The present study was unable to determine whether prenatal androgen exposure affects the cytoarchitecture of GD 90 male ovine cortex. This was mainly because pyramidal cells were indistinguishable from granule cells and the six layers that comprise of the cortex could not be differentiated enough to confidently measure each layer and then compare the results from the control and TP-treated groups. The pyramidal cells were being examined in further detail because these neurons are the primary output of the cortex and therefore have similar function to cerebellar Purkinje cells, which were altered in a variety of ways in the TP-treated group. Therefore, it was hypothesised that prenatal androgen exposure would alter pyramidal cell development. Immunohistochemistry and Nissl staining were utilised in the present study but with hindsight, perhaps Golgi staining would have been beneficial in helping answering this hypothesis. Due to time constraints, Golgi staining was not performed.

## 7.5 Plasticity during development

Developmental plasticity involves alterations to the projected development of a fetus as an adaptive response from insults to which they are exposed. Such adaptation during development may potentially modify the adult phenotype and predispose to diseases such as cardiovascular, metabolic, and neuroendocrine anomalies. The reproductive system is also acutely sensitive to the nutritional and hormonal milieus, which have been shown to induce phenotypic and functional changes. Connelly *et al.*, (2013) demonstrated that androgen over-exposure via fetal injection at GD 62 and 82 (the same conditions as the current study) resulted in altered cellular distribution in the testis. Furthermore, sons born to mothers with PCOS were found to have an increase in anti-Müllerian hormone (AMH) levels during infancy and childhood, which may indicate an increase Sertoli cell number or function. This suggests that exposure to androgens during fetal life can induce a permanent, functional effect on testicular physiology and it appears from these studies that such effects are likely to be dependent upon the timing of initial exposure to excess testosterone.

In the brain, plasticity changes are not all permanent and can change dramatically over time. When rats are placed in complex environments, there is a transient increase in dendritic length in the prefrontal cortex that can be seen after four days of complex housing, but this change disappears after 14 days. In contrast, there are no obvious changes in the sensory cortex after four days but clear, and seemingly permanent, changes after 14 days (Comeau *et al.*, 2010). The possibility that there are different chronic and transient experience-dependent changes in cerebral neurons is consistent with genetic studies showing that there are different genes expressed acutely and chronically in response to complex environments (Rampon *et al.*, 2000). The difference in how transient and persistent changes in neuronal networks relate to behavior is unknown. The observed changes in the current study show dysregulated cerebellar development in fetal male ovine, as a result of androgen exposure. Whether these changes will continue through into later life, remains unclear.

## **7.6 Delayed cerebellar development in other models**

Some chemical pollutants have the ability to act as hormone mimics. Because of a structural similarity with endogenous hormones, an ability to interact with hormone transport proteins, or an ability to disrupt hormone metabolism, these chemicals have the potential to imitate or block the effects of the endogenous hormone (Kimura-Kuroda *et al.*, 2007). These chemicals serve to disrupt the normal actions of endogenous hormones and thus have become known as “endocrine disruptors.” Because hormonally mediated events play a central role in CNS development and function, there is speculation that some of the cognitive deficits that arise from developmental exposure to environmental chemicals, and ultimately disrupted development, may be the result of endocrine disruption (Nishihara, *et al.*, 2003). Here, several different disruptors to Purkinje cells development are discussed.

### **7.6.1 Thyroid hormone**

Thyroid hormones (TH) promote the differentiation and growth of the Purkinje cell dendritic tree through the activation of the nuclear thyroid hormone receptors, TR $\alpha$ 1 and TR $\beta$ 1, and synapse formation (Boukhtouche *et al.*, 2010). A deficiency of thyroid hormones during development in humans causes cretinism which, among other abnormalities, is characterised by severe mental retardation. Rabie *et al.*, (1986) demonstrated that hypothyroidism during development can compromise the development of neurons. In cerebellar Purkinje cells of neonatal hypothyroid rats, there was a substantial delay and a permanent reduction of the size and branching of the Purkinje cell dendritic tree (Vincent *et al.*, 1982) going together with a reduction in the number of synapses in the molecular layer (Lauder, 1978; Nicholson and Altman, 1972). In the current study, we have identified through microarray analysis that *ERBA BETA1*, the gene that encodes for TR $\beta$ 1, is upregulated in GD 90 male ovine cortical tissue as a result of androgen over-exposure. It would be interesting to measure *ERBA BETA1* gene expression levels by qRT-PCR in the developing cerebellum in order to determine whether any changes in expression may contribute to the dysregulated Purkinje cell development observed in the TP-treated group.

### 7.6.2 Biphenyls

Biphenyls are organic compounds that are mainly utilised in the chemical industry, in the manufacture of textiles and dyes and of other chemicals. They are also used as heat transfer agents to produce polychlorinated biphenyls (PCBs). However, these compounds are also toxic environmental pollutants which tend to accumulate in animal tissues and go on to cause detrimental effects for the animal (Ness *et al.*, 1993). Kimura-Kuroda *et al.*, (2002) demonstrated that endocrine disruptors, PCBs and hydroxy-PCB (OH-PCB) metabolites suppress the thyroid hormone (TH)-dependent dendritic development of Purkinje cells in mouse cell cultures. T4+ and the addition of endocrine disruptors inhibited dendritic development in mouse cerebellar cell cultures. The Purkinje cells displayed abnormally shaped dendrites. Dendrites developed in the absence of T4 (T4-) but treated with a particular PCB metabolite, were immature forms similar to those *in vivo*, *i.e.* lack a primary thick shaft and have only thin branches directly coming from the soma. Bisphenol A (BPA) significantly inhibited the dendritic development of Purkinje cells in T4+. Therefore, this insult may disrupt normal brain development by both growth inhibitory and promoting effects. The literature discussed in this section points to the findings that thyroid hormone signalling is important to normal cerebellar development. Therefore, future experiments should involve examining thyroid hormone levels using radioimmunoassay and thyroid receptor gene expression levels and protein expression using qRT-PCR and immunohistochemistry, respectively, in GD 90 male ovine cerebellar tissue. It could be that our findings pose a similar mechanism to the findings of Kimura-Kuroda *et al.*, (2002) and that any type of insult, *e.g.* PCB exposure, steroid over-exposure or hypoxia, can have detrimental consequences to cerebellar development and ultimately motor and cognitive function.

### 7.6.3 SRC-1

SRC-1 is a nuclear receptor coactivator that plays a key role in transactivation of steroid hormone receptors (Sheppard *et al.*, 2001). Disruption of the SRC-1 gene in mice delays the generation of Purkinje cell precursors at an early embryonic stage and further delays the maturation of Purkinje cells after birth. Nishihara *et al.*, (2003) compared the brain function of SRC-1<sup>-/-</sup> mice with age-matched WT mice using a battery of well established behaviour tests. The results suggested that SRC-1<sup>-/-</sup> mice may have a dysfunction of motor strength,

motor learning and the motor performance are partially impaired, Motor learning tasks are mediated by Purkinje cells in the cerebellum and are frequently implicated in altered function of Purkinje cells (Linden, 1994; Raymond and Lisberger, 1996). The morphology of SRC-1<sup>-/-</sup> Purkinje cells developed to the same extent as WT Purkinje cells by PND 10, adult SRC-1<sup>-/-</sup> mice exhibited moderate motor dysfunction, suggesting that the abnormal development of Purkinje cells at early stage may have a negative impact on the cerebellar function in adulthood. SRC-1 should be examined at the mRNA level using qRT-PCR in order to determine any changes between the control and TP-treated groups and therefore establish whether this gene is involved in the dysregulated Purkinje cell development, observed in the TP-treated samples.

#### **7.6.4 Vulnerability during cerebellar development**

The literature as well as the results from the present study, indicate that cerebellar development is a critically vulnerable period and an array of insults can affect the morphology of the cerebellum that can have consequences for later life. What needs to be established is whether the insult inflicted on the fetally-injected ovine model used in the present study (TP administration) mirrors the cognitive deficits observed for other endocrine disruptors. To assess the role of androgen over-exposure in cerebellar function, a battery of behavioural tests should be performed to compare specific neural functions between the control and TP-treated groups. Motor coordination, strength, and balance could be assessed. Tests that examine executive and cognitive function, such as discrimination and reversal learning, attentional set shifting and intra- and extradimensional set shifting tasks could also be performed. These tests have already been assessed in sheep and it was concluded that sheep can not only perform these tasks but also display executive and cognitive function (Mortan and Avanzo, 2011). Further histological examination at later timepoints *i.e.* neonatal, pre and post-pubertal would also ascertain whether the delayed cerebellar development observed reaches the typical cytoarchitecture of a developed cerebellum.

### **7.7 Are the observed changes carried on in later life – the effect of disruption during cerebellar development**

The observed findings of altered cerebellar cytoarchitecture due to androgen over-exposure have brought further questions that need to be answered. One in particular is whether delayed development reaches the typical cerebellar cytoarchitecture at a later stage. Examining ovine cerebellar samples from a later gestational period, postnatally and into adulthood would give insight into this. The literature indeeds supports this conclusion as this delayed development is observed in autism (Bauman and Kemper 1985 and 1993; Ritvo *et al.*, 1986; Fehlow *et al.*, 1993), schizophrenia (Reyes and Gordon, 1981; Ritvo *et al.*, 1998) and thyroid deficiency (Vincent *et al.*, 1983; Kimura-Kuroda *et al.*, 2002).

From alterations at the mRNA level for genes involved in steroid signalling to changes to the cerebellar cytoarchitecture, the current study has demonstrated the vast effects that prenatal androgen over-exposure has on male ovine cerebellar development. What these changes during ovine mid-gestation mean for future life, in terms of development and cognitive function, still remain unclear, however this study has gained further insight and focus for further investigation.

What needs to be established are the functional consequences of the changes observed in the current study and whether they are detrimental to the typical role of the cerebellum in later life. These findings indicate the delayed development and migratory pattern of Purkinje cells as a consequence of androgen exposure. Therefore, we have established the structural effects of developmental hormone treatment on the cerebellum. Whether this has an impact on motor and/or cognitive function in later life remains unclear. If cerebellar development never catches up then this may pose detrimental consequences on cognitive and/or motor function as the cerebellum plays a vital role in these areas. Behavioural studies would establish whether higher function is affected as a result from androgen over-exposure.

Gene and protein expression, the cytoarchitecture and motor/cognitive function (by utilising behavioural studies aforementioned previously) should be examined in samples from late gestation, the neonatal, pre and post pubertal periods and adulthood. This would help identify whether the early insult of

excess androgen exposure *in utero* completely reconfigures the developed cerebellum from the changes outlined throughout this thesis.

## References

- Abbott, D.H., Dumesic, D.A., Eisner, J.R., Colman, R.J. and Kemnitz, J.W. (1998). Insights into the development of PCOS from studies of prenatally androgenized female rhesus monkeys. *Trends in Endocrinology and Metabolism*, **9**, 62–67.
- Abbott, D.H., Dumesic, D.A. and Franks, S. (2002). Developmental origin of polycystic ovary syndrome - a hypothesis. *Journal of Endocrinology*, **174**, 1-5.
- Abraham, H., Tornoczky, T., Kosztolanyi, G. & Seress, L. (2001). Cell formation in the cortical layers of the developing human cerebellum. *International Journal of Developmental Neuroscience*, **19**(1), 53-62.
- Adam, C. L., Findlay, P. A., Kyle, C. E., Young, P. & Mercer, J. G. (1997). Effect of chronic food restriction on pulsatile luteinizing hormone secretion and hypothalamic neuropeptide Y gene expression in castrate male sheep. *Journal of Endocrinology*, **152**(2), 329-337.
- Agís-Balboa, R. C., Pinna, G., Zhubi, A., Maloku, E., Veldic, M., Costa, E. & Guidotti, A. (2006). Characterization of brain neurons that express enzymes mediating neurosteroid biosynthesis. *Proceedings of the National Academy of Sciences*, **103**(39), 14602-14607.
- Ahlbom, E., Prins, G. S. & Ceccatelli, S. (2001). Testosterone protects cerebellar granule cells from oxidative stress-induced cell death through a receptor mediated mechanism. *Brain Research*, **892**(2), 255-262.
- Ali, N. S., Keller-Wood, M. & Wood, C. E. (2005). Ontogenetic changes in the extra-pituitary expression of pro-opiomelanocortin in the developing ovine fetus. *Peptides*, **26**(2), 301-306.
- Allen, G., Buxton, R. B., Wong, E. C. & Courchesne, E. (1997). Attentional activation of the cerebellum independent of motor involvement. *Science*, **275**(5308), 1940-1943.
- Altenor, A. & DeYoe, E. A. (1977). The effects of DL-p-chlorophenylalanine on learned helplessness in the rat. *Behaviour and Biology*, **20**(1), 111-115.
- Altman, J. (1972). Postnatal development of the cerebellar cortex in the rat. I. The external germinal layer and the transitional molecular layer. *The Journal of Comparative Neurology*, **145**(3), 353-397.
- Altman, J. & Bayer, S. A. (1997). *Development of the Cerebellar System in Relation to its Evolution, Structure, and Functions*. New York: CRC Press.
- Alzheimer's Association, (2014) Alzheimer's Disease Facts and Figures, *Alzheimer's & Dementia*, **10**(2).



- Andrews, W., Barber, M., Hernandez-Miranda, L. R., Xian, J., Rakic, S., Sundaresan, V., Rabbitts, T. H., Pannell, R., Rabbitts, P., Thompson, H., Erskine, L., Murakami, F. & Parnavelas, J. G. (2008). The role of Slit-Robo signaling in the generation, migration and morphological differentiation of cortical interneurons. *Developmental Biology*, **313**(2), 648-658.
- Arnold, A. P. & Gorski, R. A. (1984). Gonadal steroid induction of structural sex differences in the central nervous system. *Annual Reviews of Neuroscience*, **7**, 413-442.
- Bailey, A., Luthert, P., Dean, A., Harding, B., Janota, I., Montgomery, M., Rutter, M. & Lantos, P. (1998). A clinicopathological study of autism. *Brain*, **121** ( Pt 5), 889-905.
- Balthazart, J. & Foidart, A. (1993). Brain aromatase and the control of male sexual behavior. *The Journal of Steroid Biochemistry and Molecular Biology*, **44**(4-6), 521-540.
- Barber, M., Di Meglio, T., Andrews, W. D., Hernandez-Miranda, L. R., Murakami, F., Chedotal, A. & Parnavelas, J. G. (2009). The role of Robo3 in the development of cortical interneurons. *Cerebral Cortex*, **19** Suppl 1, 22-31.
- Barker, D. J. (1998). In utero programming of chronic disease. *Clinical Science (Lond)*, **95**(2), 115-128.
- Barker, D. J., Bull, A. R., Osmond, C. & Simmonds, S. J. (1990). Fetal and placental size and risk of hypertension in adult life. *British Medical Journal*, **301**(6746), 259-262.
- Barnard, E. A., Skolnick, P., Olsen, R. W., Mohler, H., Sieghart, W., Biggio, G., Braestrup, C., Bateson, A. N. & Langer, S. Z. (1998). International Union of Pharmacology. XV. Subtypes of  $\gamma$ -Aminobutyric AcidA Receptors: Classification on the Basis of Subunit Structure and Receptor Function. *Pharmacological Reviews*, **50**(2), 291-314.
- Baron-Cohen, S. (2002). The extreme male brain theory of autism. *Trends in Cognitive Sciences*, **6**(6), 248-254.
- Barry, J. S. & Anthony, R. V. (2008). The pregnant sheep as a model for human pregnancy. *Theriogenology*, **69**(1), 55-67.
- Bastianelli, E. (2003). Distribution of calcium-binding proteins in the cerebellum. *The Cerebellum*, **2**(4), 242-262.
- Baulieu, E. E. (1991). Neurosteroids: a new function in the brain. *Biology of Cells*, **71**(1-2), 3-10.
- Baulieu, E. E. (1998). NEUROSTEROIDS: A NOVEL FUNCTION OF THE BRAIN. *Psychoneuroendocrinology*, **23**(8), 963-987.

- Bauman, M. & Kemper, T. L. (1985). Histoanatomic observations of the brain in early infantile autism. *Neurology*, **35**(6), 866-874.
- Bayer, S. A., Altman, J., Russo, R. J., Dai, X. F. & Simmons, J. A. (1991). Cell migration in the rat embryonic neocortex. *J Comp Neurol*, **307**(3), 499-516.
- Beato, M. (1989). Gene regulation by steroid hormones. *Cell*, **56**(3), 335-344.
- Belcher, S. M. (1999). Regulated expression of estrogen receptor alpha and beta mRNA in granule cells during development of the rat cerebellum. *Brain Res Dev Brain Res*, **115**(1), 57-69.
- Belelli, D. & Lambert, J. J. (2005). Neurosteroids: endogenous regulators of the GABAA receptor. *Nat Rev Neurosci*, **6**(7), 565-575.
- Benes, F. M. & Berretta, S. (2001). GABAergic interneurons: implications for understanding schizophrenia and bipolar disorder. *Neuropsychopharmacology*, **25**(1), 1-27.
- Bering, E. A., Jr. (1955). Studies on the role of the choroid plexus in tracer exchanges between blood and cerebrospinal fluid. *J Neurosurg*, **12**(4), 385-392.
- Beyer, C. C. (1999). Estrogen and the developing mammalian brain. *Anatomy and Embryology*, **199**(5), 379-390.
- Bhatheja, K. and Field, J. (2006). Schwann cells: Origins and role in axonal maintenance and regeneration. *The International Journal of Biochemistry & Cell Biology*, **38**(12), 1995-1999.
- Biamonte, F., Assenza, G., Marino, R., D'Amelio, M., Panteri, R., Caruso, D., Scurati, S., Yague, J. G., Garcia-Segura, L. M., Cesa, R., Strata, P., Melcangi, R. C. & Keller, F. (2009). Interactions between neuroactive steroids and reelin haploinsufficiency in Purkinje cell survival. *Neurobiology of Disease*, **36**(1), 103-115.
- Biran, V., Verney, C. and Ferriero, D.M. (2011). Perinatal Cerebellar Injury in Human and Animal Models. *Neurology Research International*, **2012**, 1-9.
- Blomgren, K., Leist, M. & Groc, L. (2007). Pathological apoptosis in the developing brain. *Apoptosis*, **12**(5), 993-1010.
- Bohen, S. P., Kralli, A. & Yamamoto, K. R. (1995). Hold 'em and fold 'em: chaperones and signal transduction. *Science*, **268**(5215), 1303-1304.
- Boise, L. H., Gonzalez-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, X., Nunez, G. & Thompson, C. B. (1993). bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell*, **74**(4), 597-608.

- Bonora, E., Beyer, K. S., Lamb, J. A., Parr, J. R., Klauck, S. M., Benner, A., Paolucci, M., Abbott, A., Ragoussis, I., Poustka, A., Bailey, A. J. & Monaco, A. P. (2003). Analysis of reelin as a candidate gene for autism. *Molecular Psychiatry*, **8**(10), 885-892.
- Borghesani, P. R., Peyrin, J. M., Klein, R., Rubin, J., Carter, A. R., Schwartz, P. M., Luster, A., Corfas, G. & Segal, R. A. (2002). BDNF stimulates migration of cerebellar granule cells. *Development*, **129**(6), 1435-1442.
- Bottjer, S. W. & Johnson, F. (1992). Matters of life and death in the songbird forebrain. *Journal of Neurobiology*, **23**(9), 1172-1191.
- Boukhtouche, F., Brugg, B., Wehrle, R., Bois-Joyeux, B., Danan, J. L., Dusart, I. & Mariani, J. (2010). Induction of early Purkinje cell dendritic differentiation by thyroid hormone requires RORalpha. *Neural Development*, **5**, 18.
- Bourgeois, J. P. & Rakic, P. (1993). Changes of synaptic density in the primary visual cortex of the macaque monkey from fetal to adult stage. *Journal of Neuroscience*, **13**(7), 2801-2820.
- Bovolín, P., Santi, M. R., Puia, G., Costa, E. & Grayson, D. (1992). Expression patterns of gamma-aminobutyric acid type A receptor subunit mRNAs in primary cultures of granule neurons and astrocytes from neonatal rat cerebella. *Proceedings of the National Academy of Sciences*, **89**(19), 9344-9348.
- Bowen, I. D. (1993). Apoptosis or programmed cell death? *Cell Biology International*, **17**(4), 365-380.
- Breedlove, S. M. (1992). Sexual dimorphism in the vertebrate nervous system. *Journal of Neuroscience*, **12**(11), 4133-4142.
- Breedlove, S. M. (1994). Sexual differentiation of the human nervous system. *Annual Reviews of Psychology*, **45**, 389-418.
- Brodal, P. (2010). *The Central Nervous System: Structure and Function*. New York: NY: Oxford University Press.
- Brown, M., Keynes, R., et al. (2001). *The Developing Brain*. Oxford: Oxford University Press.
- Brunskill, E. W., Ehrman, L. A., Williams, M. T., Klanke, J., Hammer, D., Schaefer, T. L., Sah, R., Dorn, G. W., 2nd, Potter, S. S. & Vorhees, C. V. (2005). Abnormal neurodevelopment, neurosignaling and behaviour in Npas3-deficient mice. *European Journal of Neuroscience*, **22**(6), 1265-1276.
- Buchanan, R. W., Vadar, K., Barta, P. E. & Pearlson, G. D. (1998). Structural evaluation of the prefrontal cortex in schizophrenia. *American Journal of Psychiatry*, **155**(8), 1049-1055.

- Bulfone, A., Smiga, S. M., Shimamura, K., Peterson, A., Puellas, L. & Rubenstein, J. L. (1995). T-brain-1: a homolog of Brachyury whose expression defines molecularly distinct domains within the cerebral cortex. *Neuron*, **15**(1), 63-78.
- Bunone, G., Briand, P. A., Miksicek, R. J. & Picard, D. (1996). Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *Embo Journal*, **15**(9), 2174-2183.
- Burton, T., Liang, B., Dibrov, A. & Amara, F. (2002). Transcriptional activation and increase in expression of Alzheimer's beta-amyloid precursor protein gene is mediated by TGF-beta in normal human astrocytes. *Biochemical and Biophysical Research Communications*, **295**(3), 702-712.
- Buss, R. R., Sun, W. & Oppenheim, R. W. (2006). Adaptive roles of programmed cell death during nervous system development. *Annual Reviews of Neuroscience*, **29**, 1-35.
- Bustin, S. A., Benes, V., Garson, J. A., Hellems, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J. & Wittwer, C. T. (2009). The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry*, **55**(4), 611-622.
- Caron, K. M., Ikeda, Y., Soo, S. C., Stocco, D. M., Parker, K. L. & Clark, B. J. (1997). Characterization of the promoter region of the mouse gene encoding the steroidogenic acute regulatory protein. *Molecular Endocrinology*, **11**(2), 138-147.
- Chan-Palay, V., Nilaver, G., Palay, S. L., Beinfeld, M. C., Zimmerman, E. A., Wu, J. Y. & O'Donohue, T. L. (1981). Chemical heterogeneity in cerebellar Purkinje cells: existence and coexistence of glutamic acid decarboxylase-like and motilin-like immunoreactivities. *Proceedings of the National Academy of Sciences*, **78**(12), 7787-7791.
- Chan, S., Kachilele, S., McCabe, C. J., Tannahill, L. A., Boelaert, K., Gittoes, N. J., Visser, T. J., Franklyn, J. A. & Kilby, M. D. (2002). Early expression of thyroid hormone deiodinases and receptors in human fetal cerebral cortex. *Developmental Brain Research*, **138**(2), 109-116.
- Chang, C. C., Saltzman, A. A., Yeh, S. S., Young, W. W., Keller, E. E., Lee, H. J. H., Wang, C. C. & Mizokami, A. A. (1995). Androgen receptor: an overview. *Critical Reviews in Eukaryotic Gene Expression*, **5**(2), 97-125.
- Changeux, J. P. & Danchin, A. (1976). Selective stabilisation of developing synapses as a mechanism for the specification of neuronal networks. *Nature*, **264**(5588), 705-712.

- Chapman, E., Baron-Cohen, S., Auyeung, B., Knickmeyer, R., Taylor, K. & Hackett, G. (2006). Fetal testosterone and empathy: evidence from the empathy quotient (EQ) and the "reading the mind in the eyes" test. *Society of Neuroscience*, **1**(2), 135-148.
- Chen, K., Li, H.-Z., Ye, N., Zhang, J. & Wang, J.-J. (2005). Role of GABAB receptors in GABA and baclofen-induced inhibition of adult rat cerebellar interpositus nucleus neurons in vitro. *Brain Research Bulletin*, **67**(4), 310-318.
- Chen, S., Ren, Y. Q., Bing, G. Y. & Hillman, D. E. (1998). Transient c-fos gene expression in cerebellar development and functional stimulation. *Brain Research*, **795**(1-2), 87-97.
- Cherubini, E., Gaiarsa, J.L. and Ben-Ari, Y. (1991). GABA: an excitatory transmitter in early postnatal life. *Trends in Neuroscience*, **14**(12), 515-519.
- Chomczynski, P. (1993). A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biological Techniques*, **15**(3), 532-534, 536-537.
- Chung, S. H., Marzban, H. & Hawkes, R. (2009). Compartmentation of the cerebellar nuclei of the mouse. *Neuroscience*, **161**(1), 123-138.
- Clancy, B., Darlington, R. B. & Finlay, B. L. (2001). Translating developmental time across mammalian species. *Neuroscience*, **105**(1), 7-17.
- Clark, B. J., Wells, J., King, S. R. & Stocco, D. M. (1994). The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). *Journal of Biological Chemistry*, **269**(45), 28314-28322.
- Colborn, T., vom Saal, F. S. & Soto, A. M. (1993). Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environment and Health Perspectives*, **101**(5), 378-384.
- Colmers, W. F. & Bleakman, D. (1994). Effects of neuropeptide Y on the electrical properties of neurons. *Trends in Neuroscience*, **17**(9), 373-379.
- Colquhoun, D. & Sivilotti, L. G. (2004). Function and structure in glycine receptors and some of their relatives. *Trends in Neuroscience*, **27**(6), 337-344.
- Comeau, W.L., McDonald, R. and Kolb, B. (2010). Learning-induced alterations in prefrontal cortical circuitry. *Behavioural Brain Research*, **214**, 91–101.
- Compagnone, N. A. & Mellon, S. H. (2000). Neurosteroids: Biosynthesis and Function of These Novel Neuromodulators. *Frontiers in Neuroendocrinology*, **21**(1), 1-56.

- Connolly, F., Rae, M.T., Bittner, L., Hogg, K., McNeilly, A.S. and Duncan, W.C. (2013). Excess Androgens in Utero Alters Fetal Testis Development). *Endocrinology*, **154**(5), 1921-1933..
- Cooper, J. A. (2008). A mechanism for inside-out lamination in the neocortex. *Trends in Neuroscience*, **31**(3), 113-119.
- Cowan, W. M., Fawcett, J. W., O'Leary, D. D. & Stanfield, B. B. (1984). Regressive events in neurogenesis. *Science*, **225**(4668), 1258-1265.
- Cowley, M. A., Smart, J. L., Rubinstein, M., Cerdan, M. G., Diano, S., Horvath, T. L., Cone, R. D. & Low, M. J. (2001). Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature*, **411**(6836), 480-484.
- Cresswell, J.L., Barker, D.J., Osmond, C., Egger., P., Phillips, D.I and Fraser, R.B. (1997). Fetal growth, length of gestation and polycystic ovaries in adult life. *Lancet*, **350**, 1131-1135.
- Crepel, F. & Mariani, J. (1975). Anatomical, physiological and biochemical studies of the cerebellum from mutant mice. I. Electrophysiological analysis of cerebellar cortical neurons in the staggerer mouse. *Brain Research*, **98**(1), 135-147.
- Dangata, Y.Y. and Kaufman, M.H. (1997). Myelinogenesis in the optic nerve of (C57BL × CBA)F<sub>1</sub> hybrid mice: a morphometric analysis. *European Journal of Morphology*, **35**, 3–17.
- D'Arcangelo, G., G. Miao, G., Chen, S.-C., Scares, H. D., Morgan, J. I. & Curran, T. (1995). A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. *Nature*, **374**(6524), 719-723.
- Dean, B., Scarr, E. & McLeod, M. (2005). Changes in hippocampal GABAA receptor subunit composition in bipolar 1 disorder. *Molecular Brain Research*, **138**(2), 145-155.
- Dean, S. & McCarthy, M. (2008). Steroids, sex and the cerebellar cortex: implications for human disease. *The Cerebellum*, **7**(1), 38-47.
- Decety, J. & Ingvar, D. H. (1990). Brain structures participating in mental simulation of motor behavior: a neuropsychological interpretation. *Acta Psychologica (Amst)*, **73**(1), 13-34.
- Denton, C. J., Lamb, A. H., Wilson, P. & Mark, R. F. (1985). Innervation pattern of muscles of one-legged *Xenopus laevis* supplied by motoneurons from both sides of the spinal cord. *Brain Research*, **349**(1-2), 85-94.
- Deoni, S. C., Mercure, E., Blasi, A., Gasston, D., Thomson, A., Johnson, M., Williams, S. C. & Murphy, D. G. (2011). Mapping infant brain myelination with magnetic resonance imaging. *Journal of Neuroscience*, **31**(2), 784-791.

- Dewing, P., Shi, T., Horvath, S. & Vilain, E. (2003). Sexually dimorphic gene expression in mouse brain precedes gonadal differentiation. *Molecular Brain Research*, **118**(1-2), 82-90.
- Diamond, A. (2000). Close Interrelation of Motor Development and Cognitive Development and of the Cerebellum and Prefrontal Cortex. *Child Development*, **71**(1), 44-56.
- Diamond, M. I., Miner, J. N., Yoshinaga, S. K. & Yamamoto, K. R. (1990). Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. *Science*, **249**(4974), 1266-1272.
- Dias, R., Robbins, T. W. & Roberts, A. C. (1996). Dissociation in prefrontal cortex of affective and attentional shifts. *Nature*, **380**(6569), 69-72.
- Dickson, B. J. & Gilestro, G. F. (2006). Regulation of commissural axon pathfinding by slit and its Robo receptors. *Annual Reviews of Cell Development and Biology*, **22**, 651-675.
- Dougherty, S. E., Reeves, J. L., Lesort, M., Detloff, P. J. & Cowell, R. M. (2013). Purkinje cell dysfunction and loss in a knock-in mouse model of Huntington Disease. *Experimental Neurology*, **240**(0), 96-102.
- Duncan, J. R., Cock, M. L., Harding, R. & Rees, S. M. (2004). Neurotrophin expression in the hippocampus and cerebellum is affected by chronic placental insufficiency in the late gestational ovine fetus. *Developmental Brain Research*, **153**(2), 243-250.
- Durand, B., Saunders, M., Gaudon, C., Roy, B., Losson, R. & Chambon, P. (1994). Activation function 2 (AF-2) of retinoic acid receptor and 9-cis retinoic acid receptor: presence of a conserved autonomous constitutive activating domain and influence of the nature of the response element on AF-2 activity. *EMBO Journal*, **13**(22), 5370-5382.
- Durany, N., Michel, T., Kurt, J., Cruz-Sanchez, F. F., Cervos-Navarro, J. & Riederer, P. (2000). Brain-derived neurotrophic factor and neurotrophin-3 levels in Alzheimer's disease brains. *International Journal of Developmental Neuroscience*, **18**(8), 807-813.
- Dussault, I., Fawcett, D., Matthyssen, A., Bader, J. A. & Giguere, V. (1998). Orphan nuclear receptor ROR alpha-deficient mice display the cerebellar defects of staggerer. *Mechanisms of Development*, **70**(1-2), 147-153.
- Edmondson, J. C. & Hatten, M. E. (1987). Glial-guided granule neuron migration in vitro: a high-resolution time-lapse video microscopic study. *The Journal of Neuroscience*, **7**(6), 1928-1934.
- Ehrmann, D.A., Barnes, R.B., Rosenfield, R.L., Cavaghan, M.K. and Imperial, J. (1999). Prevalence to impaired glucose tolerance and diabetes in women with polycystic ovary syndrome. *Diabetes Care*, **22**, 141-146.

- Elbaggari, A., McDonald, K., Alburo, A. and Martin, C. (2008). Tech note 5809: Imaging of chemiluminescent Western blots: comparison of digital imaging and X-ray film. In B. Laboratories (Ed.). California.
- Elston, G. N. (2003). Cortex, cognition and the cell: new insights into the pyramidal neuron and prefrontal function. *Cerebral Cortex*, **13**(11), 1124-1138.
- Elston, G. N., Oga, T. & Fujita, I. (2009). Spinogenesis and pruning scales across functional hierarchies. *Journal of Neuroscience*, **29**(10), 3271-3275.
- Eng, L. F., Ghirnikar, R. S. & Lee, Y. L. (2000). Glial Fibrillary Acidic Protein: GFAP-Thirty-One Years (1969–2000). *Neurochemical Research*, **25**(9), 1439-1451.
- Erlander, M. G., Tillakaratne, N. J. K., Feldblum, S., Patel, N. & Tobin, A. J. (1991). Two genes encode distinct glutamate decarboxylases. *Neuron*, **7**(1), 91-100.
- Farrow, S. N., White, J. H., Martinou, I., Raven, T., Pun, K. T., Grinham, C. J., Martinou, J. C. & Brown, R. (1995). Cloning of a bcl-2 homologue by interaction with adenovirus E1B 19K. *Nature*, **374**(6524), 731-733.
- Fatemi, S., Reutiman, T., Folsom, T., Rooney, R., Patel, D. & Thuras, P. (2010). mRNA and Protein Levels for GABA and Its Receptors are Altered in Brains from Subjects with Autism. *Journal of Autism and Developmental Disorders*, **40**(6), 743-750.
- Fatemi, S. H., Halt, A. R., Realmuto, G., Earle, J., Kist, D. A., Thuras, P. & Merz, A. (2002). Purkinje cell size is reduced in cerebellum of patients with autism. *Cell and Molecular Neurobiology*, **22**(2), 171-175.
- Fatemi, S. H., Pearce, D. A., Brooks, A. I. & Sidwell, R. W. (2005). Prenatal viral infection in mouse causes differential expression of genes in brains of mouse progeny: A potential animal model for schizophrenia and autism. *Synapse*, **57**(2), 91-99.
- Favor, J., Gloeckner, C. J., Janik, D., Klempt, M., Neuhauser-Klaus, A., Pretsch, W., Schmahl, W. & Quintanilla-Fend, L. (2007). Type IV procollagen missense mutations associated with defects of the eye, vascular stability, the brain, kidney function and embryonic or postnatal viability in the mouse, *Mus musculus*: an extension of the Col4a1 allelic series and the identification of the first two Col4a2 mutant alleles. *Genetics*, **175**(2), 725-736.
- Fehlow, P., Bernstein, K., Tennstedt, A. & Walther, F. (1993). Early infantile autism and excessive aerophagy with symptomatic megacolon and ileus in a case of Ehlers-Danlos syndrome. *Padiatrie Grenzgebiete*, **31**(4), 259-267.



- Feist, G. & Schreck, C. B. (1996). Brain-pituitary-gonadal axis during early development and sexual differentiation in the rainbow trout, *Oncorhynchus mykiss*. *General and Comparative Endocrinology*, **102**(3), 394-409.
- Feng, Q. P., Sun, X. Y. & Hedner, T. (1996). Cardiovascular responses and interactions by neuropeptide Y in rats with congestive heart failure. *Blood Press*, **5**(5), 312-318.
- Fink, A. J., Englund, C., Daza, R. A. M., Pham, D., Lau, C., Nivison, M., Kowalczyk, T. & Hevner, R. F. (2006). Development of the Deep Cerebellar Nuclei: Transcription Factors and Cell Migration from the Rhombic Lip. *The Journal of Neuroscience*, **26**(11), 3066-3076.
- Finlay, B. L. (1992). Cell death and the creation of regional differences in neuronal numbers. *Journal of Neurobiology*, **23**(9), 1159-1171.
- Fiore, M., Korf, J., Antonelli, A., Talamini, L. and Aloe, L. (2002). Long-lasting effects of prenatal MAM treatment on water maze performance in rats: associations with altered brain development and neurotrophin levels. *Neurotoxicology and Teratology*, **24**(2), 179-191.
- Fischer, A. H., Jacobson, K. A., Rose, J. & Zeller, R. (2008). Hematoxylin and Eosin Staining of Tissue and Cell Sections. *Cold Spring Harbor Protocols*, **2008**(5), pdb.prot4986.
- Fleige, S. & Pfaffl, M. W. (2006). RNA integrity and the effect on the real-time qRT-PCR performance. *Molecular Aspects in Medicine*, **27**(2-3), 126-139.
- Flood, J. F., Hernandez, E. N. & Morley, J. E. (1987). Modulation of memory processing by neuropeptide Y. *Brain Research*, **421**(1-2), 280-290.
- Forger, N. G., Fishman, R. B. & Breedlove, S. M. (1992). Differential effects of testosterone metabolites upon the size of sexually dimorphic motoneurons in adulthood. *Hormones and Behaviour*, **26**(2), 204-213.
- Franks, S., Mason, H. and Willis, D. (2000). Follicular dynamics and anovulation in polycystic ovary syndrome. *Molecular and Cellular Endocrinology*, **163**, 49-52.
- Furukawa, A., Miyatake, A., Ohnishi, T. & Ichikawa, Y. (1998). Steroidogenic Acute Regulatory Protein (StAR) Transcripts Constitutively Expressed in the Adult Rat Central Nervous System: Colocalization of StAR, Cytochrome P-450SCC (CYP XIA1), and 3 $\beta$ -Hydroxysteroid Dehydrogenase in the Rat Brain. *Journal of Neurochemistry*, **71**(6), 2231-2238.
- Fuster, J. M. (1997). Network memory. *Trends in Neuroscience*, **20**(10), 451-459.

- Fuster, J. M. (2002). Frontal lobe and cognitive development. *Journal of Neurocytology* **31**(3-5), 373-385.
- Fuwa, K. & Vallee, B. L. (1966). A multichannel flame spectrometer. I. Instrumental design. *Annals of Biochemistry*, **17**(3), 444-455.
- Gagliardini, V., Fernandez, P. A., Lee, R. K., Drexler, H. C., Rotello, R. J., Fishman, M. C. & Yuan, J. (1994). Prevention of vertebrate neuronal death by the crmA gene. *Science*, **263**(5148), 826-828.
- Gassmann, M., Grenacher, B., Rohde, B. & Vogel, J. (2009). Quantifying Western blots: pitfalls of densitometry. *Electrophoresis*, **30**(11), 1845-1855.
- Gates, M. A., Thomas, L. B., Howard, E. M., Laywell, E. D., Sajin, B., Faissner, A., Gotz, B., Silver, J. & Steindler, D. A. (1995). Cell and molecular analysis of the developing and adult mouse subventricular zone of the cerebral hemispheres. *Journal of Comparative Neurobiology*, **361**(2), 249-266.
- Ghose, A. & Van Vactor, D. (2002). GAPs in Slit-Robo signaling. *Bioessays*, **24**(5), 401-404.
- Gimenez., D. and Rodning, S. (2007) Reproductive management of sheep and goats. Alabama A&M and Auburn Universities, ANR-1316.
- Glantz, L. A. & Lewis, D. A. (2000). Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia. *Archives of General Psychiatry*, **57**(1), 65-73.
- Godwin, J., Hartman, V., Nag, P. & Crews, D. (2000). Androgenic regulation of steroid hormone receptor mRNAs in the brain of whiptail lizards. *Journal of Neuroendocrinology*, **12**(7), 599-606.
- Gogtay, N., Sporn, A., Clasen, L. S., Nugent, T. F., 3rd, Greenstein, D., Nicolson, R., Giedd, J. N., Lenane, M., Gochman, P., Evans, A. & Rapoport, J. L. (2004). Comparison of progressive cortical gray matter loss in childhood-onset schizophrenia with that in childhood-onset atypical psychoses. *Archives of General Psychiatry*, **61**(1), 17-22.
- Gold, D. A., Baek, S. H., Schork, N. J., Rose, D. W., Larsen, D. D., Sachs, B. D., Rosenfeld, M. G. & Hamilton, B. A. (2003). ROR $\alpha$  Coordinates Reciprocal Signaling in Cerebellar Development through Sonic hedgehog and Calcium-Dependent Pathways. *Neuron*, **40**(6), 1119-1131.
- Goto, J., Inoue, T., Kuruma, A. and Mikoshiba, K. (2006). Short-term potentiation at the parallel fiber-Purkinje cell synapse. *Neuroscience Research*, **55**, 28-33.

- Grobin, A. C., Heenan, E. J., Lieberman, J. A. & Morrow, A. L. (2003). Perinatal neurosteroid levels influence GABAergic interneuron localization in adult rat prefrontal cortex. *Journal of Neuroscience*, **23**(5), 1832-1839.
- Grundemar, L., Wahlestedt, C. & Reis, D. J. (1991). Neuropeptide Y acts at an atypical receptor to evoke cardiovascular depression and to inhibit glutamate responsiveness in the brainstem. *Journal of Pharmacology and Experimental Therapeutics*, **258**(2), 633-638.
- Guenoun, R., Fiddes, R. J., Gouézou, M., Lombès, M. & Baulieu, E. E. (1995). A key enzyme in the biosynthesis of neurosteroids, 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta$ 5- $\Delta$ 4-isomerase (3 $\beta$ -HSD), is expressed in rat brain. *Molecular Brain Research*, **30**(2), 287-300.
- Guerin, P., Lyon, G., Barthelemy, C., Sostak, E., Chevrollier, V., Garreau, B. & Lelord, G. (1996). Neuropathological study of a case of autistic syndrome with severe mental retardation. *Developmental Medicine and Child Neurology*, **38**(3), 203-211.
- Guerra-Araiza, C., Coyoy-Salgado, A. & Camacho-Arroyo, I. (2002). Sex differences in the regulation of progesterone receptor isoforms expression in the rat brain. *Brain Research Bulletin*, **59**(2), 105-109.
- Gupta, S. K. & Pillarisetti, K. (1999). Cutting edge: CXCR4-Lo: molecular cloning and functional expression of a novel human CXCR4 splice variant. *Journal of Immunology*, **163**(5), 2368-2372.
- Hales, C. N. & Barker, D. J. (2001). The thrifty phenotype hypothesis. *British Medical Bulletin*, **60**, 5-20.
- Hashimoto, K. and Kano, M. (2005). Postnatal development and synapse elimination of climbing fiber to Purkinje cell projection in the cerebellum. *Neuroscience Research*, **53**, 221–228.
- Hatten, M. E. & Heintz, N. (1995). Mechanisms of neural patterning and specification in the developing cerebellum. *Annual Reviews in Neuroscience*, **18**, 385-408.
- Hecht, J., Kuhl, H., Haas, S. A., Bauer, S., Poustka, A. J., Lienau, J., Schell, H., Stiege, A. C., Seitz, V., Reinhardt, R., Duda, G. N., Mundlos, S. & Robinson, P. N. (2006). Gene identification and analysis of transcripts differentially regulated in fracture healing by EST sequencing in the domestic sheep. *BMC Genomics*, **7**, 172.
- Heijnen, C. J., Zijlstra, J., Kavelaars, A., Croiset, G. & Ballieux, R. E. (1987). Modulation of the immune response by POMC-derived peptides. I. Influence on proliferation of human lymphocytes. *Brain, Behaviour and Immunity*, **1**(4), 284-291.

- Heilig, M., Wahlestedt, C., Ekman, R. & Widerlov, E. (1988). Antidepressant drugs increase the concentration of neuropeptide Y (NPY)-like immunoreactivity in the rat brain. *European Journal of Pharmacology*, **147**(3), 465-467.
- Hengartner, M. O. & Horvitz, H. R. (1994). The ins and outs of programmed cell death during *C. elegans* development. *Philosophical Transactions of the Royal Society of London B Biological Sciences*, **345**(1313), 243-246.
- Herman, R. A., Jones, B., Mann, D. & Wallen, K. (2000). Timing of Prenatal Androgen Exposure: Anatomical and Endocrine Effects on Juvenile Male and Female Rhesus Monkeys. *Hormones and Behavior*, **38**(1), 52-66.
- Herzog, H., Hort, Y. J., Shine, J. & Selbie, L. A. (1993). Molecular cloning, characterization, and localization of the human homolog to the reported bovine NPY Y3 receptor: lack of NPY binding and activation. *DNA Cell Biology* **12**(6), 465-471.
- Hill, R. A., Wu, Y. W. C., Kwek, P. & Buuse, M. (2012). Modulatory Effects of Sex Steroid Hormones on Brain-Derived Neurotrophic Factor-Tyrosine Kinase B Expression during Adolescent Development in C57Bl/6 Mice. *Journal of Neuroendocrinology*, **24**(5), 774-788.
- Hines, M. & Alexander, G. M. (2008). Monkeys, girls, boys and toys: a confirmation Letter regarding "Sex differences in toy preferences: striking parallels between monkeys and humans". *Hormones and Behaviour*, **54**(3), 478-479; author reply 480-471.
- Hines, M., Golombok, S., Rust, J., Johnston, K. J. & Golding, J. (2002). Testosterone during pregnancy and gender role behavior of preschool children: a longitudinal, population study. *Child Development*, **73**(6), 1678-1687.
- Hines, M. & Kaufman, F. R. (1994). Androgen and the development of human sex-typical behavior: rough-and-tumble play and sex of preferred playmates in children with congenital adrenal hyperplasia (CAH). *Child Development*, **65**(4), 1042-1053.
- Hirst, J. J., Walker, D. W., Yawno, T. & Palliser, H. K. (2009). Stress in Pregnancy: A Role for Neuroactive Steroids in Protecting the Fetal and Neonatal Brain. *Developmental Neuroscience*, **31**(5), 363-377.
- Hoek, H. W., Susser, E., Buck, K. A., Lumey, L. H., Lin, S. P. & Gorman, J. M. (1996). Schizoid personality disorder after prenatal exposure to famine. *American Journal of Psychiatry*, **153**(12), 1637-1639.
- Hoffman, G. E., Smith, M. S. & Verbalis, J. G. (1993). c-Fos and Related Immediate Early Gene Products as Markers of Activity in Neuroendocrine Systems. *Frontiers in Neuroendocrinology*, **14**(3), 173-213.

- Hogg, K., Young, J. M., Oliver, E. M., Souza, C. J., McNeilly, A. S. & Duncan, W. C. (2012). Enhanced Thecal Androgen Production Is Prenatally Programmed in an Ovine Model of Polycystic Ovary Syndrome. *Endocrinology*, **153**(1), 450-461.
- Hogg, K. K., McNeilly, A. S. A. S. & Duncan, W. C. W. C. (2011). Prenatal androgen exposure leads to alterations in gene and protein expression in the ovine fetal ovary. *Endocrinology*, **152**(5), 2048-2048-2059.
- Holmberg, A., Blomstergren, A., Nord, O., Lukacs, M., Lundeberg, J. & Uhlen, M. (2005). The biotin-streptavidin interaction can be reversibly broken using water at elevated temperatures. *Electrophoresis*, **26**(3), 501-510.
- Houk, C. P., Pearson, E. J., Martinelle, N., Donahoe, P. K. & Teixeira, J. (2004). Feedback inhibition of steroidogenic acute regulatory protein expression in vitro and in vivo by androgens. *Endocrinology*, **145**(3), 1269-1275.
- Hu, H. (1999). Chemorepulsion of neuronal migration by Slit2 in the developing mammalian forebrain. *Neuron*, **23**(4), 703-711.
- Huang, E. J. & Reichardt, L. F. (2001). Neurotrophins: roles in neuronal development and function. *Annual Reviews of Neuroscience*, **24**, 677-736.
- Hummon, A. B. A. B., Lim, S. R. S. R., Difilippantonio, M. J. M. J. & Ried, T. T. (2007). Isolation and solubilization of proteins after TRIzol extraction of RNA and DNA from patient material following prolonged storage. *Biology Techniques*, **42**(4), 467-467-470, 472.
- Huttenlocher, P. R. (1979). Synaptic density in human frontal cortex - developmental changes and effects of aging. *Brain Research*, **163**(2), 195-205.
- Huttenlocher, P. R. & Dabholkar, A. S. (1997). Regional differences in synaptogenesis in human cerebral cortex. *Journal of Comparative Neurobiology*, **387**(2), 167-178.
- Ibanez, L., Vallis, C., Ferrer, A., Ong, K., Dunger, D.B. and Zegher, F. (2002). Additive effects of insulin-sensitizing and anti-androgen treatment in young nonobese women with hyperinsulinism, hyperandrogenism, dyslipidemia and anovulation. *The Journal of Endocrinology and Metabolism*, **87**(6), 2870-2874.
- Ito, M. (2001). Cerebellar long-term depression: Characterization, signal transduction, and functional roles. *Physiology Reviews*, **81**, 1143-1195.
- Ito, M. (2002). The molecular organization of cerebellar long term depression. *Nature Reviews Neuroscience*, **3**, 896-902.
- Ito, M. (2006). Cerebellar circuitry as a neuronal machine. *Progress In Neurobiology*, **78**, 272-303.

- Jackson, R. S., Creemers, J. W., Ohagi, S., Raffin-Sanson, M. L., Sanders, L., Montague, C. T., Hutton, J. C. & O'Rahilly, S. (1997). Obesity and impaired prohormone processing associated with mutations in the human prohormone convertase 1 gene. *Nature Genetics*, **16**(3), 303-306.
- Jacobsen, J. C., Bawden, C.S., Rudiger, S. R., McLaughlan, C. J., Reid, S. J., Waldvogel, H. J., MacDonald, M. E., Gusella, J. F., Walker, S. K., Kelly, J. M., Webb, G. C., Faull, R. L., Rees, M. I. & Snell, R. G. (2010). An ovine transgenic Huntington's disease model. *Human Molecular Genetics*, **19**(10), 1873-1873-1882.
- Jacobson, M. (1997). *Developmental Neurobiology* (3rd edition ed.). New York: Telnum.
- Jiang, Y. et al., (2014). The sheep genome illuminates biology of the rumen and lipid metabolism. *Science*, **344**, 1168-1173.
- Janmaat, S., Akwa, Y., Doulazmi, M., Bakouche, J., Gautheron, V., Liere, P., Eychenne, B., Pianos, A., Luiten, P., Groothuis, T., Baulieu, E. E., Mariani, J., Sherrard, R. M. & Frederic, F. (2011). Age-related Purkinje cell death is steroid dependent: RORalpha haplo-insufficiency impairs plasma and cerebellar steroids and Purkinje cell survival. *Age*, **33**(4), 565-578.
- Jensen, P., Zoghbi, H. Y. & Goldowitz, D. (2002). Dissection of the cellular and molecular events that position cerebellar Purkinje cells: a study of the math1 null-mutant mouse. *Journal of Neuroscience*, **22**(18), 8110-8116.
- Jenson, S. D., Robetorye, R. S., Bohling, S. D., Schumacher, J. A., Morgan, J. W., Lim, M. S. & Elenitoba-Johnson, K. S. (2003). Validation of cDNA microarray gene expression data obtained from linearly amplified RNA. *Molecular Pathology*, **56**(6), 307-312.
- Johnsen, L., Kongsted, A. H. & Nielsen, M. O. (2013). Prenatal undernutrition and postnatal overnutrition alter thyroid hormone axis function in sheep. *Journal of Endocrinology*, **216**(3), 389-402.
- Jones, K. J., Kinderman, N. B. & Oblinger, M. M. (1997). Alterations in Glial Fibrillary Acidic Protein (GFAP) mRNA Levels in the Hamster Facial Motor Nucleus: Effects of Axotomy and Testosterone. *Neurochemical Research*, **22**(11), 1359-1366.
- Ju, Y. T., Chang, A. C., She, B. R., Tsaur, M. L., Hwang, H. M., Chao, C. C., Cohen, S. N. & Lin-Chao, S. (1998). gas7: A gene expressed preferentially in growth-arrested fibroblasts and terminally differentiated Purkinje neurons affects neurite formation. *Proceedings of National Acad Science U. S. A*, **95**(19), 11423-11428.
- Jung-Testas, I. and Baulieu, E.E. (1998). Steroid hormone receptors and steroid action in rat glial cells of the central and peripheral nervous system. *Journal of Steroid Biochemistry and Molecular Biology*, **65**(1-6), 243-251.

- Juraska, J. M., Sisk, C. L. & DonCarlos, L. L. (2013). Sexual differentiation of the adolescent rodent brain: hormonal influences and developmental mechanisms. *Hormones and Behaviour*, **64**(2), 203-210.
- Kano M, Rexhausen U, Dreessen J, Konnerth A. (1992). Synaptic excitation produces a long-lasting rebound potentiation of inhibitory synaptic signals in cerebellar Purkinje cells. *Nature*, **356**, 601-604.
- Karlsson, C. (2014). Autism and Other Neurodevelopmental Disorders *American Journal of Psychiatry*, **171**, 232-233.
- Karolak, J. A., Kulinska, K., Nowak, D. M., Pitarque, J. A., Molinari, A., Rydzanicz, M., Bejjani, B. A. & Gajicka, M. (2011). Sequence variants in COL4A1 and COL4A2 genes in Ecuadorian families with keratoconus. *Molecular Vision*, **17**, 827-843.
- Karolczak, M., Küppers, E. & Beyer, C. (1998). Developmental Expression and Regulation of Aromatase- and 5 $\alpha$ -Reductase Type 1 mRNA in the Male and Female Mouse Hypothalamus. *Journal of Neuroendocrinology*, **10**(4), 267-274.
- Kemper, T. L. & Bauman, M. L. (1993). The contribution of neuropathologic studies to the understanding of autism. *Clinical Neurology*, **11**(1), 175-187.
- Kendrick, K. M., da Costa, A. P., Leigh, A. E., Hinton, M. R. & Peirce, J. W. (2001). Sheep don't forget a face. *Nature*, **414**(6860), 165-166.
- Kerr, J. F., Winterford, C. M. & Harmon, B. V. (1994). Apoptosis. Its significance in cancer and cancer therapy. *Cancer*, **73**(8), 2013-2026.
- Kim, J. J. & Thompson, R. F. (1997). Cerebellar circuits and synaptic mechanisms involved in classical eyeblink conditioning. *Trends in Neuroscience*, **20**(4), 177-181.
- Kimura-Kuroda, J., Nagata, I., Negishi-Kato, M. & Kuroda, Y. (2002). Thyroid hormone-dependent development of mouse cerebellar Purkinje cells in vitro. *Developmental Brain Research*, **137**(1), 55-65.
- King, S. R., Ginsberg, S. D., Ishii, T., Smith, R. G., Parker, K. L. & Lamb, D. J. (2004). The Steroidogenic Acute Regulatory Protein Is Expressed in Steroidogenic Cells of the Day-Old Brain. *Endocrinology*, **145**(10), 4775-4780.
- King, S. R., Manna, P. R., Ishii, T., Syapin, P. J., Ginsberg, S. D., Wilson, K., Walsh, L. P., Parker, K. L., Stocco, D. M., Smith, R. G. & Lamb, D. J. (2002). An Essential Component in Steroid Synthesis, the Steroidogenic Acute Regulatory Protein, Is Expressed in Discrete Regions of the Brain. *The Journal of Neuroscience*, **22**(24), 10613-10620.

- Kirsch, L., Liscovitch, N. & Chechik, G. (2012). Localizing genes to cerebellar layers by classifying ISH images. *PLoS Computational Biology*, **8**(12), e1002790.
- Koibuchi, N. & Chin, W. W. (2000). Thyroid hormone action and brain development. *Trends in Endocrinology and Metabolism*, **11**(4), 123-128.
- Koibuchi, N., Yamaoka, S. & Chin, W. W. (2001). Effect of altered thyroid status on neurotrophin gene expression during postnatal development of the mouse cerebellum. *Thyroid*, **11**(3), 205-210.
- Kolb, B., Mychasiuka, R., Muhammada, A., Lia, Y., Frost, D. and Gibb, R. (2012). Experience and the developing prefrontal cortex. *Proceedings of the National Academy of Sciences*, **109**(2), 17186-17193.
- Kosik, K. S. & Krichevsky, A. M. (2002). The message and the messenger: delivering RNA in neurons. *Sci STKE*, **2002**(126), pe16.
- Kraus, W. L., Weis, K. E. & Katzenellenbogen, B. S. (1997). Determinants for the repression of estrogen receptor transcriptional activity by ligand-occupied progesterin receptors. *Journal of Steroid Biochemistry and Molecular Biology*, **63**(4-6), 175-188.
- Krude, H., Biebermann, H., Luck, W., Horn, R., Brabant, G. & Gruters, A. (1998). Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans. *Nature Genetics*, **19**(2), 155-157.
- Kujawa, K. A., Emeric, E. & Jones, K. J. (1991). Testosterone differentially regulates the regenerative properties of injured hamster facial motoneurons. *Journal of Neuroscience*, **11**(12), 3898-3906.
- Kumar, R. K., Chapple, C. C. & Hunter, N. (1999). Improved double immunofluorescence for confocal laser scanning microscopy. *Journal of Histochemistry and Cytochemistry*, **47**(9), 1213-1218.
- Kurien, B. T. & Scofield, R. H. (2009). A brief review of other notable protein detection methods on blots. *Methods of Molecular Biology*, **536**, 557-571.
- Kurz, E. M., Sengelaub, D. R. & Arnold, A. P. (1986). Androgens regulate the dendritic length of mammalian motoneurons in adulthood. *Science*, **232**(4748), 395-398.
- Lakatosova, S., Schmidtová, E., Celec, P., Janega, P., Kubranská, A., Durdiaková, J. & Ostatníková, D. (2010). Prenatal Testosterone Influence on Reelin Expression in Association with Autism. *Activitas Nervosa Superior Rediviva*, **52**(247-252).
- Lamb, A. H. (1979). Evidence that some developing limb motoneurons die for reasons other than peripheral competition. *Development and Biology*, **71**(1), 8-21.



- Lamb, A. H. (1981). Selective bilateral motor innervation in *Xenopus* tadpoles with one hind limb. *Journal of Embryology and Experimental Morphology*, **65**, 149-163.
- Lanser, M. E. & Fallon, J. F. (1984). Development of the lateral motor column in the limbless mutant chick embryo. *Journal of Neuroscience*, **4**(8), 2043-2050.
- Lanser, M. E. & Fallon, J. F. (1987). Development of the brachial lateral motor column in the wingless mutant chick embryo: motoneuron survival under varying degrees of peripheral load. *Journal of Comparative Neurobiology*, **261**(3), 423-434.
- Lauber, M. E., Sarasin, A. & Lichtensteiger, W. (1997). Sex differences and androgen-dependent regulation of aromatase (CYP19) mRNA expression in the developing and adult rat brain. *Journal of Steroid Biochemistry and Molecular Biology*, **61**(3-6), 359-364.
- Lauder, J. M. (1978). Effects of early hypo- and hyperthyroidism on development of rat cerebellar cortex. IV. The parallel fibers. *Brain Research*, **142**(1), 25-39.
- Laurie, D. J., Wisden, W. & Seeburg, P. H. (1992). The distribution of thirteen GABAA receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. *Journal of Neuroscience*, **12**(11), 4151-4172.
- Leavitt, W. W., Chen, T. J. & Allen, T. C. (1977). Regulation of progesterone receptor formation by estrogen action. *Annals of the New York Academy of Sciences*, **286**, 210-225.
- Leder, E. H. & Silverstein, J. T. (2006). The pro-opiomelanocortin genes in rainbow trout (*Oncorhynchus mykiss*): duplications, splice variants, and differential expression. *Journal of Endocrinology*, **188**(2), 355-363.
- Lees, J.A., Fawell, S. E. & Parker, M. G. (1989). Identification of two transactivation domains in the mouse oestrogen receptor. *Nucleic Acids Research*, **17**, 5477-5488.
- Legro, R.S., Bentley-Lewis, R., Driscoll, D., Wang, S.C. and Dunaif, A. (2002). Insulin resistance in the sisters of women with polycystic ovary syndrome: Association with hyperandrogenemia rather than menstrual irregularity. *Journal of Clinical Endocrinology and Metabolism*, **87**, 2128-2133.
- Legro, R.S., Driscoll, D., Strauss, J.F. and Dunaif, A. (1998). Evidence for a genetic basis for hyperandrogenemia in polycystic ovary syndrome. *Proceedings of National Sciences of United States of America*, **95**, 14956-14960.
- Lephart, E. D., Ladle, D. R., Jacobson, N. A. & Rhees, R. W. (1996). Inhibition of brain 5 alpha-reductase in pregnant rats: effects on enzymatic and behavioral activity. *Brain Research*, **739**(1-2), 356-360.

- Levi-Montalcini, R. (1964). The nerve growth factor. *Annals of the New York Academy of Sciences*, **118**, 149-170.
- Levy, E., Prelli, F. & Frangione, B. (2006). Studies on the first described Alzheimer's disease amyloid beta mutant, the Dutch variant. *Journal of Alzheimers Disease*, **9**(3 Suppl), 329-339.
- Lewis, D. A. & Levitt, P. (2002). SCHIZOPHRENIA AS A DISORDER OF NEURODEVELOPMENT. *Annual Review of Neuroscience*, **25**(1), 409-432.
- Lieberman, S., Greenfield, N. J. & Wolfson, A. (1984). A heuristic proposal for understanding steroidogenic processes. *Endocrine Reviews*, **5**(1), 128-148.
- Lightfoot, S. (2002). Quantitation comparison of total RNA using the Agilent 2100 bioanalyzer, ribogreen analysis and UV spectrometry. In Agilent Technologies (Ed.), *5988-7650EN: Agilent Application Note*.
- Linden, D. J. (1994). Input-specific induction of cerebellar long-term depression does not require presynaptic alteration. *Learning and Memory*, **1**(2), 121-128.
- Little, M., Rumballe, B., Georgas, K., Yamada, T. & Teasdale, R. D. (2002). Conserved modularity and potential for alternate splicing in mouse and human Slit genes. *International Journal of Developmental Biology*, **46**(4), 385-391.
- Liu, X., Robertson, E. & Miall, R. C. (2003). Neuronal activity related to the visual representation of arm movements in the lateral cerebellar cortex. *Journal of Neurophysiology*, **89**(3), 1223-1237.
- Luine, V. & Rodriguez, M. (1994). Effects of estradiol on radial arm maze performance of young and aged rats. *Behavioural and Neural Biology*, **62**(3), 230-236.
- Luo, L., Chen, H. & Zirkin, B. R. (2001). Leydig cell aging: steroidogenic acute regulatory protein (StAR) and cholesterol side-chain cleavage enzyme. *Journal of Andrology*, **22**(1), 149-156.
- MacLusky, N. J., Clark, A. S., Naftolin, F. & Goldman-Rakic, P. S. (1987). Estrogen formation in the mammalian brain: possible role of aromatase in sexual differentiation of the hippocampus and neocortex. *Steroids*, **50**(4-6), 459-474.
- Mahmood, T. & Yang, P. C. (2012). Western blot: technique, theory, and trouble shooting. *National American Journal of Medical Science*, **4**(9), 429-434.
- Mallard, E. C., Rees, S., Stringer, M., Cock, M. L. & Harding, R. (1998). Effects of chronic placental insufficiency on brain development in fetal sheep. *Pediatric Research*, **43**(2), 262-270.

- Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P. & Evans, R. M. (1995). The nuclear receptor superfamily: the second decade. *Cell*, **83**(6), 835-839.
- Manna, P. R., Dyson, M. T. & Stocco, D. M. (2009). Regulation of the steroidogenic acute regulatory protein gene expression: present and future perspectives. *Molecular Human Reproduction*, **15**(6), 321-333.
- Manna, P. R., Wang, X. J. & Stocco, D. M. (2003). Involvement of multiple transcription factors in the regulation of steroidogenic acute regulatory protein gene expression. *Steroids*, **68**(14), 1125-1134.
- Manning, J. T., Scutt, D., Wilson, J. & Lewis-Jones, D. I. (1998). The ratio of 2nd to 4th digit length: a predictor of sperm numbers and concentrations of testosterone, luteinizing hormone and oestrogen. *Human Reproduction*, **13**(11), 3000-3004.
- Manning, J. T. J., Baron-Cohen, S. S., Wheelwright, S. S. & Sanders, G. G. (2001). The 2nd to 4th digit ratio and autism. *Developmental Medicine and Child Neurology*, **43**(3), 160-160-164.
- Mansouri-Attia, N., Aubert, J., Reinaud, P., Giraud-Delville, C., Taghouti, G., Galio, L., Everts, R. E., Degrelle, S., Richard, C., Hue, I., Yang, X., Tian, X. C., Lewin, H. A., Renard, J. P., et al. (2009). Gene expression profiles of bovine caruncular and intercaruncular endometrium at implantation. *Physiology and Genomics*, **39**(1), 14-27.
- Marcinkiewicz, M. & Seidah, N. G. (2000). Coordinated expression of beta-amyloid precursor protein and the putative beta-secretase BACE and alpha-secretase ADAM10 in mouse and human brain. *Journal of Neurochemistry*, **75**(5), 2133-2143.
- Markham, J. A. & Koenig, J. I. (2011). Prenatal stress: role in psychotic and depressive diseases. *Psychopharmacology (Berl)*, **214**(1), 89-106.
- Mason, D. Y., Micklem, K. & Jones, M. (2000). Double immunofluorescence labelling of routinely processed paraffin sections. *Journal of Pathology*, **191**(4), 452-461.
- Matsumoto, A. (1997). Hormonally induced neuronal plasticity in the adult motoneurons. *Brain Research Bulletin*, **44**(4), 539-547.
- Matsunaga, M., Ukena, K. & Tsutsui, K. (2001). Expression and localization of cytochrome P450 17 $\alpha$ -hydroxylase/c17,20-lyase in the avian brain. *Brain Research*, **899**(1-2), 112-122.
- McConnell, S. K. & Kaznowski, C. E. (1991). Cell cycle dependence of laminar determination in developing neocortex. *Science*, **254**(5029), 282-285.

- McIntosh, G. H., Baghurst, K. I., Potter, B. J. & Hetzel, B. S. (1979). Foetal thyroidectomy and brain development in the sheep. *Neuropathology and Applied Neurobiology*, **5**(5), 363-376.
- McTigue, D. M. & Tripathi, R. B. (2008). The life, death, and replacement of oligodendrocytes in the adult CNS. *Journal of Neurochemistry*, **107**(1), 1-19.
- Meaney, M. J. & Aitken, D. H. (1985). [3H]Dexamethasone binding in rat frontal cortex. *Brain Research*, **328**(1), 176-180.
- Mensah-Nyagan, A. G., Do-Rego, J.-L., Beaujean, D., Luu-The, V., Pelletier, G. & Vaudry, H. (1999). Neurosteroids: Expression of Steroidogenic Enzymes and Regulation of Steroid Biosynthesis in the Central Nervous System. *Pharmacological Reviews*, **51**(1), 63-82
- Merlino, A. A., Welsh, T. N., Tan, H., Yi, L. J., Cannon, V., Mercer, B. M. & Mesiano, S. (2007). Nuclear progesterone receptors in the human pregnancy myometrium: evidence that parturition involves functional progesterone withdrawal mediated by increased expression of progesterone receptor-A. *Journal of Clinical Endocrinology and Metabolism*, **92**(5), 1927-1933.
- Mertsch, S., Schmitz, N., Jeibmann, A., Geng, J. G., Paulus, W. & Senner, V. (2008). Slit2 involvement in glioma cell migration is mediated by Robo1 receptor. *Journal of Neurooncology*, **87**(1), 1-7.
- Messer, A. (1988). Thyroxine injections do not cause premature induction of thymidine kinase in sg/sg mice. *Journal of Neurochemistry*, **51**(3), 888-891.
- Meyer-Bahlburg, H. F., Dolezal, C., Baker, S. W., Carlson, A. D., Obeid, J. S. & New, M. I. (2004). Cognitive and motor development of children with and without congenital adrenal hyperplasia after early-prenatal dexamethasone. *Journal of Clinical Endocrinology and Metabolism*, **89**(2), 610-614.
- Micheva, K. D., Vallée, A., Beaulieu, C., Herman, I. M. & Leclerc, N. (1998).  $\beta$ -Actin is confined to structures having high capacity of remodelling in developing and adult rat cerebellum. *European Journal of Neuroscience*, **10**(12), 3785-3798.
- Milgrom, E., Thi, L., Atger, M. & Baulieu, E. E. (1973). Mechanisms regulating the concentration and the conformation of progesterone receptor(s) in the uterus. *Journal of Biology and Chemistry*, **248**(18), 6366-6374.
- Millar, J. K., Wilson-Annan, J. C., Anderson, S., Christie, S., Taylor, M. S., Semple, C. A. M., Devon, R. S., Clair, D. M. S., Muir, W. J., Blackwood, D. H. R. & Porteous, D. J. (2000). Disruption of two novel genes by a translocation co-segregating with schizophrenia. *Human Molecular Genetics*, **9**(9), 1415-1423.

- Millen, K., J. & Gleeson, J., G. (2008). Cerebellar development and disease. *Current Opinion in Neurobiology*, **18**(1), 12-19.
- Milosevic, A. & Zecevic, N. (1998). Developmental changes in human cerebellum: Expression of intracellular calcium receptors, calcium-binding proteins, and phosphorylated and nonphosphorylated neurofilament protein. *The Journal of Comparative Neurology*, **396**(4), 442-460.
- Miyata, T., Nakajima, K., Mikoshiba, K. & Ogawa, M. (1997). Regulation of Purkinje cell alignment by reelin as revealed with CR-50 antibody. *Journal of Neuroscience*, **17**(10), 3599-3609.
- Miyata, T., Ono, Y., Okamoto, M., Masaoka, M., Sakakibara, A., Kawaguchi, A., Hashimoto, M. & Ogawa, M. (2010). Migration, early axonogenesis, and Reelin-dependent layer-forming behavior of early/posterior-born Purkinje cells in the developing mouse lateral cerebellum. *Neural Development* **5**, 23.
- Mong, J. A., Glaser, E. & McCarthy, M. M. (1999). Gonadal steroids promote glial differentiation and alter neuronal morphology in the developing hypothalamus in a regionally specific manner. *Journal of Neuroscience*, **19**(4), 1464-1472.
- Morange, M. (2001). *The Misunderstood Gene*. Cambridge, MA: Harvard University Press.
- Morey, J. S., Ryan, J. C. & Van Dolah, F. M. (2006). Microarray validation: factors influencing correlation between oligonucleotide microarrays and real-time PCR. *Biology Proceedings Online*, **8**, 175-193.
- Morrison, M. E. & Mason, C. A. (1998). Granule neuron regulation of Purkinje cell development: striking a balance between neurotrophin and glutamate signaling. *Journal of Neuroscience*, **18**(10), 3563-3573.
- Morton, A. J. & Avanzo, L. (2011). Executive Decision-Making in the Domestic Sheep. *PloS one*, **6**(1), e15752.
- Mrzljak, L., Uylings, H. B., Kostovic, I. & Van Eden, C. G. (1988). Prenatal development of neurons in the human prefrontal cortex: I. A qualitative Golgi study. *Journal of Comparative Neurology*, **271**(3), 355-386.
- Mrzljak, L., Uylings, H. B., Kostovic, I. & van Eden, C. G. (1992). Prenatal development of neurons in the human prefrontal cortex. II. A quantitative Golgi study. *Journal of Comparative Neurology*, **316**(4), 485-496.
- Mueller, O., Lightfoot, S. & Schroeder, A. (2004). RNA Integrity Number (RIN) – Standardization of RNA Quality Control. In Agilent. Technoliges (Ed.), *5989-1165EN: Agilent Application Note*.

- Mulac-Jericevic, B., Mullinax, R. A., DeMayo, F. J., Lydon, J. P. & Conneely, O. M. (2000). Subgroup of Reproductive Functions of Progesterone Mediated by Progesterone Receptor-B Isoform. *Science*, **289**(5485), 1751-1754.
- Muller, J. F., Mascagni, F. & McDonald, A. J. (2006). Pyramidal cells of the rat basolateral amygdala: synaptology and innervation by parvalbumin-immunoreactive interneurons. *Journal of Comparative Neurology*, **494**(4), 635-650.
- Munoz-Cueto, J. A., Garcia-Segura, L. M. & Ruiz-Marcos, A. (1990). Developmental sex differences and effect of ovariectomy on the number of cortical pyramidal cell dendritic spines. *Brain Research*, **515**(1-2), 64-68.
- Nadarajah, B. & Parnavelas, J. G. (2002). Modes of neuronal migration in the developing cerebral cortex. *Nature Reviews in Neuroscience*, **3**(6), 423-432.
- Nadri, C., Lipska, B. K., Kozlovsky, N., Weinberger, D. R., Belmaker, R. H. & Agam, G. (2003). Glycogen synthase kinase (GSK)-3 $\beta$  levels and activity in a neurodevelopmental rat model of schizophrenia. *Developmental Brain Research*, **141**(1-2), 33-37.
- Naftolin, F. (1994). Brain aromatization of androgens. *Journal of Reproductive Medicine*, **39**(4), 257-261.
- Neal, J. K. & Wade, J. (2007). Androgen receptor expression and morphology of forebrain and neuromuscular systems in male green anoles displaying individual differences in sexual behavior. *Hormones and Behaviour*, **52**(2), 228-236.
- Ness, D. K., Schantz, S. L., Moshtaghian, J. & Hansen, L. G. (1993). Effects of perinatal exposure to specific PCB congeners on thyroid hormone concentrations and thyroid histology in the rat. *Toxicology Letters*, **68**(3), 311-323.
- Neugebauer, R., Hoek, H. W. & Susser, E. (1999). Prenatal exposure to wartime famine and development of antisocial personality disorder in early adulthood. *JAMA*, **282**(5), 455-462.
- Nguyen, A., Rauch, T. A., Pfeifer, G. P. & Hu, V. W. (2010). Global methylation profiling of lymphoblastoid cell lines reveals epigenetic contributions to autism spectrum disorders and a novel autism candidate gene, RORA, whose protein product is reduced in autistic brain. *FASEB Journal*, **24**(8), 3036-3051.
- Nicholson, J. L. & Altman, J. (1972). Synaptogenesis in the rat cerebellum: effects of early hypo- and hyperthyroidism. *Science*, **176**(4034), 530-532.

- Nicol, M. B., Hirst, J. J. & Walker, D. (1999). Effects of pregnanolone on behavioural parameters and the responses to GABAA receptor antagonists in the late gestation fetal sheep. *Neuropharmacology*, **38**(1), 49-63.
- Nicoletto, S. F. & Rinaldi, A. (2011). In the womb's shadow. The theory of prenatal programming as the fetal origin of various adult diseases is increasingly supported by a wealth of evidence. *EMBO Reproduction*, **12**(1), 30-34.
- Nishihara, E., Yoshida-Komiya, H., Chan, C. S., Liao, L., Davis, R. L., O'Malley, B. W. & Xu, J. (2003). SRC-1 null mice exhibit moderate motor dysfunction and delayed development of cerebellar Purkinje cells. *Journal of Neuroscience*, **23**(1), 213-222.
- Numakawa, T., Yokomaku, D., Richards, M., Hori, H., Adachi, N. and Kunugi, H. (2010). Functional interactions between steroid hormones and neurotrophin BDNF. *World Journal of Biological Chemistry*, **1**(5), 133-143.
- Nunez, J. L., Huppenbauer, C. B., McAbee, M. D., Juraska, J. M. & DonCarlos, L. L. (2003). Androgen receptor expression in the developing male and female rat visual and prefrontal cortex. *Journal of Neurobiology*, **56**(3), 293-302.
- Ohyu, J., Yamanouchi, H. & Takashima, S. (1997). Immunohistochemical study of microtubule-associated protein 5 (MAP5) expression in the developing human brain. *Brain and Development*, **19**(8), 541-546.
- Oltvai, Z. N., Milliman, C. L. & Korsmeyer, S. J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell*, **74**(4), 609-619.
- Ongur, D., Ferry, A. T. & Price, J. L. (2003). Architectonic subdivision of the human orbital and medial prefrontal cortex. *Journal of Comparative Neurology*, **460**(3), 425-449.
- Oppenheim, R. W. (1989). The neurotrophic theory and naturally occurring motoneuron death. *Trends in Neuroscience*, **12**(7), 252-255.
- Oppenheim, R. W., Homma, S., Marti, E., Prevet, D., Wang, S., Yaginuma, H. & McMahon, A. P. (1999). Modulation of early but not later stages of programmed cell death in embryonic avian spinal cord by sonic hedgehog. *Molecular and Cellular Neuroscience*, **13**(5), 348-361.
- Ozdinler, P. H. & Erzurumlu, R. S. (2002). Slit2, a branching-arborization factor for sensory axons in the Mammalian CNS. *Journal of Neuroscience*, **22**(11), 4540-4549.

- Paech, K., Webb, P., Kuiper, G. G., Nilsson, S., Gustafsson, J., Kushner, P. J. & Scanlan, T. S. (1997). Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science*, **277**(5331), 1508-1510.
- Parker, M. G. (1993). Steroid and related receptors. *Current Opinions in Cell Biology*, **5**(3), 499-504.
- Pass, D. & Freeth, G. (1993). The Rat: ANZCCART Fact Sheet. *ANZCCART News*, **6**(1-4).
- Paxinos, G. a. M., J.K. (2004). *The Human Nervous System* (Second Edition ed.). London, UK: Academic Press, Elsevier.
- Payne, A. H. & Hales, D. B. (2004). Overview of Steroidogenic Enzymes in the Pathway from Cholesterol to Active Steroid Hormones. *Endocrine Reviews*, **25**(6), 947-970.
- Petanjek, Z., Judas, M., Simic, G., Rasin, M. R., Uylings, H. B., Rakic, P. & Kostovic, I. (2011). Extraordinary neoteny of synaptic spines in the human prefrontal cortex. *Proceedings of the National Academy of Science U. S. A*, **108**(32), 13281-13286.
- Petratos, S., Hirst, J. J., Mendis, S., Anikijenko, P. & Walker, D. W. (2000). Localization of P450scc and 5 $\alpha$ -reductase type-2 in the cerebellum of fetal and newborn sheep. *Developmental Brain Research*, **123**(1), 81-86.
- Pettmann, B. & Henderson, C. E. (1998). Neuronal cell death. *Neuron*, **20**(4), 633-647.
- Peytremann, A., Nicholson, W. E., Hardman, J. G. & Liddle, G. W. (1973). Effect of adrenocorticotrophic hormone on extracellular adenosine 3',5'-monophosphate in the hypophysectomized rat. *Endocrinology*, **92**(5), 1502-1506.
- Phoenix, C. H., Goy, R. W., Gerall, A. A. & Young, W. C. (1959). Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig. *Endocrinology*, **65**, 369-382.
- Pickard, B. S., Malloy, M. P., Porteous, D. J., Blackwood, D. H. R. & Muir, W. J. (2005). Disruption of a brain transcription factor, NPAS3, is associated with schizophrenia and learning disability. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*, **136B**(1), 26-32.



- Pieper, A. A., Wu, X., Han, T. W., Estill, S. J., Dang, Q., Wu, L. C., Reece-Fincannon, S., Dudley, C. A., Richardson, J. A., Brat, D. J. & McKnight, S. L. (2005). The neuronal PAS domain protein 3 transcription factor controls FGF-mediated adult hippocampal neurogenesis in mice. *Proceedings of the National Academy of Sciences of the United States of America*, **102**(39), 14052-14057.
- Pilati, N., Barker, M., Panteleimonitis, S., Donga, R. & Hamann, M. (2008). A rapid method combining Golgi and Nissl staining to study neuronal morphology and cytoarchitecture. *J Histochem Cytochem*, **56**(6), 539-550.
- Polak, J. M. & Van Noorden, S. (2003). *Introduction to Immunocytochemistry* (3rd edition ed.). Oxford, U.K.: Bios Scientific Publishers Ltd.
- Potter, B. J., Mano, M. T., Belling, G. B., McIntosh, G. H., Hua, C., Cragg, B. G., Marshall, J., Wellby, M. L. & Hetzel, B. S. (1982). RETARDED FETAL BRAIN DEVELOPMENT RESULTING FROM SEVERE DIETARY IODINE DEFICIENCY IN SHEEP. *Neuropathology and Applied Neurobiology*, **8**(4), 303-313.
- Price, R.H. and Handa, R.J. (2000) Expression of estrogen receptor-beta protein and mRNA in the cerebellum of the rat. *Neuroscience Letters*, **288**(2), 115-118.
- Pryce, C. R., Aubert, Y., Maier, C., Pearce, P. C. & Fuchs, E. (2011). The developmental impact of prenatal stress, prenatal dexamethasone and postnatal social stress on physiology, behaviour and neuroanatomy of primate offspring: studies in rhesus macaque and common marmoset. *Psychopharmacology (Berl)*, **214**(1), 33-53.
- Quadros, P. S. & Wagner, C. K. (2008). Regulation of progesterone receptor expression by estradiol is dependent on age, sex and region in the rat brain. *Endocrinology*, **149**(6), 3054-3061.
- Rabie, A., Brehier, A., Intrator, S., Clavel, M. C., Parkes, C. O., Legrand, C. & Thomasset, M. (1986). Thyroid state and cholecalciferol (calcium-binding protein) in cerebellum of the developing rat. *Brain Research*, **394**(2), 253-265.
- Rae, M.T., Grace, C., Hogg, K., Wilson, L. M., McHaffie, S. L., Ramaswamy, S., MacCallum, J., Connolly, F., McNeilly, A. S. & Duncan, C. (2013). The pancreas is altered by in utero androgen exposure: implications for clinical conditions such as polycystic ovary syndrome (PCOS). *PLoS One*, **8**(2), e56263.
- Rakic, S., & Zecevic, N. . (2000). Programmed cell death in the developing human telencephalon. *The European Journal of Neuroscience*, **12**(8), 2721-2734.
- Ramos-Vara, J. A. (2005). Technical Aspects of Immunohistochemistry. *Veterinary Pathology Online*, **42**(4), 405-426.

- Rampon, C., Jiang, C.H., Dong, H., Tang, Y.P., Lockart, D.J., Schultz, P.G. and Hu, Y. (2000). Effects of environmental enrichment on gene expression in the brain. *Proceedings of the National Academy of Science (USA)*, **97**, 12880–12884.
- Raymond, J. L. & Lisberger, S. G. (1996). Error signals in horizontal gaze velocity Purkinje cells under stimulus conditions that cause learning in the VOR. *Annals of New York Academy Science*, **781**, 686-689.
- Recabarren, S.E., Smith, R., Rios, R., Maliqueo, M., Echiburú, B., Codner, E., Cassorla, F., Rojas, P. and Sir-Petermann, T. (1998). Metabolic profile in sons of women with polycystic ovary syndrome. *Journal of Clinical Endocrinology and Metabolism*, **93**(5), 1820-1826.
- Rees, S. & Harding, R. (1988). The effects of intrauterine growth retardation on the development of the Purkinje cell dendritic tree in the cerebellar cortex of fetal sheep: A note on the ontogeny of the Purkinje cell. *International Journal of Developmental Neuroscience*, **6**(5), 461-469.
- Rees, S. & Harding, R. (2004). Brain development during fetal life: influences of the intra-uterine environment. *Neuroscience Letters*, **361**(1-3), 111-114.
- Rees, S., Stringer, M., Just, Y., Hooper, S. B. & Harding, R. (1997). The vulnerability of the fetal sheep brain to hypoxemia at mid-gestation. *Development and Brain Research*, **103**(2), 103-118.
- Reid, S. N. & Juraska, J. M. (1992). Sex differences in the gross size of the rat neocortex. *Journal of Comparative Neurology*, **321**(3), 442-447.
- Reid, S. N. & Juraska, J. M. (1995). Sex differences in the number of synaptic junctions in the binocular area of the rat visual cortex. *Journal of Comparative Neurology*, **352**(4), 560-566.
- Reyes, M. G. & Gordon, A. (1981). Cerebellar vermis in schizophrenia. *Lancet*, **2**(8248), 700-701.
- Rimland, J., Xin, W., Sweetnam, P., Saijoh, K., Nestler, E. J. & Duman, R. S. (1991). Sequence and expression of a neuropeptide Y receptor cDNA. *Molecular Pharmacology*, **40**(6), 869-875.
- Ritvo, E. R., Freeman, B. J., Scheibel, A. B., Duong, T., Robinson, H., Guthrie, D. & Ritvo, A. (1986). Lower Purkinje cell counts in the cerebella of four autistic subjects: initial findings of the UCLA-NSAC Autopsy Research Report. *American Journal of Psychiatry*, **143**(7), 862-866.
- Robson, S. L. & Wood, B. (2008). Hominin life history: reconstruction and evolution. *Journal of Anatomy*, **212**(4), 394-425.

- Rocamora, N., Garcia-Ladona, F. J., Palacios, J. M. & Mengod, G. (1993). Differential expression of brain-derived neurotrophic factor, neurotrophin-3, and low-affinity nerve growth factor receptor during the postnatal development of the rat cerebellar system. *Molecular Brain Research*, **17**(1-2), 1-8.
- Romeo, R. D., Diedrich, S. L. & Sisk, C. L. (2000). Effects of gonadal steroids during pubertal development on androgen and estrogen receptor-alpha immunoreactivity in the hypothalamus and amygdala. *Journal of Neurobiology*, **44**(3), 361-368.
- Roseboom, T., de Rooij, S. & Painter, R. (2006). The Dutch famine and its long-term consequences for adult health. *Early Human Development*, **82**(8), 485-491.
- Roselli, C., Schrunk, J., Stadelman, H., Resko, J. & Stormshak, F. (2006). The effect of aromatase inhibition on the sexual differentiation of the sheep brain. *Endocrine*, **29**(3), 501-511.
- Roselli, C. E., Resko, J. A. & Stormshak, F. (2003). Estrogen synthesis in fetal sheep brain: effect of maternal treatment with an aromatase inhibitor. *Biology and Reproduction*, **68**(2), 370-374.
- Rosellini, R. A., DeCola, J. P., Plonsky, M., Warren, D. A. & Stilman, A. J. (1984). Inescapable shock interferes with the acquisition of a free appetitive operant. *Journal of Experimental Psychology*, **10**(3), 346-359.
- Roth, K. A. & D'Sa, C. (2001). Apoptosis and brain development. *Mental Retardation and Developmental Disability Research Reviews*, **7**(4), 261-266.
- Ruiz-Marcos, A., Salas, J., Sanchez-Toscano, F., Escobar del Rey, F. & Morreale de Escobar, G. (1983). Effect of neonatal and adult-onset hypothyroidism on pyramidal cells of the rat auditory cortex. *Brain Research*, **285**(2), 205-213.
- Sakamoto, H., Shikimi, H., Ukena, K. & Tsutsui, K. (2003). Neonatal expression of progesterone receptor isoforms in the cerebellar Purkinje cell in rats. *Neuroscience Letters*, **343**(3), 163-166.
- Sakamoto, H., Ukena, K. & Tsutsui, K. (2001). Effects of Progesterone Synthesized De Novo in the Developing Purkinje Cell on Its Dendritic Growth and Synaptogenesis. *The Journal of Neuroscience*, **21**(16), 6221-6232.
- Salouci, M., Engelen, V., Gyan, M., Antoine, N., Jacqmot, O., Mignon, Y., Kirschvink, N. & Gabriel, A. (2012). Development of Purkinje Cells in the Ovine Brain. *Anatomia, Histologia, Embryologia*, **41**(3), 227-232.
- Sanchez, P., Torres, J. M., Del Moral, R. G. & Ortega, E. (2006). Effects of testosterone on brain mRNA levels of steroid 5alpha-reductase isozymes in early postnatal life of rat. *Neurochemistry International*, **49**(6), 626-630.

- Sam, S., Sung, Y.H., Legro, R.S. and Dunaif, A. (2008). Evidence for pancreatic beta-cell dysfunction in brothers of women with polycystic ovary syndrome. *Metabolism*, **57**, 84-89.
- Sang, Q., Wu, J., Rao, Y., Hsueh, Y. P. & Tan, S. S. (2002). Slit promotes branching and elongation of neurites of interneurons but not projection neurons from the developing telencephalon. *Molecular and Cellular Neuroscience*, **21**(2), 250-265.
- Santana, N., Bortolozzi, A., Serrats, J., Mengod, G. & Artigas, F. (2004). Expression of serotonin1A and serotonin2A receptors in pyramidal and GABAergic neurons of the rat prefrontal cortex. *Cerebral Cortex*, **14**(10), 1100-1109.
- Sarachana, T., Xu, M., Wu, R. C. and Hu, V. W. (2011). Sex hormones in autism: androgens and estrogens differentially and reciprocally regulate RORA, a novel candidate gene for autism. *PloS one*, **6**(2), e17116-e17116.
- Schaub, C. E., Gersting, J. A., Keller-Wood, M. & Wood, C. E. (2008). Development of ER-alpha and ER-beta expression in the developing ovine brain and pituitary. *Gene Expression Patterns*, **8**(6), 457-463.
- Schilling, K., Oberdick, J., Rossi, F. & Baader, S. (2008). Besides Purkinje cells and granule neurons: an appraisal of the cell biology of the interneurons of the cerebellar cortex. *Histochemistry and Cell Biology*, **130**(4), 601-615.
- Schulz, L. C. (2010). The Dutch Hunger Winter and the developmental origins of health and disease. *Proceedings of the National Academy of Sciences U. S. A.*, **107**(39), 16757-16758.
- Schurov, I. L., Handford, E. J., Brandon, N. J. & Whiting, P. J. (2004). Expression of disrupted in schizophrenia 1 (DISC1) protein in the adult and developing mouse brain indicates its role in neurodevelopment. *Molecular Psychiatry*, **9**(12), 1100-1110.
- Schwartz, H. L. (1983). Effect of Thyroid Hormone on Growth and Development. In J. H. Oppenheimer (Ed.), *Molecular Basis of Thyroid Hormone Action*. New York: Academic Press Inc.
- Seidler, R. D., Purushotham, A., Kim, S. G., Ugurbil, K., Willingham, D. & Ashe, J. (2002). Cerebellum activation associated with performance change but not motor learning. *Science*, **296**(5575), 2043-2046.
- Seligman, M. E. & Maier, S. F. (1967). Failure to escape traumatic shock. *Journal of Experimental Psychology*, **74**(1), 1-9.
- Serajee, F. J., Zhong, H. & Mahbubul Huq, A. H. M. (2006). Association of Reelin gene polymorphisms with autism. *Genomics*, **87**(1), 75-83.

- Sharma, P. K. & Thakur, M. K. (2006). Expression of estrogen receptor (ER) alpha and beta in mouse cerebral cortex: effect of age, sex and gonadal steroids. *Neurobiology and Aging*, **27**(6), 880-887.
- Sheppard, H. M., Harries, J. C., Hussain, S., Bevan, C. & Heery, D. M. (2001). Analysis of the steroid receptor coactivator 1 (SRC1)-CREB binding protein interaction interface and its importance for the function of SRC1. *Molecular and Cellular Biology*, **21**(1), 39-50.
- Shughrue, P. J., Stumpf, W. E., MacLusky, N. J., Zielinski, J. E. & Hochberg, R. B. (1990). Developmental changes in estrogen receptors in mouse cerebral cortex between birth and postweaning: studied by autoradiography with 11 beta-methoxy-16 alpha-[125I]iodoestradiol. *Endocrinology*, **126**(2), 1112-1124.
- Sieghart, W. & Sperk, G. (2002). Subunit Composition, Distribution and Function of GABA-A Receptor Subtypes. *Current Topics in Medicinal Chemistry*, **2**(8), 795-816.
- Sierra, A. (2004). Neurosteroids: The StAR Protein in the Brain. *Journal of Neuroendocrinology*, **16**(9), 787-793.
- Sir-Petermann, T., Maliqueo, M., Angel, B., Lara, H.E., Perez-Bravo, F. and Recabarren, S.E. (2002). Maternal serum androgens in pregnant women with polycystic ovary syndrome: possible implications in prenatal androgenization. *Human Reproduction*, **17**, 2573-2579.
- Smith, D. F. & Toft, D. O. (1993). Steroid receptors and their associated proteins. *Molecular Endocrinology*, **7**(1), 4-11.
- Sotelo, C. (1978). Purkinje cell ontogeny: formation and maintenance of spines. *Progressive Brain Research*, **48**, 149-170.
- Sotelo, C. (2004). Cellular and genetic regulation of the development of the cerebellar system. *Progress in Neurobiology*, **72**(5), 295-339.
- Sowell, E.R., Peterson, B.S., Thompson, P.M., Welcome, S.E., Henkenius, A.L. and Toga, AW. (2003). Mapping Cortical Change Across the Human Life Span. *Nature Neuroscience*, **6**, 309-315.
- Spritzer, M. D. & Galea, L. A. (2007). Testosterone and dihydrotestosterone, but not estradiol, enhance survival of new hippocampal neurons in adult male rats. *Developmental Neurobiology*, **67**(10), 1321-1333.
- Squire, L. R., Bloom, F. E., McConnell, S. K., Roberts, J. L., Spitzer, N. C. & Zigmond, M. J. (2003). *Cerebellum In: Fundamental Neuroscience* New York: Academic Press.
- Stiles, J. (2008). *The Fundamentals of Brain Development: Integrating Nature and Nurture*. Cambridge: MA: Harvard University Press.

- Stiles, J. & Jernigan, T. (2010). The Basics of Brain Development. *Neuropsychology Review*, **20**(4), 327-348.
- Stocco, D. M. & Clark, B. J. (1996). Regulation of the Acute Production of Steroids in Steroidogenic Cells. *Endocrine Reviews*, **17**(3), 221-244.
- Strait, K. A., Schwartz, H. L., Seybold, V. S., Ling, N. C. & Oppenheimer, J. H. (1991). Immunofluorescence localization of thyroid hormone receptor protein beta 1 and variant alpha 2 in selected tissues: cerebellar Purkinje cells as a model for beta 1 receptor-mediated developmental effects of thyroid hormone in brain. *Proceedings of the National Academy of Science U.S.A.*, **88**(9), 3887-3891.
- Suarez-Vega, A., Gutierrez-Gil, B., Cuchillo-Ibanez, I., Saez-Valero, J., Perez, V., Garcia-Gamez, E., Benavides, J. & Arranz, J. J. (2013). Identification of a 31-bp deletion in the RELN gene causing lissencephaly with cerebellar hypoplasia in sheep. *PLoS One*, **8**(11), e81072.
- Suddath, R. L., Christison, G. W., Torrey, E. F., Casanova, M. F. & Weinberger, D. R. (1990). Anatomical abnormalities in the brains of monozygotic twins discordant for schizophrenia. *N Engl J Med*, **322**(12), 789-794.
- Symonds, M. E., Sebert, S. P., Hyatt, M. A. & Budge, H. (2009). Nutritional programming of the metabolic syndrome. *Nature Reviews Endocrinology*, **5**(11), 604-610.
- Talge, N. M., Neal, C. & Glover, V. (2007). Antenatal maternal stress and long-term effects on child neurodevelopment: how and why? *Journal of Child Psychology and Psychiatry*, **48**(3-4), 245-261.
- Tanaka, H. & Landmesser, L. T. (1986). Cell death of lumbosacral motoneurons in chick, quail, and chick-quail chimera embryos: a test of the quantitative matching hypothesis of neuronal cell death. *Journal of Neuroscience*, **6**(10), 2889-2899.
- Takagi, K., Ito, S., Miyazaki, T., Miki, Y., Shibahara, Y., Ishida, T., Watanabe, M., Inoue, S., Sasano, H. and Suzuki, T. (2013). Amyloid precursor protein in human breast cancer: An androgen-induced gene associated with cell proliferation. *Cancer Science*, **104**(11), 1532-1538.
- Teffer, K. & Semendeferi, K. (2012). Human prefrontal cortex: evolution, development, and pathology. *Progressive Brain Research*, **195**, 191-218.
- Terry, R. D. & Katzman, R. (1983). Senile dementia of the Alzheimer type. *Annals of Neurology*, **14**(5), 497-506.
- Thigpen, A. E., Silver, R. I., Guileyardo, J. M., Casey, M. L., McConnell, J. D. & Russell, D. W. (1993). Tissue distribution and ontogeny of steroid 5 alpha-reductase isozyme expression. *Journal of Clinical Investigation*, **92**(2), 903-910.

- Thomaidou, D., Mione, M. C., Cavanagh, J. F. & Parnavelas, J. G. (1997). Apoptosis and its relation to the cell cycle in the developing cerebral cortex. *Journal of Neuroscience*, **17**(3), 1075-1085.
- Thompson, J. & Meyer, H. (1994). Body Condition Scoring of Sheep. In O. S. University (Ed.) (Vol. 1433). Oregon: Oregon State University Extension Services.
- Thomson, P. A., Harris, S. E., Starr, J. M., Whalley, L. J., Porteous, D. J. & Deary, I. J. (2005). Association between genotype at an exonic SNP in DISC1 and normal cognitive aging. *Neuroscience Letters*, **389**(1), 41-45.
- Tilson, H. A. (1990). Neurotoxicology in the 1990s. *Neurotoxicology and Teratology*, **12**(4), 293-300.
- Tindall, E. A., Petersen, D. C., Woodbridge, P., Schipany, K. & Hayes, V. M. (2009). Assessing high-resolution melt curve analysis for accurate detection of gene variants in complex DNA fragments. *Human Mutations*, **30**(6), 876-883.
- Tirassa, P., Thiblin, I., Agren, G., Vigneti, E., Aloe, L. & Stenfors, C. (1997). High-dose anabolic androgenic steroids modulate concentrations of nerve growth factor and expression of its low affinity receptor (p75-NGFr) in male rat brain. *Journal of Neuroscience Research*, **47**(2), 198-207.
- Tora, L., Gronemeyer, H., Turcotte, B., Gaub, M.-P. & Chambon, P. (1988). The N-terminal region of the chicken progesterone receptor specifies target gene activation. *Nature*, **333**(6169), 185-188.
- Toran-Allerand, C. D., Gerlach, J. L. & McEwen, B. S. (1980). Autoradiographic localization of [3H]estradiol related to steroid responsiveness in cultures of the newborn mouse hypothalamus and preoptic area. *Brain Research*, **184**(2), 517-522.
- Toyooka, K., Asama, K., Watanabe, Y., Muratake, T., Takahashi, M., Someya, T. & Nawa, H. (2002). Decreased levels of brain-derived neurotrophic factor in serum of chronic schizophrenic patients. *Psychiatry Research*, **110**(3), 249-257.
- Tran, K. D., Smutzer, G. S., Doty, R. L. & Arnold, S. E. (1998). Reduced Purkinje cell size in the cerebellar vermis of elderly patients with schizophrenia. *American Journal of Psychiatry*, **155**(9), 1288-1290.
- Tsujimoto, S. (2008). The prefrontal cortex: functional neural development during early childhood. *Neuroscientist*, **14**(4), 345-358.
- Tsutsui, K. (2006). Biosynthesis and organizing action of neurosteroids in the developing Purkinje cell. *The Cerebellum*, **5**(2), 89-96.
- Tsutsui, K. (2008). Neurosteroids in the Purkinje Cell: Biosynthesis, Mode of Action and Functional Significance. *Molecular Neurobiology*, **37**(2), 116-125.

- Tsutsui, K., Ukena, K., Sakamoto, H., Okuyama, S. & Haraguchi, S. (2011). Biosynthesis, mode of action, and functional significance of neurosteroids in the purkinje cell. *Frontiers in Endocrinology (Lausanne)*, **2**, 61.
- Tsutsui, K., Ukena, K., Usui, M., Sakamoto, H. & Takase, M. (2000). Novel brain function: biosynthesis and actions of neurosteroids in neurons. *Neuroscience Research*, **36**(4), 261-273.
- Ullian, E. M., Christopherson, K. S. & Barres, B. A. (2004). Role for glia in synaptogenesis. *Glia*, **47**(3), 209-216.
- Valnes, K. & Brandtzaeg, P. (1984). Paired indirect immunoenzyme staining with primary antibodies from the same species. Application of horseradish peroxidase and alkaline phosphatase as sequential labels. *Histochemistry Journal*, **16**(5), 477-487.
- Van der Loos, C. (1999). *Immunoenzyme multiple staining techniques*. Oxford, UK: BIOS Scientific Publishers.
- Van der Loos, C. M. (2008). Multiple immunoenzyme staining: methods and visualizations for the observation with spectral imaging. *Journal of Histochemistry and Cytochemistry*, **56**(4), 313-328.
- Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, **3**(7), 1-12.
- Ventura, J., Nuechterlein, K. H., Lukoff, D. & Hardesty, J. P. (1989). A prospective study of stressful life events and schizophrenic relapse. *Journal of Abnormal Psychology*, **98**(4), 407-411.
- Vincent, J., Legrand, C., Rabie, A. & Legrand, J. (1982). Effects of thyroid hormone on synaptogenesis in the molecular layer of the developing rat cerebellum. *Journal of Physiology (Paris)*, **78**(8), 729-738.
- Vogel, M. W., McInnes, M., Zanjani, H. S. & Herrup, K. (1991). Cerebellar Purkinje cells provide target support over a limited spatial range: evidence from lurcher chimeric mice. *Development and Brain Research*, **64**(1-2), 87-94.
- Vogel, M. W., Sunter, K. & Herrup, K. (1989). Numerical matching between granule and Purkinje cells in lurcher chimeric mice: a hypothesis for the trophic rescue of granule cells from target-related cell death. *Journal of Neuroscience*, **9**(10), 3454-3462.
- Voogd, J. & Glickstein, M. (1998). The anatomy of the cerebellum. *Trends in Cognitive Sciences*, **2**(9), 307-313.



- Wahlestedt, C., Regunathan, S. & Reis, D. J. (1992). Identification of cultured cells selectively expressing Y1-, Y2-, or Y3-type receptors for neuropeptide Y/peptide YY. *Life Sciences*, **50**(4), P17-12.
- Wall, M. J. (2003). Endogenous nitric oxide modulates GABAergic transmission to granule cells in adult rat cerebellum. *European Journal of Neuroscience*, **18**(4), 869-878.
- Wang, D. D. & Kriegstein, A. R. (2009). Defining the role of GABA in cortical development. *The Journal of Physiology*, **587**(9), 1873-1879.
- Wang, K. H., Brose, K., Arnott, D., Kidd, T., Goodman, C. S., Henzel, W. & Tessier-Lavigne, M. (1999). Biochemical purification of a mammalian slit protein as a positive regulator of sensory axon elongation and branching. *Cell*, **96**(6), 771-784.
- Wang, Q., Wang, M. & Whim, M. D. (2013). Neuropeptide y gates a stress-induced, long-lasting plasticity in the sympathetic nervous system. *Journal of Neuroscience*, **33**(31), 12705-12717.
- Wang, W., Stock, R. E., Gronostajski, R. M., Wong, Y. W., Schachner, M. & Kilpatrick, D. L. (2004). A role for nuclear factor I in the intrinsic control of cerebellar granule neuron gene expression. *Journal of Biology and Chemistry*, **279**(51), 53491-53497.
- Webb, P., Lopez, G. N., Uht, R. M. & Kushner, P. J. (1995). Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. *Molecular Endocrinology*, **9**(4), 443-456.
- Wechsler-Reya, R. J. & Scott, M. P. (1999). Control of Neuronal Precursor Proliferation in the Cerebellum by Sonic Hedgehog. *Neuron*, **22**(1), 103-114.
- Weiss, L. A., Arking, D. E., Daly, M. J. & Chakravarti, A. (2009). A genome-wide linkage and association scan reveals novel loci for autism. *Nature*, **461**(7265), 802-808.
- Wellman, C. L. (2001). Dendritic reorganization in pyramidal neurons in medial prefrontal cortex after chronic corticosterone administration. *Journal of Neurobiology*, **49**(3), 245-253.
- Westmoreland, J. J., Hancock, C. R. & Condie, B. G. (2001). Neuronal Development of Embryonic Stem Cells: A Model of GABAergic Neuron Differentiation. *Biochemical and Biophysical Research Communications*, **284**(3), 674-680.
- White, R. & Parker, M. G. (1998). Molecular Mechanisms of Steroid Hormone Action. *Endocrine-Related Cancer*, **5**, 1-14.

- Williams, C. L. & Meck, W. H. (1991). The organizational effects of gonadal steroids on sexually dimorphic spatial ability. *Psychoneuroendocrinology*, **16**(1-3), 155-176.
- Williams, R. W. & Herrup, K. (1988). The control of neuron number. *Annual Reviews in Neuroscience*, **11**, 423-453.
- Wingate, R. J. (2001). The rhombic lip and early cerebellar development. *Curr Opin Neurobiol*, **11**(1), 82-88.
- Wong, M. L. & Medrano, J. F. (2005). Real-Time PCR for mRNA Quantification. *Biological Techniques*, **39**, 75-85.
- Wright, C. L., Schwarz, J. S., Dean, S. L. & McCarthy, M. M. (2010). Cellular mechanisms of estradiol-mediated sexual differentiation of the brain. *Trends in Endocrinology and Metabolism*, **21**(9), 553-561.
- Wright, L. L. & Smolen, A. J. (1983). Neonatal testosterone treatment increases neuron and synapse numbers in male rat superior cervical ganglion. *Brain Research*, **284**(2-3), 145-153.
- Yawno, T., Yan, E. B., Walker, D. W. & Hirst, J. J. (2007). Inhibition of neurosteroid synthesis increases asphyxia-induced brain injury in the late gestation fetal sheep. *Neuroscience*, **146**(4), 1726-1733.
- Yuan, S., Trachtenberg, J., Mills, G. B., Brown, T. J., Xu, F. & Keating, A. (1993). Androgen-induced inhibition of cell proliferation in an androgen-insensitive prostate cancer cell line (PC-3) transfected with a human androgen receptor complementary DNA. *Cancer Research*, **53**(6), 1304-1311.
- Yuan, W., Zhou, L., Chen, J. H., Wu, J. Y., Rao, Y. & Ornitz, D. M. (1999). The mouse SLIT family: secreted ligands for ROBO expressed in patterns that suggest a role in morphogenesis and axon guidance. *Development and Biology*, **212**(2), 290-306.
- Yuasa, S., Kitoh, J., Oda, S. & Kawamura, K. (1993). Obstructed migration of Purkinje cells in the developing cerebellum of the reeler mutant mouse. *Anat Embryology (Berl)*, **188**(4), 317-329.
- Zhang, L., Chang, Y.H., Barker, J.L., Hu, Q., Maric, D., Li, B.S. and Rubinow, D.R. (2000). Testosterone and estrogen affect neuronal differentiation but not proliferation in early embryonic cortex of the rat: the possible roles of androgen and estrogen receptors. *Neuroscience Letters*, **281**, 57-60.
- Zhao, Y., Kwan, K.-M., Mailloux, C. M., Lee, W.-K., Grinberg, A., Wurst, W., Behringer, R. R. & Westphal, H. (2007). LIM-homeodomain proteins Lhx1 and Lhx5, and their cofactor Ldb1, control Purkinje cell differentiation in the developing cerebellum. *Proceedings of the National Academy of Sciences*, **104**(32), 13182-13186.

