Exploring a Novel Mechanism Instructing Maternal Care and Influencing Offspring Outcomes.

— **Hugo David James Creeth** —

Supervisors: Prof. Rosalind John and Prof. Anthony Isles

Submitted for consideration for award of PhD

December 2016

School of Biosciences, Cardiff University

creethh@cardiff.ac.uk

Student Number: 0920762

LaTeX

What is this life if, full of care,
We have no time to stand and stare.

No time to stand beneath the boughs,
And stare as long as sheep or cows.

No time to see, when woods we pass,
Where squirrels hide their nuts in grass.

No time to see, in broad daylight,
Streams full of stars, like skies at night.

No time to turn at Beauty's glance,
And watch her feet, how they can dance.

No time to wait till her mouth can
Enrich that smile her eyes began.

A poor life this if, full of care,
We have no time to stand and stare.

— **W. H. Davies**

Contents

Acknowledgements

What a journey! None of this would have been possible without the help and support of Ros and Anthony. Thank you for taking a gamble on me and this project. The hours you spent trying to find the funding to keep me employed and able continue to work on the research that is (still!) so close to my heart will always be remembered and appreciated.

Thank you to Simon, a truly inspirational man and, even if he won't admit it, a great friend. He often said "I am carrying you through your PhD!", and at times he wasn't far wrong. The constant belittling and teasing, was only matched by the support he provided not only with work but with life. Thank you.

I want to thank those friends I have made along the way Jess (who started me on this path), Jordan, Claire, Gráinne, Hannah and Anna. Seeing you all go through the trials of a PhD before me not only terrified me, but taught me that it pays to be determined. I want to thank my friends with proper jobs who kept me entertained, grounded, poor, fed and happy. You mean more to me than you know. Soon I will have a proper job too, I promise. To my sister, who has never stopped believing in me from the day I was born (at least after she forgave me for being a boy), your support and encouragement was vital.

I have to say an enormous thank you to Jon whom I met during my masters and who quickly became a close friend. Not only the endless trips climbing and walking through the Brecon Beacons, but the time and money we spent drinking Costa coffee on the "Thinking Wall" during my first year and now whenever you visit, played a massive part in helping me get to where I am today.

The staff and technicians in psychology and BIOSI, thank you for the time and effort you put in daily to make the University what it is, and making my job run so smoothly.

Annabel, where do I begin? From feeding me and housing me during my masters, supporting me when I chose to do a PhD, sticking by me throughout it all and even agreeing to marry me. Thank you. You are my rock I could never have done this without your never ending love and support. I don't deserve you but I am so happy to have you.

Finally, I want to thank my parents. It still amazes me how you managed to turn an atopic, hyperactive child into who I am today. Thank you for instilling in me an inquisitive mind and for encouraging me to always ask questions and to constantly try to better myself. You have always been there for me whenever I needed you and, perhaps more importantly, even when I didn't realise that I did. This is for you...

Declaration

Initial training in techniques and laboratory practice and subsequent mentoring:

- **Professor Rosalind John:** conception and design of the experiment, general advice and discussion with reference to imprinting, the placenta, genetics, methodology and general genetics advice and discussion.
- **Professor Anthony Isles:** experimental design of behavioural tests, general advice and discussion with reference to neuroscience, behavioural neuroscience, behavioural genetics, laboratory techniques and general genetics advice and discussion.
- **Dr Simon Tunster:** practical assistance with setting up of biomolecular procedures and general troubleshooting advice.
- **Dr Jessica Eddy:** practical assistance with setting up behavioural tasks and general advice with reference to behavioural testing paradigms.
- **Dr Dominic Dwyer:** practical assistance with setting up the Lick Cluster Analysis behavioural assay and general advice with reference to behavioural testing paradigms and statistics.
- **Jonathan Davies:** provided coding advice and help regarding bioinformatic analysis.

Data produced jointly:

- **Dr Simon Tunster and Dr Gráinne MacNamara:** were jointly involved in the generation of tissue for prolactin receptor model.

Data/materials provided by someone else:

- **Dr Jing Xia:** performed the HPLC on adult brain samples.
- **Derek Scarborough:** performed paraffin embedding and sectioning of adult brain samples.

Published Work:

The work presented in the chapter entitled "Placental Endocrine Lineage Analysis" (Chapter 3) has been published in *Developmental Biology*. So has the paper mentioned at the end of that chapter in the note. These papers are provided as supplements at the end of the thesis. All the other work is yet to be published.

Note: Any illustrations taken from external sources are explicitly acknowledged in the text of this thesis.

Funding:

This work was funded by:

- **The Ewen Maclean Scholarship**
- **The Waterloo Foundation**
- **The Biotechnology and Biological Sciences Research Council (BBSRC)**

Abstract

Pregnancy induces numerous changes in the physiology of the mother as she adapts to the metabolically demanding fetus developing within her womb. It is during this period of intense physiological stress that she begins to develop the maternal behaviours that will ultimately support the nurturing of the offspring after parturition and protect them from harm. In human pregnancies, women are particularly susceptible to mood disorders which may reflect an enhanced vulnerability induced by pregnancy. The placenta is the key organ of pregnancy driving the physiological and potentially the behavioural changes required for a successful pregnancy. Placental dysfunction may contribute to maternal mood disorders by mis-programming maternal behaviour, a relationship that can be explored in a tractable animal model.

Imprinted genes are expressed in the placenta and have been implicated in the regulation of key endocrine lineage of the mouse placenta. *Phlda2* is a maternally expressed imprinted gene that regulates one such lineage, the Spongiotrophoblast (SpT), which is a source of placental lactogens and a number of other hormones. Placental lactogens belong to the same hormone family as prolactin and some members of this family mediate their action via the Prolactin Receptor (Prlr). Both prolactin and the Prlr have been directly shown to be required for the establishment of maternal care behaviour in rodents. This led to the hypothesis that *Phlda2* might modulate maternal care in rodents by regulating the size of the placental endocrine compartment.

To test this hypothesis, we used three cohorts of Wildtype (WT) dams carrying fetuses that possessed either a 50% reduction in the SpT, resulting from a double dose

of *Phlda2* (Mouseline: *Phlda2* $^{+/+\ BACx1}$, TG; 2X) or a 200% increase in the SpT, resulting from a null dose of *Phlda2* (Mouseline: *Phlda2* $^{-/+}$), KO; 0X) or 100% of the WT size of the SpT, resulting from a single dose of *Phlda2* (Mouseline: *Phlda2* $^{+/+}$, WT; 1X) generated using Recipient Embryo Transfer (RET). Maternal behaviour was studied postpartum and a biomolecular characterisation was performed during pregnancy using microarray and Quantitative Polymerase Chain Reaction (qPCR) to look at changes in maternal gene expression and a histological approach used to examine maternal neurogenesis in the Sub-ventricular Zone (SVZ) of the lateral ventricles and Sub-Granular Zone (SGZ) of the hippocampus. Additionally, a study was made of male offspring carrying the *Phlda2* transgene (TG), which were previously shown to be low birth weight, their non-transgenic litter mates (NON-TG) and a fully WT cohort of mice to ask whether placental endocrine dysfunction *in utero* programmed altered behaviour later in life.

The results of this first study showed distinct changes in pup retrieval, nest building and the dam's grooming and licking behaviour with dams exposed to the smaller endocrine compartment spending less time with their pups. Specific maternal brain regions showed altered transcriptional profiles at Embryonic Day (E) 16.5 of pregnancy and there was a significant reduction in neurogenesis. While there were no differences in anxiety or locomotor activity levels in the offspring cohorts, there were significant changes in hedonic response in both the TG and NON-TG offspring.

Together, these data provide the first evidence that imprinted genes can influence both maternal care behaviour and offspring behavioural outcomes via the placental endocrine compartment. This work has wider implications since human studies have shown that elevated placental *PHLDA2* is a common features of human growth restricted pregnancies. There is a a co-occurrence of low birth weight and maternal mood disorders with mothers experiencing prenatal depression having a three-fold increased risk of a Low Birth Weight (LBW) baby. Aberrant imprinting in the placenta could account for this co-occurrence.

List of Abbreviations

ACTH Adrenocorticotropic hormone

ADH Vasopressin

ADHD Attention Deficit Hyperactivity Disorder

ALI Average Inter-Lick Interval

aNSCs Adult Neural Stem Cells

ASR Acoustic Startle Response

Ascl2 Achaete-Scute Family bHLH Transcription Factor 2

BAC Bacterial Artificial Chromosome

BD Bipolar Disorder

BL6 Black 6 Mice

BrdU Brdu

CBS Central Biotechnology Services

Cdkn1c Cyclin Dependent Kinase Inhibitor 1C

Ch-TGC Channel Trophoblast Giant Cells

CICS Cardiff Infant Contentiousness Scale

CRH Corticotropin releasing hormone

CSH1 Chorionic Somatomammotropin Hormone 1

CSH2 Chorionic Somatomammotropin Hormone 2

DAVID Database for Annotation Visualization and Integrated Discovery

DCX Doublecortin

DG Dentate Gyrus

E Embryonic Day

ELISA Enzyme-Linked Immunosorbent Assay

EPM Elevated Plus Maze

EtOH Ethanol

EZM Elevated Zero Maze

GH Growth Hormone

Grb10 Growth Factor Receptor Bound Protein 10

HPA Hypothalamic-Pituitary-Adrenal

hPL Human Placental Lactogens

HPLC High Performance Liquid Chromatography

IC1 Imprinting Centre 1

IC2 Imprinting Centre 2

ICEs Imprint Control Elements

ICM Inner Cell Mass

ICRs Imprinting Control Regions

ICs Imprinting Centres

Igf2 Insulin-like Growth Factor 2

Igf2r Insulin-like Growth Factor 2 Receptor

IUGR Intra Uterine Growth Restriction

LBW Low Birth Weight

LCA Lick Cluster Analysis

LCS Lick Cluster Size

LMA Locomotor Activity

LOI Loss of Imprinting

LSE London School of Economics

LV Lateral Ventricles

MDD Major Depressive Disorder

mPoA Medial Pre-Optic Area

Nestin Neuroectodermal Stem Cell Marker

OB Olfactory Bulb

OF Open Field

OXT Oxytocin

P Postnatal Day

PCR Polymerase Chain Reaction

PEG1 Paternally Expressed Gene 1

Peg3 Paternally Expressed Gene 3

Phlda2 Pleckstrin Homology Like Domain family A member 2

Phlda3 Pleckstrin Homology Like Domain family A member 3

PLI Placental Lactogen I

PLII Placental Lactogen II

PND Postnatal Depression

PP Puerperal Psychosis

PPI Pre-Pulse Inhibition

Prlr Prolactin Receptor

Prls Prolactin-like Proteins

Psgs Placental Specific Glycoproteins

PTSD Post Traumatic Stress Disorder

PVN Paraventricular Nucleus

PWS Prader-Willi Syndrome

qPCR Quantitative Polymerase Chain Reaction

RET Recipient Embryo Transfer

RIN RNA Integrity Number

RMS Rostral Migratory Stream

SGA Small for Gestational Age

SGZ Sub-Granular Zone

Slc22a18 Solute Carrier Family 22 Member 18

SpT Spongiotrophoblast

SRIF Science Research Infrastructure Fund

SRS Silver Russell Syndrome

sTGC Sinusoidal Trophoblast Giant Cells

SVZ Sub-ventricular Zone

SynTI Syncitiotrophoblast Layer I

SynTII Syncytiotrophoblast Layer II

TGC Trophoblast Giant Cells

VS Ventral Striatum

WT Wildtype

List of Figures

List of Tables

General Introduction 1

1.1 Pregnancy

We all begin our lives as the products of pregnancy. Events during pregnancy can influence an individual's phenotype throughout their life. There are well documented studies that implicate early life events *in utero* and the postpartum period on the life of the offspring. There is less research or at least less understanding about the short term and lasting effects that pregnancy has upon the mother's health. The two are most likely intimately linked as demonstrated by the co-occurrence of IUGR, LBW and psychiatric illness. Therefore understanding and investigating the novel origins of the adverse mental health effects during and after pregnancy on both the mother and the offspring is vital, and is the focus of this research.

The point of conception signifies the beginning of an exceptionally challenging period of time for a mother. In the human condition the developing embryo becomes known as a fetus at around 8 weeks gestation. This is the stage when mineralization occurs and bone marrow forms. At this point numerous changes have already occurred to the mother's physiology in order to accommodate for this new metabolically demanding group of cells (Brunton and Russell, 2008, Grattan, 2011, Shingo, 2003). These changes continue throughout pregnancy and encompass both physiological alterations as well as behavioural adaptations (**Figure** 1.1).

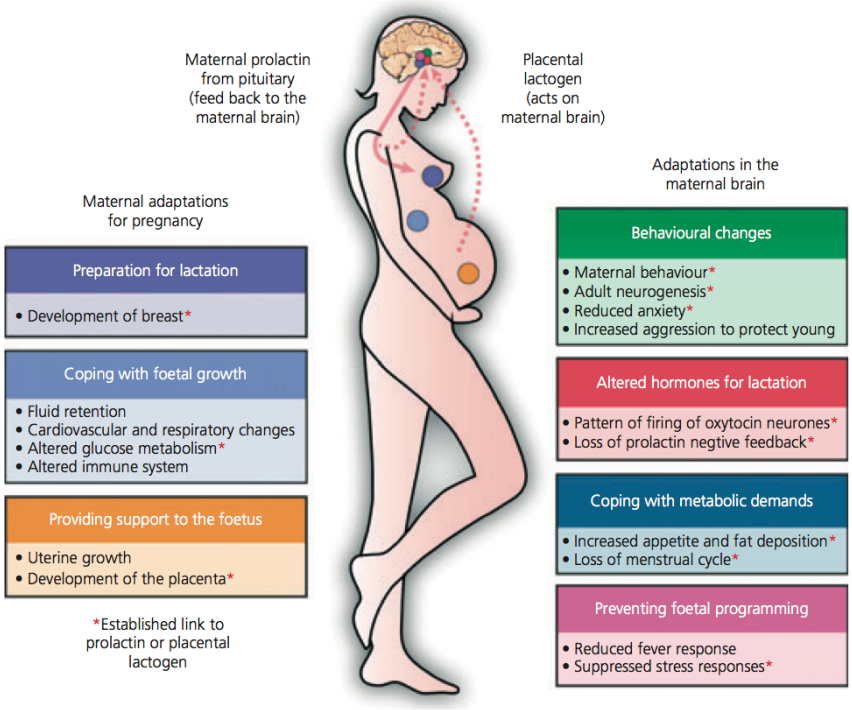


Figure 1.1: Pregnancy Adaptations. The many adaptations, both physiological and behavioural, that take place in a mother during pregnancy. Taken from Grattan (2011)

1.2 Maternal Behaviour and Maternal Mood Disorders

Maternal behaviour is broadly defined as "the pattern of a mother's behaviour that appears to enhance her offspring's survival and reproductive success" (Saltzman and Maestripieri, 2011). The natural process of transitioning from being a nulliparous female to a mother irreversibly alters the motivational and behavioural repertoire of the female, initiating maternal behaviour. The care given as a result of the initiation of this maternal behaviour significantly affects the developmental trajectory of her offspring, thus making these behavioural responses of the mother to her newborn offspring vital to understand. In humans there are differences in the levels and quality of maternal behaviour provided to the offspring. These differences are often based on factors such as: social economic status, lifestyle choices and cultural differences of the

mother and her family (Atif *et al.*, 2015). Irrespective of these potentially confounding factors there is an underlying set of nurturing behaviours that a mother must possess in order to care for her offspring effectively. The pregnant female has an increased appetite throughout pregnancy to support the developing fetus (Grattan, 2011). She will also show increased aggression and reduced anxiety postpartum (Hahn-Holbrook *et al.*, 2011) (**Figure** 1.1). These behaviours are evident throughout gestation but are most obvious after parturition, when direct mother-offspring interactions can occur. As already mentioned, pregnancy is hugely challenging for any mother due to the unrelenting physiological and psychological changes that must take place. It is therefore not surprising that during pregnancy sometimes these basic maternal behaviours can become disrupted, this combined with the enormous emotional upheaval associated with this transitioning period to motherhood means that a new mother is increasingly vulnerable to developing specific psychiatric illnesses.

Mood disorders are a far-reaching class of psychiatric illnesses. They affect between 1 in 4 and 1 in 10 people during their lifetime and are classified clinically as "a disturbance in a person's mood" (Somers *et al.*, 2006, Waraich *et al.*, 2004). Originally known as "affective" disorders the switch to the term "mood" is an effort to more accurately describe the underlying emotional instability of those suffering from these disorders (American Psychiatric Association *et al.*, 2013). There are two major categories of mood disorders that are grouped together based on the presence of either manic or hypomanic episodes. The two categories are Major Depressive Disorder (MDD) and Bipolar Disorder (BD). As with most psychiatric illnesses MDD and BD represent a sliding scale of syndromes with a variety of symptoms and severities. This mosaic of syndromes and symptoms offers a challenge to clinicians and researchers alike. In particular when trying to identify the origin and appropriate treatment for these disorders. It is therefore vital that research into this area is conducted, so to improve understanding and ultimately lead to the better treatment of these increasingly prevalent disorders (Gaynes *et al.*, 2005, Musters *et al.*, 2008).

There is a known link between emotionality instability and mental health, specifically

in relation to mood disorders. The increased plasticity across the body systems of the mother during pregnancy allows the body systems to adapt to ensure they function in the optimum ways to provide the ideal conditions for pregnancy. This increased plasticity of the body systems is thought to be one reason why new mothers are prone to developing psychiatric disorders (O'Hara and Wisner, 2014, Perani and Slattery, 2014). The increased susceptibility during this period has given rise to a new class of mood disorders, known collectively as the "Maternal Mood Disorders". These are thought to represent a general failure of the mother to undergo the appropriate behavioural changes associated with motherhood, leading to emotional instability and psychopathology (American Psychiatric Association *et al.*, 2013).

It has been shown that around 50% of women experience the "baby blues" in the immediate stages after birth (Gaynes *et al.*, 2005, Harris *et al.*, 1994). Although the "baby blues" are not considered as clinically relevant, more severe behavioural disorders, like Postnatal Depression (PND) and Puerperal Psychosis (PP), are. Both PND and PP are often seen within a large proportion of those mothers impacted by the "baby blues" (Stewart *et al.*, 2003). The effects of PND and PP can have detrimental effects to both the mother and the offspring for many years after birth (Capron *et al.*, 2015). Depression is a common psychiatric disorder that is known to have substantial morbidity (Cox *et al.*, 1993, Halbreich, 2005, Liu *et al.*, 2012, Murray, 1992). PND is thought to affect 1 in 10 new mothers with estimates of prevalence being averaged at 13% (Gaynes *et al.*, 2005, Musters *et al.*, 2008). Some estimates have been as large as 85%, although this study has since been dismissed as being indiscriminate in its diagnosis of PND (Henshaw, 2003, Pearlstein *et al.*, 2009). Symptoms include mood swings, confusion, irritability and fatigue (American Psychiatric Association *et al.*, 2013). PP, is a more severe condition that can develop shortly after the woman has given birth. PP occurs in 1 in 1000 women and is characterised by the emergence of severe hallucinations. PP due to the danger the condition poses to both the mother and child, is considered a psychiatric emergency (American Psychiatric Association *et al.*, 2013, Musters *et al.*, 2008). As mentioned

already PND and PP not only have an affect on the mother's health, but also significantly impact upon the development of mother-infant bonds (Dubber *et al.*, 2015, Stein *et al.*, 1991). It is surprising therefore that despite the evidence linking maternal mood disorders to a range of other morbidities and adverse outcomes for mother and child, there is remarkably little known about the causes of these depressive symptoms.

Historically, research has focussed on the impact prenatal stress during pregnancy, lifestyle choices or previous susceptibility of a mother to mental health issues has upon the mother and child (Bale *et al.*, 2010b, Bourke *et al.*, 2013, Hanley *et al.*, 2013). Prenatal depression and perinatal anxiety is reported to affect between 12-20% of women during pregnancy (O'Hara and Wisner, 2014). In monetary terms a study by the London School of Economics (LSE) has estimated the total economic cost of perinatal anxiety and depression at between £7-8 billion a year (Bauer *et al.*, 2016). The high prevalence of prenatal and PND and the consequences for the health of both the mother and her offspring highlight the need to better understand the origins of maternal mood disorders. Emotional or physiological changes that may occur during gestation may potentiate existing mental health disorders in vulnerable women, consistent with the observation that women with BD have a 50% risk of developing PP (Jones and Craddock, 2001). The baseline female adult population risk for PP is 1-2/1000; with the risk rising dramatically to 1:7 for women with just one previous episode of PP (Brockington, 2004, Jones and Craddock, 2001). Some studies have even suggested the prevalence to increase to 1 in 2 women, within those that have a history of BD or previous episodes of PP (Munk-Olsen *et al.*, 2012, Musters *et al.*, 2008). Further work in this area substantiates the importance of these findings. Jones and Craddock (2001) have showed that PP affects 74% of mothers with BD and a first-degree relative who had PP, compared with only 30% of BD women without any family history of PP. Taken together with the increased risk that maternal lifestyle has upon the mental health outcomes of the mother, this suggests that epigenetic events during pregnancy may underlie both prenatal depression and

PND (Janssen *et al.*, 2015, 2016).

Similarly, it is known that early life environmental differences can play a vital role on the developmental trajectory of the offspring as they grow up. In particular it has been shown that postnatal maternal separation can produce altered emotion-related behaviour responses, which in humans increases the risk of psychiatric disorders and anti-social behaviour, such as aggression (Haller *et al.*, 2014, Nishi *et al.*, 2014, Paksarian *et al.*, 2015). Similarly, early life stress has been linked to increased risk of schizophrenia and substance abuse (Agid *et al.*, 1999, Kendler *et al.*, 2000). This early life stress is often associated with neglect. Evidence from several studies suggests that the effect of early life neglect constitutes one of the major risk factors for the development and persistence of mental disorders including depression, Post Traumatic Stress Disorder (PTSD), and Attention Deficit Hyperactivity Disorder (ADHD) (Famularo *et al.*, 1992, Pelcovitz *et al.*, 1994). The mechanisms behind what causes these later life pathologies is not well understood in humans, with preclinical studies suggest that early life stress may be a factor that results in increased stress responsiveness (Drake, 2014, Nemeroff, 2002, Zeng *et al.*, 2015).

1.3 IUGR and LBW

IUGR and LBW are both associated with poor perinatal outcomes. Although there are two types of IUGR, symmetric and asymmetric the accepted definition for the pathology by the Royal College of Obstetricians and Gynaecologists (RCOG) is "a fetus that fails to reach its potential growth" (Fiander, 2015). In contrast a LBW baby, also known as a Small for Gestational Age (SGA) baby, is defined as a baby having a birth weight below a given 10th percentile for gestational age (Fiander, 2015). The two conditions are not synonymous but are both important clinical problems which carry complications for both the mother and the offspring during

pregnancy and into later life.

1.3.1 Maternal Outcomes

LBW has long been used as a measure of overall fetal and maternal health (Halbreich, 2005). Prenatal depression has often been associated with LBW (Glynn and Sandman, 2011, Liu *et al.*, 2012, Monk *et al.*, 2012). In the past studies have focussed primarily on the social development and outcome of the offspring and not upon the psychiatric health of the mothers. These studies have helped attribute future adult psychiatric illness of the offspring to pre-natal stress, drug abuse and other similar lifestyle choices (see subsection 1.3.2). Very little work has been done to look at the impact that fetal development has upon the mother's behaviour and whether there is any link between LBW and maternal mood disorders. There are reports that indicate the psychological distress that can occur in mothers of LBW babies (Singer *et al.*, 1999), including cases of PTSD (Kersting *et al.*, 2004). Another study carried out by Liu *et al.* (2012) highlighted a 3-fold increase in depressed mood in mothers with LBW children. This has previously been reported in a meta analysis study, Grote *et al.* (2010) concluded that women with depression during pregnancy are at increased risk for preterm birth and LBW, although they noted that the magnitude of the effect varies according to the depression measurement, country location and socioeconomic status. These studies have often focussed on LBW and IUGR being a direct result of the maternal depressive state during pregnancy. There is however growing evidence that these depressive symptoms may originate as a result of the changes brought about from carrying an IUGR baby.

The placenta is the key organ of pregnancy and plays a nourishing role in the development of the embryo. This means that placental defects can have severe consequences for embryonic growth and development. In humans, placental defects result in complications including pre-eclampsia and IUGR (Szymonowicz and Yu,

1987, Valero De Bernabé *et al.*, 2004). One study by Evans *et al.* (2001) of 12,059 women found there to be an increase in reported depressive symptoms in the third trimester (13.6%) compared to the second trimester (11.8%). The second and third trimesters are when placental hormones are signalling most strongly (Samaan *et al.*, 1966). While the general consensus has been that changes in the term placenta is associated with prenatal depression as a consequence of the condition (Olivier *et al.*, 2014), it is also possible that placental endocrine dysfunction drives prenatal depression (Janssen *et al.*, 2016). It follows that these placental differences may contribute to the pathology of postpartum mood disorders.

1.3.2 Offspring Outcomes

In humans the offspring of mothers with suboptimal *in utero* environments often have several bodily systems that are compromised. These include cardiovascular fitness (Barker, 1995, Lindblom *et al.*, 2015), metabolic function (Entringer, 2013) and behavioural responses (Bale *et al.*, 2010b). In humans, maternal stress (Weinstock, 2008), poor diet (Brown *et al.*, 2000) and depression (Rice *et al.*, 2007) throughout pregnancy have been linked to LBW and a subsequent range of physiological and behavioural outcomes in the offspring. Such as delayed fetal growth, structural alterations to the neural system, impaired cognitive performance, social abnormalities and an increased risk of psychiatric illnesses later in life (Sandman *et al.*, 2011). In particular, prenatal stress in LBW mothers has been related to an increased risk for major depression in adulthood (Hulshoff Pol *et al.*, 2000). LBW, IUGR and preterm birth have been linked to behavioural problems, such as hyperactivity during school (McCormick *et al.*, 1990). In one study, known as the Helsinki study, in which they followed up very low-birth-weight (VLBW) babies ($<$ 1500 g) into young adulthood (20 years of age) they highlighted the link between IUGR and risk of depression in young adulthood (Räikkönen *et al.*, 2008).

1.4 Pregnancy Hormones

The plethora of changes that are brought about through the pregnant state in humans and other mammals are predominately initiated and ultimately controlled through the endocrine system. Maternal and placental hormones act to bring about the appropriate physiological responses to cope with the developing fetus (Fiander, 2015).

Hormones in the general sense are "chemical signals that serve to communicate messages to multiple internal sites simultaneously. Hormones are released by a gland. They are then picked up by receptors at multiple other sites, thereby affecting them in a way specific to the site" (Gangestad and Grebe, 2016). There are many hormones that are prevalent throughout pregnancy and after birth. These all act in distinct ways in order to maintain the pregnant state or prepare the female for the physiological demands of pregnancy and onward challenges of motherhood. The hormones that have been most abundantly studied in relation to pregnancy are the protein hormones and the steroid hormones. There are too many to discuss them all in detail, but the major hormones are briefly outlined in the following sections and their functions in humans and rodents summarised in **Table** 1.1 and **Table** 1.2.

1.4.1 Steroid Hormones

These hormones are secreted from the gonads, adrenal cortex and placenta. They include progesterone, a member of the progestins. Progesterone is a key hormone produced by the corpus luteum up until around 10 weeks of gestation in humans, before the placenta produces enough progesterone to maintain pregnancy independently until term (Kumar and Magon, 2012). Progesterone is sometimes referred to as the hormone of pregnancy, due to its critical role in the maintenance and establishment of the pregnant state, through the support of the endometrium, which is critical

for the survival of the conceptus and suppression of ovulation (Sonigo *et al.*, 2014, Wright and Johnson, 2008). Progesterone also helps suppress the smooth muscles of the myometrium near the end of gestation, however as oestrogen levels rise this suppression is reduced encouraging parturition.

The other key group of steroid hormones that are found at high levels during pregnancy are the oestrogens. The levels of oestrogens rise continually throughout gestation, reaching their maximum at term. The principle effects of oestrogens on the pregnant state are their antagonising effects upon progesterone at term and the stimulation of the growth of the myometrium (Pi *et al.*, 2003). Like progesterone the oestrogens help maintain the pregnant state through suppression of gonadotropin hormones from the pituitary. Finally oestrogens are amongst the key hormones that help in mammary gland development (Brisken and O'Malley, 2010).

1.4.2 Protein Hormones

Protein/peptide hormones are extremely abundant during gestation and play a key role in a number of pregnancy processes. Corticotropin releasing hormone (CRH), is the main modulator of the Hypothalamic-Pituitary-Adrenal (HPA) axis by acting upon Adrenocorticotropic hormone (ACTH) and acting in its release into the circulation (Nephew and Murgatroyd, 2013). CRH is released from the Paraventricular Nucleus (PVN) of the hypothalamus stimulating the pituitary synthesis of ACTH. ACTH is released in high levels during pregnancy leaving the mother in a state of hypercortisolism (Mastorakos and Ilias, 2003). Interestingly, oestrogen increases the sensitivity of the adrenal gland to ACTH, an example how the hormones of pregnancy often work together for a given outcome, in this case hypercortisolism (Waddell and Atkinson, 1994). Vasopressin (ADH) is most commonly known for its more general role in the kidney where it is vital for helping the body regulate the retention of water (Robertson, 1983). However, it has emerged that ADH is also a critical hormone in

pregnancy, where it acts in parallel with CRH in mediating the stress response in mammals during pregnancy and lactation modulating the HPA axis (Goland *et al.*, 1991, Ma *et al.*, 2005, Mastorakos and Ilias, 2003). Oxytocin (OXT) is secreted from the posterior pituitary, the PVN of the hypothalamus and the placenta. OXT is essential for lactation, as it it stimulates milk ejections (Brunton and Russell, 2008, Russell *et al.*, 2003). It is also important in promoting parturition. It does this by being released in pulses a few minutes apart, stimulating uterine contractions. Prolactin is released from the pituitary in humans and placental lactogens from the placenta. It is most well known for its vital role in lactation and milk production (Nephew and Murgatroyd, 2013).

1.5 Modelling Maternal Behaviour

In order to further our understanding of the processes that induce and maintain maternal behaviour, processes that may be disrupted in maternal mood disorders, rodents can provide a useful tool. Both mice and rats are commonly used in scientific research for numerous reasons when modelling human disease. They provide a tractable system that allows the continual monitoring of the behavioural and biochemical changes occurring during a defined period, as well as allowing genetic and non-genetic manipulation. Their short gestation and weaning time allows longitudinal and generational studies to be carried out over a much shorter time frame than in the human equivalent. Thus it is possible to look at the offsprings development as a consequence of a compromised *in utero* and/or early life environment.

Crucially for this study, mice and rats elicit maternal behaviours that are predictable and testable, making them the ideal choice of animal model for this kind of research (Bridges *et al.*, 1990). The behaviours that a new dam must exhibit during pregnancy encompass finding and remembering food stores, water sources, nest sites and danger zones (Franks *et al.*, 2011). These locations once processed must either be avoided

Table 1.1: Pregnancy Hormones in Humans and their Functions

Hormone/s	**Tissue/s excreted from**	**Function/s**	**Reference/s**
Progesterone	Gonads/adrenal glands/placenta	Establishment and maintenance of pregnancy. Inhibits lactogenesis.	Kumar and Magon (2012), Macias and Hinck (2012), Sonigo *et al.* (2014)
Oestrogens	Gonads	Antagonising effect upon progesterone at parturition. Stimulates the growth of the myometrium. Helps maintain the pregnant state by suppressing gonadotropin hormones. Promotes mammary development.	Brisken and O'Malley (2010), Macias and Hinck (2012)
Oxytocin	Placenta/hypoth - alamus/pituitary	Stimulates contractions of uterus and cervix. Vital in lactation and milk ejection. Helps with bonding and emotionality. Promotes prolactin production.	Apter-Levi *et al.* (2014), Bachner-Melman and Ebstein (2014), Dale (1906)
Corticotropin releasing hormone	Hypothalamus	Determines length of gestation. Mediates vasopressin release.	Wadhwa *et al.* (1998)
Vasopressin	Pituitary	Increases anxiety and increases agression.	Heinrichs *et al.* (2009)
Prolactin	Pituitary/placenta	Milk production and lactation. Maternal metabolism. Suppression of the stress response. Suppresses ovulation and maintains pregnancy.	Freeman *et al.* (2000)
Placental Lactogens	Placenta	Lactation and milk production. Maternal metabolism.	HANDWERGER (1991), Walker *et al.* (1990)

Table 1.2: Pregnancy Hormones in Rodents and their Functions

Hormone/s	**Tissue/s excreted from**	**Function/s**	**Reference/s**
Progesterone	Gonads/adrenal glands/placenta	Helps establish and maintain pregnancy and inhibits lactogenesis.	Bazer (1998), López-Fontana *et al.* (2012)
Oestrogens	Gonads	Helps establish implantation and maintain pregnancy.	Gidley-Baird (1981), Pi *et al.* (2003)
Oxytocin	Placenta/hypo - thalamus/pituitary	Initiates parturition and promotes social behaviours. Induces maternal behaviour	Champagne *et al.* (2001), Champagne and Meaney (2006), Li *et al.* (1999)
Corticotropin releasing hormone	Hypothalamus/ placenta	Stimulates the production of ACTH and main modulator of HPA axis. Inhibits maternal behaviour.	Klampfl *et al.* (2013), Pedersen *et al.* (1991)
Vasopressin	Pituitary	Works with CRH to modulate release of ACTH during pregnancy.	Ma *et al.* (2005), Mastorakos and Ilias (2003)
Prolactin	Pituitary/placenta	Promotes lactation and mammary growth. Suppression of the immune and stress response. Suppresses ovulation and maintains pregnancy. Increases maternal neurogenesis.	Freeman *et al.* (2000), Hu and Cross (2010), Nephew and Murgatroyd (2013), Shingo (2003), Torner (2016)
Placental Lactogens	Placenta	Main lactogenic hormone at mid-gestation. Lactation and milk production. Involved in maternal behaviour.	Bridges (1994), Bridges and Grattan (2003), Lee and Voogt (1999), Lucas *et al.* (1998)

or exploited to ensure the survival of her offspring. Nests, even if already built, must be re-constructed in a more elaborate and functional way for the young, to provide protection and warmth (Lisk *et al.*, 1969). Critically, the new dam must engage in discrete acts of maternal care. These include retrieving, grouping, crouching over and licking/grooming each individual pup (Franks *et al.*, 2011). These mother-pup interactions are thought to be vital in the development of the offspring. The pressure of having offspring will force the female body into a more time efficient routine for eating and sleeping. Maximising the care and fitness for both herself and her young. Thus maternal behavioural testing is often used in animal models to determine whether the dam has undergone the appropriate transition into motherhood. These assays check that the dam has developed behaviours that allow her to effectively care for her offspring. The lack of specific maternal behaviours point towards certain maternal behavioural deficits that may suggest that either the dam is compromised or that the offspring are.

1.5.1 Maternal Care and Offspring Outcome

Maternal care experience by offspring during postnatal development predicts their long-term neurobiology and behavioural outcomes (Franks *et al.*, 2015). This study by Franks *et al.* (2015) indicated that lower postnatal care can be associated with less frequent maternal behaviour during the pre-weaning period. Additionally, naturally occurring variations in maternal care have been shown to alter the expression of genes that regulate behavioural and endocrine responses to stress, as well as hippocampal synaptic development (Meaney, 2001). Meaney (2001) revealed that parental care can act as a key mediator for the effects of environmental adversity on neural and behavioural development. It is widely understood that postnatal maternal separation can produce lasting abnormalities in emotion-related behaviour and neuroendocrine responses to stress in rodents (Lehmann *et al.*, 1999, Millstein and Holmes, 2007). There is increasing evidence that maternal care can play a role in the developing

epigenome (Monk *et al.*, 2012). Despite this the mechanisms behind what causes these behavioural outcomes in the offspring are not well understood but it is most likely a combination of factors stemming from both the *in utero* and early life environments.

1.6 Modelling IUGR and LBW

LBW and IUGR have both been successfully been modelled in mice. These models provide invaluable research tools for furthering the understanding of what causes these conditions (Tunster *et al.*, 2010). They also offer the unique opportunity to study the co-occurrence of both LBW and maternal mood disorders (Janssen *et al.*, 2016).

The effects that both prenatal adversity and early life adversity has upon the postnatal outcomes of the offspring has been well documented in both humans and mice. There has been less research into whether there is a link between the *in utero* environment and the subsequent maternal care in the early stages postpartum and what effect this may have upon offspring behavioural development. This research presents a novel mouse model that was used to assess both these facets in the development of the offspring postnatally.

1.6.1 The *in utero* Environment and Offspring Outcomes

In rodents the same patterns, that link the prenatal stressors found in human *in utero* environments, also cause behavioural and neural abnormalities in these model systems (Hausknecht *et al.*, 2013). The *in utero* environment can often be a direct

consequence of maternal lifestyle, but can also be due to the effects of dosage of imprinted genes in the placenta. Changes in the expression of *Insulin-like Growth Factor 2 (Igf2)* has been shown to have programming effects on later life adult behaviour, where it has been shown to induce an anxiety phenotype in adult mice (Mikaelsson *et al.*, 2013). Similarly the changes in the expression of the paternally expressed gene *Peg3* has been associated with the co-adaptive behaviour in both the dam and offspring. Including the suckling behaviour of the pups and nurturing provision given by the dams during the pre-weaning postnatal period (Curley *et al.*, 2004).

The role the placenta plays in initiating the physiological changes that are necessary for a successful pregnancy, as well as the placental origin of IUGR and LBW means that gaining a better understanding of the placenta and its role during pregnancy is essential.

1.7 The Placenta

The placenta is the first organ that begins to form early on in pregnancy, and is the most important. The word "placenta", which means "flat cake" in latin aptly describes it underwhelming appearance, but fails to exemplify its extremely important functional role during pregnancy (Cross, 2005, Watson and Cross, 2005). Placental development begins at the point of implantation of the blastocyst into the maternal uterine wall. This occurs at around day 6 or 7 post conception in humans, and on E 3.5 in mice, just before implantation (Rossant and Cross, 2001). The placenta plays an active role as a selective delivery interface for nutrients between mother and offspring from its early days, but becomes most active as an endocrine organ between weeks 12/13 and term in humans and between E10.5 and birth in mice (Watson and Cross, 2005).

The placenta features both a maternal and a fetal interface that allow it to perform a variety of functions. In humans the maternally derived placenta is called the decidua basalis and the fetally derived placenta is the chorion frondosum (Georgiades *et al.*, 2002). It is primarily comprised of extra-embryonic trophoblast cells and permeated by blood vessels initiating from the umbilical cord and the maternal uterus. The highly vascularised nature of the placenta is an essential component that allows it to perform its vital role in the continued nourishment of the fetus. The placenta's role in the endocrine system during pregnancy is critical. It helps in the regulation of hormones that bring about many of the physiological changes in the expectant mother. The trophoblast cells that are critical for the development of the multitude of roles for the placenta means that defects during their differentiation are associated with a vast array of pregnancy complications, including: infertility, miscarriage, pre-eclampsia and IUGR (Jauniaux *et al.*, 2010).

The functional roles of the placenta have consequently been incorporated to define the two broad functional categories:

1. **Transport and Barrier:** This functional spectrum begins from implantation when the placenta prevents immune rejection of the semi-allogeneic fetus. The exact mechanisms behind the protection that the placenta provides against the maternal immune system are not fully understood (Georgiades *et al.*, 2002). Recent advancements in immunology however have begun to uncover how the intricate process occurs. The increasing evidence suggests that decidual macrophages plus regulatory T cells are key regulators of fetal tolerance by the mother (Svensson-Arvelund *et al.*, 2015). This work by Svensson-Arvelund *et al.* (2015) has shown that these regulatory T cells are likely induced by the human fetal placenta itself, mainly through the trophoblast cells. Subsequently, the placenta is able to transport sufficient nutrients to the developing embryo and remove toxic waste products whilst maintaining the protection of the fetus from the maternal immune response.

2. **Invasive and Endocrine:** Foundational to this is the vascularised nature of the placenta. The fetally derived cells augment the modification of the maternal arterial vasculature increasing the volume of maternal blood available for undisturbed transfer (Georgiades *et al.*, 2002, Watson and Cross, 2005). This feto-maternal interface is a dynamic system that changes throughout gestation in order to increase efficiency of the placenta (Moll, 1985). This "vascular invasion" not only allows for transport and removal of the waste, but also renders the placenta an important endocrine organ. The invasion of the uterine wall results in the anchoring of the placenta and enables it to regulate the release of hormones and cytokines into the maternal blood (Hu and Cross, 2010). These hormones act to alter the mother's metabolism and re-programme the mother's physiology in order to favour embryonic growth through heightened blood flow to the placenta (Tunster *et al.*, 2013, Watson and Cross, 2005).

1.7.1 The Human Vs The Mouse Mature Placenta

To study placental function in humans presents a number of challenges. Genetic heterogeneity requires studies on large populations, examination of placental structure and function during pregnancy although not impossible is limited in its application and, for ethical reasons, intervention studies are a considerable undertaking. Nonetheless, some progress has been made using randomised control trials such as those done by Heazell *et al.* (2013) and Rai *et al.* (1997). These difficulties and the need to understand the relationship between the structure and subsequent function of the placenta means the use of a model system is still vital. Mice offer a genetically tractable model system through which specific genes can be assessed via genetic modifications and breeding programmes (Malassiné *et al.*, 2003). Subsequently it is critical to understand the differences between the two species placenta in order to be able to relate the findings these mouse models to what we know in humans.

The gross anatomy of mammalian placenta varies greatly between species (Leiser and Dantzer, 1994, Perry, 1981). The same is true for the gross architecture of the human and mouse placenta. However the core functional similarities between the cell types, overall structure and the molecular mechanisms underlying placental development are in fact quite similar between the two species (Cross *et al.*, 2003). The key tissues and their analogous cell types between the species are summarised in **Table** 1.3.

In mice, placental development begins in the blastocyst at E3.5 when the trophectoderm layer is set aside from the inner cell mass, slightly earlier than the equivalent in humans. The overall shape of both human and mouse placenta are discoid. They are both able to be split into three distinct structural layers (Georgiades *et al.*, 2002). These layers are known by different names between the species and are based on their underlying structural function. The three categories are: the labyrinth in mice and fetal placenta in humans, second is the junctional zone and the basal plate respectively, thirdly is the murine decidua basalis or the placental bed in humans, all of which are interspersed with Trophoblast Giant Cells (TGC).

Implantation occurs on E4.5 in mice, when the mural trophectoderm cells form. This simple trophectoderm surrounding the blastocyst ultimately goes on to differentiate into a variety of different trophoblast cell subtypes, with specific functions (Georgiades *et al.*, 2002, Watson and Cross, 2005). The trophectoderm cells that are not in direct contact with the Inner Cell Mass (ICM), become TGC. The TGC are analogous to the extravillous cytotrophoblast cells in the human placenta. In both species these cells stop dividing, but still continue to replicate DNA in a process known as "endoreduplication", becoming polyploid.

In contrast, two diploid cell types emerge from the polar trophectoderm and are in direct contact with the ICM. These are the extraembryonic ectoderm and the ectoplacental cone. The extramembryonic ectoderm will ultimately become, the chorion layer and, finally, the labyrinth in mice. The ectoplacental cone contains the *trophoblast specific protein alpha (Tpbpa)*-positive progenitors that give rise to

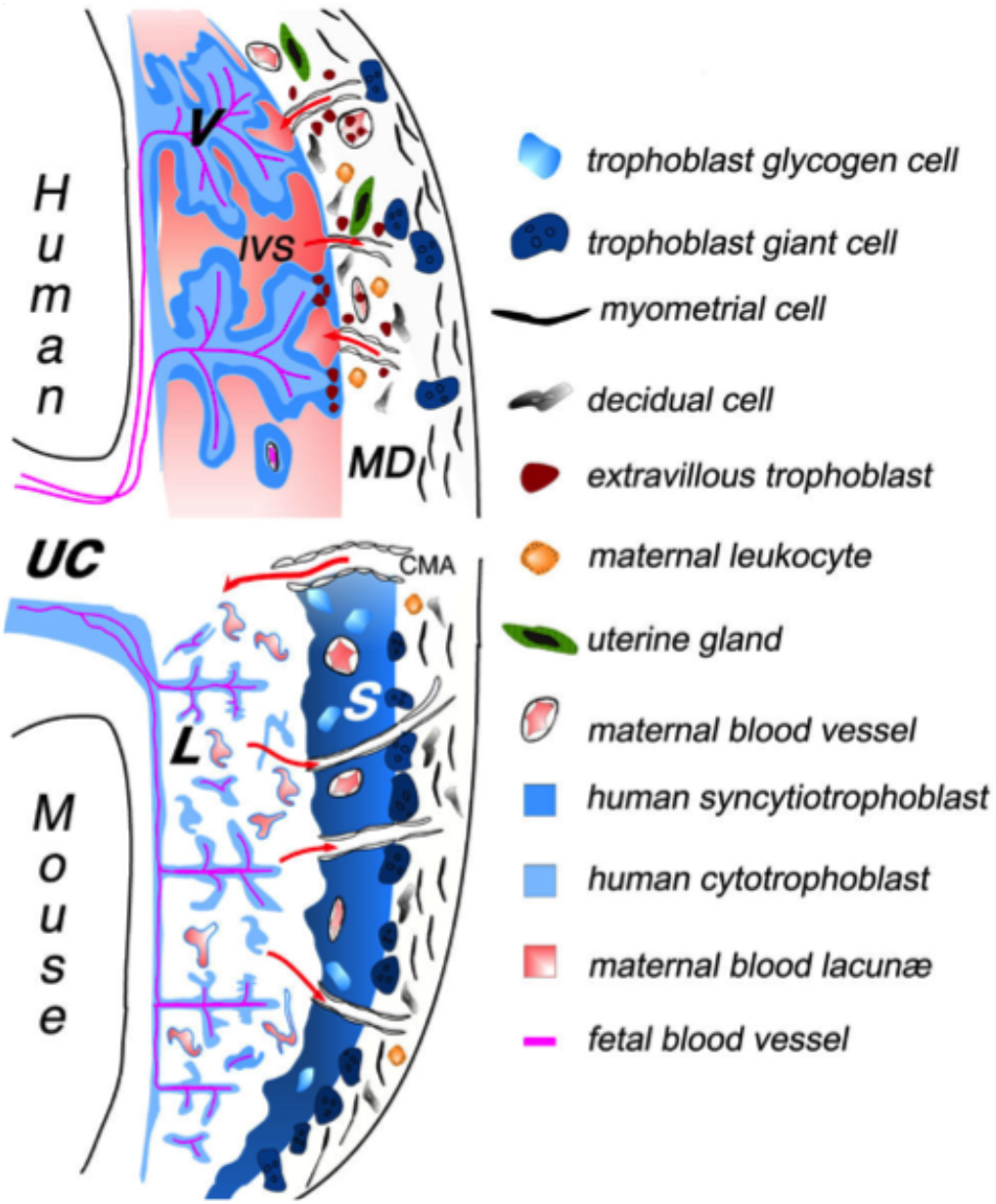


Figure 1.2: The Human Placenta Vs Mouse Mature Placenta. A simplified schematic demonstrating the differences and similarities of the human and mouse placenta. The labyrinth (L) region of the mouse placenta is the equivalent of the fetal placenta or inter villous space (IVS) found in humans. Some of the fundamental differences between the structure of the mouse and human placenta is found in the fetal vasculature. Humans have a villous (V) structure with a series of branching and sub-branching. The same region in mice is interconnected and more similar to a maze or labyrinth. The consequence of this difference is that the blood sinuses found in the mouse placenta are considerably smaller than those in the human placenta. Above the labyrinth/fetal placenta region there is a distinct group of cytotrophoblast cells. These cells are referred to as the spongiotrophoblast (S) in mice and the basal plate or maternal decidua (MD) in humans. A final layer of giant cells covers the cytotrophoblast cells is similar in both humans and mice. Adapted from Kanellopoulos-Langevin *et al.* (2003)

the SpT, the glycogen cells and four of the six TGC subtypes of the mature mouse placenta (Rai and Cross, 2014, Simmons *et al.*, 2008). It is the SpT that goes onto functionally support the developing labyrinth.

The SpT itself is a compact layer of cells sandwiched between the labyrinth and the outer giant cell layer. It corresponds to the column cytotrophoblast of the human placenta. This layer is known collectively as the junctional zone in mice and the basal plate in humans (Rossant and Cross, 2001, Watson and Cross, 2005). This region is known to be a key endocrine region in both species. It secretes hormones associated with pregnancy, including Prolactin-like Proteins (Prls) in mice and the Chorionic Somatomammotropin Hormone 1 (CSH1) and Chorionic Somatomammotropin Hormone 2 (CSH2) in humans, known collectively as Human Placental Lactogens (hPL). As gestation continues the SpT begins to contain glycogen trophoblast cells that have differentiated within this layer. It is the glycogen cells that diffusely invade the uterine wall. There has been some speculation that they could influence the secretory behaviour of the SpT due to there close association with them and because they express *calcyclin*, an inducer of Prls secretion by trophoblast (Farnsworth and Talamantes, 1998)

The vascular portion of the placenta is derived from extra-embryonic mesoderm at E8.0 in mice. At this stage the allantois forms. This then extends from the posterior end of the embryo and joins the chorion (the umbilical chord), in a process known as chorioallantoic attachment. The chorion is the outer fetal membrane that together with the amnion form the amniotic sac. Once the chorioallantoic attachment is formed, the chorion begins to fold to form the villi (Rossant and Cross, 2001, Watson and Cross, 2005). These villi help create space into which the fetal blood vessels grow from the allantois. The chorionic trophoblast cells start their differentiation into three labyrinth cell types. The first and second are the multi-nucleated syncytiotrophoblast cell layers I and II, formed by the fusion of trophoblast cells (Georgiades *et al.*, 2002, Rossant and Cross, 2001). Mice display extensive branching of the villi in the labyrinth. In humans this is comparable to the chorionic villi, both however become

larger and more extensive right up until birth (Adamson *et al.*, 2002). Unlike mice however, the human chorionic trophoblast form a single syncytiotrophoblast layer making it monochorial, compared to the trichorial nature of the mouse chorionic trophoblast (Georgiades *et al.*, 2002). In both however the maternal and fetal blood flows past each other in a countercurrent way through the labyrinth and fetal placenta in order to maximise the functional exchange of nutrients and waste between the mother and offspring.

So overall the similarities between the mouse and mature human placenta are numerous. The main differences are found within the fetal vasculature. The human placenta is villous, the chorionic villi maintain a tree-like pattern with innumerable branches and sub-branches, all having villous like extremities. In contrast this region in mice is interconnected and much more like a maze, this difference contributes to a considerably larger intervillous space in humans, or smaller blood sinuses/lacunae in mice. These differences are based on general appearance rather than function, as the functionality and origins of the cell types are the same (Carter, 2007). Therefore, the mouse offers a model that has a short gestation time, large litters and immature offspring (altricial young). Mice are also phylogenetically similar to humans/primates meaning they share many similarly conserved features of pregnancy and development. The emerging importance of imprinted genes in placental development and structure cement the mouse as an ideal model organism to use when studying placental development due to its genetic tractability and readily available embryonic stem cells, that are crucial for gene targeting and transgenic model development (Carter, 2007, Watson and Cross, 2005).

Table 1.3: Human vs Mouse Placenta

Mouse	Human
Labyrinth	Fetal Placenta
Maternal Blood Space/Lacunae	Intervillous
Junctional Zone -Spongiotrophoblast and Glycogen Cells	Basal Plate - Cytotrophoblast and Glycogen Cells
Giant Cells	Giant Cells (invasive extravillous trophoblast)
Decidua	Placental Bed

1.8 Prolactin and the Placental Hormones

In mice the various endocrine cell types and their respective functions have been recently reviewed by John (2013) and are summarised in **Table** 1.4 and **Figure** 1.3. The SpT, TGC and the glycogen cell lineage all express *Prls* as well as other hormones (Simmons *et al.*, 2008). This complex group of hormones exert both local and systemic endocrine effects on the mother including, amongst others, the suppression of the maternal immune system and preparing the mother's body for pregnancy (Bridges, 1994, Hu and Cross, 2010). The known functions are summarised in **Table** 1.1, **Table** 1.2 and in **Figure** 1.1.

Prolactin and *Growth Hormone (GH)* genes arose from a common ancestral gene (Forsyth and Wallis, 2002). They have since expanded into clusters via a species dependent mechanism, with humans and primates focussing on the *GH* locus. In rodents this expansion occurred for the *prolactin* gene locus, which has become highly evolved. It is now known to contain 22 genes (**Table** 1.5) in mice. Most of the *Prls*, are expressed in the placenta but some are expressed in the decidua (Hu and Cross, 2010, Simmons *et al.*, 2008, Wiemers *et al.*, 2003). In humans the prolactin that is expressed in the decidua is indistinguishable from pituitary prolactin. The large number of Prls in mice means that mouse prolactin is distinguishable from the Prls (Freeman *et al.*, 2000). Prolactin has important roles in the metabolic adaptations of

Table 1.4: Mouse Placental Cell Types

Trophoblast Cell Type	Location	Propoese Function
Sinusoidal TGC (sTGC)	Labyrinth, exposed directly to maternal blood	Nutrient, gas and waste exchange between mother and fetus
Syncitiotrophoblast Layer I (SynTI)	Labyrinth, between sTGC and SynTII	Nutrient, gas and waste exchange between mother and fetus
Syncytiotrophoblast Layer II (SynTII)	Labyrinth, closest to fetal endothelial cells	Nutrient, gas and waste exchange between mother and fetus
Parietal trophoblast giant cells	Bordering maternal decidua layer	Hormone production (together with the other TGC)
Canal-associated TGC	Decidua and junctional zone, surrounding maternal blood canals	Vasodilation, hormone production
Channel TGCs Ch-TGC	Line the small channels of the labyrinth	Collect deoxygenated blood
Spiral artery-associated TGC	Decidua	Invasion, spiral artery remodelling, vasodilation, production of angiogenic and anti-coagulative factors
Spongiotrophoblast cells	Junctional zone	Endocrine signalling to sustain pregnancy and induce maternal physiological adaptations, together with TGC.
Glycogen cells	Junctional zone, maternal decidua (by migration)	Function unknown âĂŞ enhancing maternal blood flow, sustaining late embryonic growth

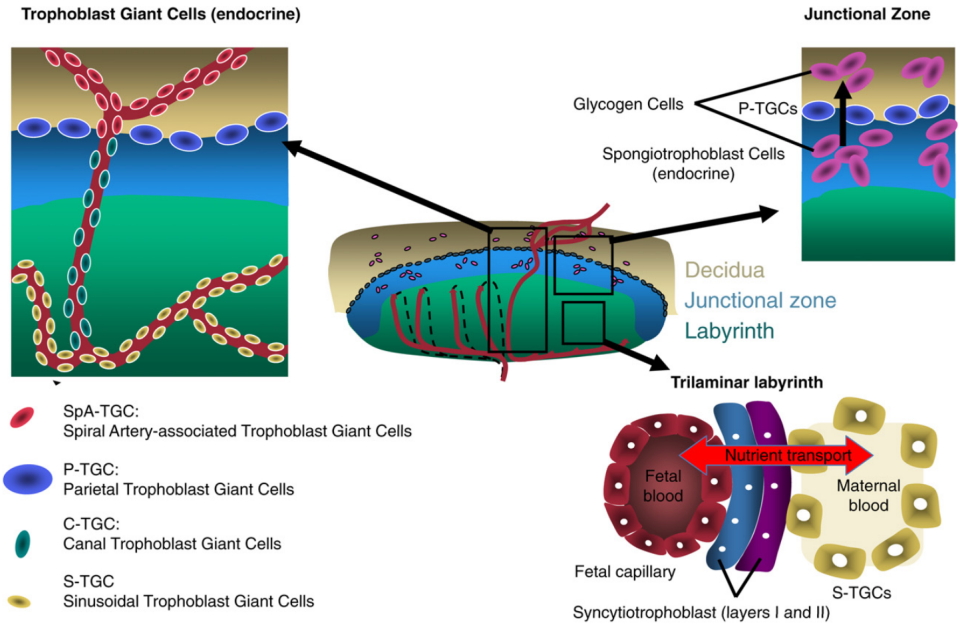


Figure 1.3: Placental Cell Types. A diagram showing the names and locations of the different cell types present in the murine placenta. Taken from John and Hemberger (2012)

the mother, contributing to the induction of leptin resistance in increased food intake during pregnancy (Grattan *et al.*, 2007). Prolactin is involved in the suppression of the stress response (Torner, 2016, Torner and Neumann, 2002), as well as showing inhibitory effect on the HPA axis (Torner *et al.*, 2002), by inducing changes in activity of OXT neurons (Kokay *et al.*, 2006), and in suppressing ovulation (Freeman *et al.*, 2000). Prolactin is elevated throughout pregnancy, parturition and lactation. The expression levels however do show distinct patterns, peaking initially in rodents before dropping off during mid-gestation and then peaking once more before parturition. This is in contrast to the Prls, which show a steady increase throughout pregnancy (Soares, 2004).

A subset of the Prls act on the Prlr much like prolactin (Bu *et al.*, 2016, Linzer and Fisher, 1999, Tunster *et al.*, 2016). It has also become evident that Prls act upon a wide range of other tissues to induce changes required for a successful pregnancy (Grattan, 2011, Grattan and Kokay, 2008). The original *Prls* discovered in rodents were named *Placental Lactogen I (PLI)* and *Placental Lactogen II (PLII)*. Since this

PLI has been shown to contain three genes that encode distinct *Prls* in mice. The three genes for *PLI* are the *Prl3d*'s, whilst *PLII* encodes one *Prl*, *Prl3b* (Table 1.5). These three Prls are most well understood with regards to their lutenizing effects on dams whilst the remaining Prls functions are not so well understood and many of their receptors are still not known. In humans these *Prls* are known as *CSH1* (with three isoforms: α, β and γ) and *CSH2*. These are homologous to the *Prl3d* and *Prl3b* genes in mice (Power, 2012).

The Prl3d's (PLI) and Prl3b (PLII) have a wide spectrum of activities and act upon numerous target tissues, independently of the mammary gland. These hormones act on the ovary of the mother, where they act alongside progesterone and oestrogen to maintain the corpus luteum of pregnancy. Specifically certain Prls actually stimulate luteal progesterone production to help maintain pregnancy. In early pregnancy pituitary prolactin is depended upon for progesterone production and luteal maintenance (Forsyth and Wallis, 2002). Once the Prl3d levels reach sufficient amounts after implantation, pituitary prolactin is functionally replaced by the Prl3d's (Simmons *et al.*, 2008, Talamantes and Ogren, 1988). At around mid gestation the synthesis of Prl3d's decrease, making way for an increase in *Prl3b* expression (Rosenfeld, 2015, Talamantes and Ogren, 1988).

1.8.1 Hormones and Maternal Behaviour

There is considerable evidence in rodents that hormonal priming of maternal behaviour occurs not only throughout pregnancy but as it ends. Many postnatal behaviours have been hypothesised to be from the sudden removal of the placenta and the associated hormones that are released from it throughout pregnancy to prepare the mother and protect the fetus (Grattan, 2011, Shingo, 2003, Woodside, 2016). These fluctuations in pregnancy related hormones may result in dysregulated brain responses that cause dramatic changes in maternal behaviour after birth, and

Table 1.5: Mouse *Prl*/PL Family

PRL Family Gene Name	**Official Symbol (Human Analogue)**
Prolactin	*Prl* (*PRL*)
Prolactin family 2, subfamily a, member 1	*Prl2a1*
Prolactin family 2, subfamily b, member 1	*Prl2b1*
Prolactin family 2, subfamily c, member 2	*Prl2c2*
Prolactin family 2, subfamily c, member 3	*Prl2c3*
Prolactin family 2, subfamily c, member 4	*Prl2c4*
Prolactin family 2, subfamily c, member 5	*Prl2c5*
Prolactin family 3, subfamily a, member 1	*Prl3a1*
Prolactin family 3, subfamily b, member 1	*Prl3b1* (*CSH2*)
Prolactin family 3, subfamily c, member 1	*Prl3c1*
Prolactin family 3, subfamily d, member 1	*Prl3d1* (*CSH1*α)
Prolactin family 3, subfamily d, member 2	*Prl3d2* (*CSH1*β)
Prolactin family 3, subfamily d, member 3	*Prl3d3* (*CSH1*γ)
Prolactin family 4, subfamily a, member 1	*Prl4a1*
Prolactin family 5, subfamily a, member 1	*Prl5a1*
Prolactin family 6, subfamily a, member 1	*Prl6a1*
Prolactin family 7, subfamily a, member 1	*Prl7a1*
Prolactin family 7, subfamily a, member 2	*Prl7a2*
Prolactin family 7, subfamily b, member 1	*Prl7b1*
Prolactin family 7, subfamily c, member 1	*Prl7c1*
Prolactin family 7, subfamily d, member 1	*Prl7d1*
Prolactin family 8, subfamily a, member 1	*Prl8a1*
Prolactin family 8, subfamily a, member 2	*Prl8a2*
Prolactin family 8, subfamily a, member 6	*Prl8a6*
Prolactin family 8, subfamily a, member 8	*Prl8a8*
Prolactin family 8, subfamily a, member 9	*Prl8a9*

any disruption to this neuro chemical cocktail may predispose new mothers to altered behaviour that may ultimately lead to mood disorders (Tan and Tan, 2013) (**Figure** 1.4).

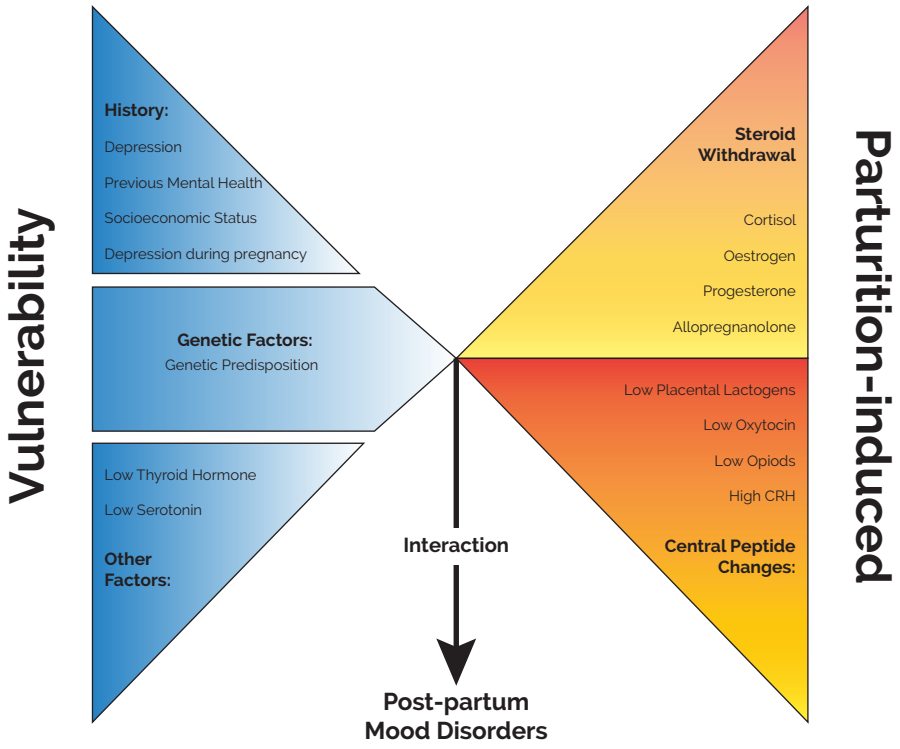


Figure 1.4: Hormonal Interactions. Factors that may predispose mothers to postpartum mood disorders. These hormonal interactions could account for pre-natal depression, which show a co-occurrence with post-natal mood disorders. Thus it follows that any disturbance in this early neuro chemical cocktail may result in altered prenatal and postpartum behavioural outcomes, thus linking this array of conditions. Adapted from Brunton and Russell (2008).

At the point of parturition, progesterone withdrawal combined with increasing oestradiol levels activates Medial Pre-Optic Area (mPoA) neurons, as does pup-seeking behaviour or postpartum exposure to pups (Champagne *et al.*, 2001, Numan *et al.*, 2005). The mPoA has been linked to improved parenting behaviour in mice (Wu *et al.*, 2014). Previously Modney *et al.* (1990) suggested that neuronal changes are caused by stimulation provided by pups alone. Numan (2006) demonstrated that this so called sensitisation was possible, renewing the debate that asks whether it is possible for sensory stimulation from newborn pups alone is enough to influence the learning and memory of a dam or non-pregnant female. Further evidence has

since indicated an increase in dendritic spine concentrations (improving synaptic signalling) in lactating females, whose circulating hormone profile is vastly different from the late-pregnant one, suggesting an influence of the pups. This work has subsequently been supported through the experimental work in rodents that showed how central prolactin infusion alone could stimulate maternal behaviour in steroid primed female rats (Bridges *et al.*, 1990). Similarly, Svare and Gandelman (1976) reported that suckling stimulation, and olfactory cues from pups, maintain maternal aggressive behaviour (Garland and Svare, 1988, Svare and Gandelman, 1976). Taken together this may indicate that there is a combined role of hormonal priming and the reinforcement of neuronal restructuring and plasticity brought about through the combined affect of hormonal release and exposure to pup provided stimuli.

It therefore follows that any disruption to either of these key points in pregnancy and early life will have an impact upon the maternal postnatal phenotype. Numan (2006) demonstrated that changes in the brain of pregnant rats allows the establishment of maternal behaviour; behaviour that will disappear unless reinforced through maternal experience postpartum. In sheep olfactory memory is essential for bonding (Kendrick, 2000). Research using non-human primates that focussed on neural and endocrine regulation have all produced converging results (Keverne and Kendrick, 1994, Saltzman and Maestripieri, 2011). This ensures confidence when applying discoveries about maternal behaviour onto the human condition, and mammals in general (Numan, 1994, 2006, Pryce, 1992). This is because although outwardly different the behavioural adaptations that occur in human pregnancies are fundamentally very similar in non human species and are likely mediated by similar systems, such as the maternal hormones originating from the both the placenta and the brain (Kristal, 2009).

1.8.2 Prolactin and Neurogenesis

Prolactin and the Prls not only help indicate the reproductive state, they have been shown to indirectly coordinate a range of neuronal and neuroendocrine adaptations to pregnancy including neurogenesis (Bridges and Grattan, 2003, Larsen and Grattan, 2010). Interestingly, as the levels of oestrogens get higher during gestation, it facilitates the entry of prolactin into the brain of the mothers by increasing the production of the Prlr in the choroid plexus (Pi *et al.*, 2003), suggesting the importance of prolactin and the Prls signalling in the brain. An idea that is substantiated by the indirect evidence that prolactin is essential for normal maternal behaviour (Bridges, 1994, Larsen and Grattan, 2010), aiding in the transition to motherhood. Shingo (2003) were first to show the exciting work that indicates that prolactin stimulates neurogenesis in adult female mice. New neurons stimulated initially by the act of mating, continue to be produced in the forebrain during pregnancy and lactation and migrate to the olfactory bulb where they likely participate in processing olfactory cues received by the new dam as she adapts to the challenges of raising young (Alvarez-Buylla and Garcia-Verdugo, 2002, Alvarez-Buylla and Lim, 2004, Shapiro *et al.*, 2009). Brunton and Russell (2008) in their review highlighted the different dimensions of gestation that are known to contribute to maternal behaviour. Research by Lucas *et al.* (1998) further implicated a role of prolactin, their paper showed that heterozygous *Prlr* KO mice have severe maternal-behaviour deficits, demonstrating the essential role of prolactin and/or Prls in initiating maternal behaviour.

As the placenta is a source of hormones, in particular the Prls (John, 2013, Tunster *et al.*, 2015), it is hypothesised that the placenta may be involved in the initiation of maternal neurogenesis. The hormones generated by the placenta during pregnancy have previously been described in section 1.8, and are manufactured by TGC and SpT lineages in mice. Prolactin is a closely related hormone that has been implemented in the generation of specific maternal behaviours. The fact that Prls far outweigh prolactin levels at midgestation (Lee and Voogt, 1999, Soares, 2004), and their action

on the Prlr makes them key candidates for playing a key role in the development of maternal behaviour. Prolactin is periodically released by the pituitary in response to pregnancy (Freeman *et al.*, 2000, Soares, 2004). It is known for its involvement in inducing multiple maternal behaviours and lactation, its involvement in stimulating adult neurogenesis in the SVZ of the forebrain lateral ventricles and SGZ of the Dentate Gyrus (DG) within the hippocampus is relatively new (Shingo, 2003). The hippocampus has been shown in rodent models to be vital for memory and learning (O'Keefe and Nadel, 1978). The SVZ being a presiding contributor to the olfactory system of the brain and thus olfactory reception. This therefore shows the need for a functional SVZ in normal postnatal maternal behaviour including grooming and cleaning in mice (Larsen and Grattan, 2010, Shingo, 2003).

Studies have focussed primarily on the impact of prolactin and have been promising. The results, caused by changing levels of these hormones, caused reduced pup retrieval scores. When loss of function of the *Prlr* models were used for both homo- and hetero- zygous nulliparous female rats, they went on to develop a defect in the olfactory dependent foster pup induced maternal behaviour (Lucas *et al.*, 1998). A model where the experimentally reduced prolactin levels had the effect of increasing postnatal anxiety and decreased pup retrieval (Lucas *et al.*, 1998). This is consistant with Shingo (2003)'s findings that a heterozygous loss in the *Prlr* gene in pregnant mothers causes a 50% reduction in forebrain SVZ neurogenesis on GD7. The way that prolactin appears to work is through increasing hippocampal precursors/interneurons, seen through neurosphere assays (Walker *et al.*, 2012, 2013).

There are no reports that look at adult neurogenesis status in the SVZ and SGZ of the maternal brain during late gestation when the placenta is signalling most intensely. The female rodents blood stream during pregnancy contains many times more Prls compared to pituitary prolactin in the later stages of gestation (Newbern and Freemark, 2011, Soares, 2004, Soares *et al.*, 2007). This may imply that there is a shared contribution between Prls and prolactin in maternal neurogenesis, leading to the hypothesis that altering levels of either of these hormones will result in changes in

maternal behaviour. The transient decrease in neurogenesis seen in the hippocampus on E11.5 and E12.5, indicated through BrdU/DCX positive cells by Rolls *et al.* (2007) coincide with a decrease in signalling before a further spike in Prls that have previously been widely ignored.

Maternal behavioural adaptations are sparsely studied in detail in humans outside of psychology (Saltzman and Maestripieri, 2011). Meaning the processes surrounding the induction of these behaviours are not well understood, and are subject to ongoing debate and research. In rodents it is known that certain aspects of maternal behaviour once established do persist, whilst others can revert back to the pre-pregnancy state. Those that do persist, do so independently from hormonal regulation and are thus considered permanent. This suggests structural alterations in the neurons in the maternal brain (Bridges *et al.*, 1990). The indirect support from rodent studies that links lactogenic hormones, in particular prolactin and the Prls, to maternal behavioural alterations suggests that these hormones are critical signals to the maternal brain(Grattan and Kokay, 2008). Taken together it is likely that the initial neurogenesis and early behavioural phenotype may be dictated by hormones but maintained through mother-pup interaction.

1.9 IMPRINTING

Imprinted genes, which are expressed from one parental chromosome as a consequence of epigenetic events initiated in the germline (Surani *et al.*, 1993) have been proposed to play a role in regulating placental hormones (Haig, 1993). This monoallelic parent of origin dependent expression is what is known as "genomic imprinting" (Surani *et al.*, 1984). Genomically imprinted genes are genes that are subject to complex epigenetic processes and have been linked to key roles in the regulation of fetal growth and placental development (Crossey *et al.*, 2002, Fowden *et al.*, 2006, Georgiades *et al.*, 2002, Tunster *et al.*, 2012). Imprinted genes only make up approximately 0.4% of

the mammalian genome, but have a disproportionately important influence on early mammalian development (Tycko and Morison, 2002). In diploid organisms there are two copies of each autosomal chromosome. These chromosomes are homologous and are a result of the inheritance of a complete haploid set of chromosomes from each parent at the point of conception. Autosomal genes are represented by two alleles that are usually capable of being transcribed (Watanabe and Barlow, 1996). This biallelic expression is hypothesised to be vital in protecting the genome from harmful genetic mutations such as random insertions and deletions (Otto and Goldstein, 1992). In spite of the proposed advantages of biallelic expression at least 100 protein coding mammalian genes are imprinted genes that are monoallelically expressed (Cross, 2005, Isles and Holland, 2005).

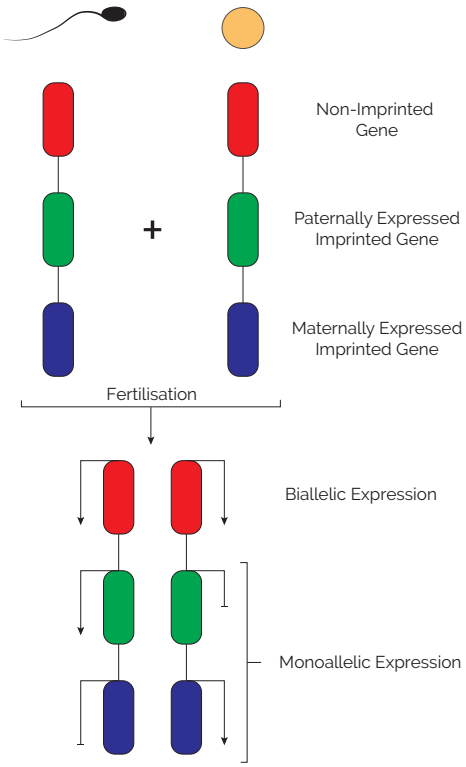


Figure 1.5: Biallelic and Monoallelic Expression of Genes. A basic schematic showing the difference between biallelic expression of a gene and monoallelic expression for a paternally expressed gene (green) and maternally expressed gene (blue).

The term genomic imprinting was initially used in the context of experiments in mammals examining parthenogenetic and androgenetic development in mice

(Mann and Lovell-Badge, 1984, Surani *et al.*, 1984). Parthenogenesis is an asexual process where an embryo develops without fertilisation by a male. It is common in invertebrates but extremely rare in vertebrates occurring exclusively in fish, reptiles and birds (Chapman *et al.*, 2007, Rougier and Werb, 2001). Parthenogenetic individuals usually contain duplicate copies of maternal DNA. Micromanipulation of newly fertilised mouse embryos was used to generate individuals with either two maternal genomes or two paternal genomes (Surani *et al.*, 1984). Parthenogenetic embryos were shown not to be viable, dying by E9.5. Androgenetic conceptuses (two copies of paternal DNA) were found to die earlier with developmental delay leading to the hypothesis that both parental genomes are vital for development in mammals. Importantly, parthenogenetic and androgenetic trophoblasts were phenotypically abnormal. The associated extraembryonic tissue of parthenogenetic embryos was almost non-existent. This was seen to be a likely contributing factor to the observed embryonic lethality (Thomson and Solter, 1988).

These reciprocal phenotypes, associated with gynogenetic/parthenogenetic and androgenetic conceptuses therefore not only suggested complementary roles for the two parental genomes in the development of the embryo, but also in the development of the placenta. Although imprinted genes were not yet discovered the observation that large amounts of extraembryonic tissue was present in androgenetic embryos, pointed to idea that the expression of paternal genes were required for placental development. Whilst those controlling stages of embryonic development were expressed from the maternal genome (Barton *et al.*, 1984). It wasn't until further experiments using chimeric mice, generated from a mixture of parthenogenetic/gynogenetic and androgenetic cells, that the striking pattern of segregation of the two cell types was revealed. Trophoblast tissue was shown to tend to consist of androgenetic cells and the embryo being formed largely from parthenogenetic/gynogenetic cells (Surani *et al.*, 1987, Thomson and Solter, 1988). These chimeras also failed to survive to term. This indicated that there was still an incomplete complement of gene expression. Therefore it was proposed that some placental-specific genes are

also maternally expressed and similarly there are some embryo specific genes that are paternally expressed. A theory that has been shown to be the case. Despite this, these experiments importantly identified a critical role for imprinted genes in placental development.

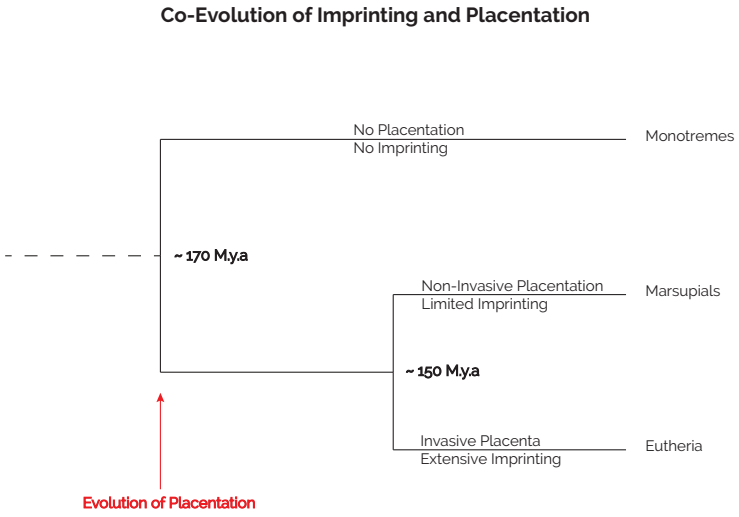


Figure 1.6: Co-Evolution of Imprinting and Placentation. Placentation evolved 170 million years ago. Marsupials and Eutherians diverged around 150 M.y.a. It was at this point that the imprinting of specific genes enabled the evolution of the more invasive eutherian placenta after diverging from marsupials around 150 M.y.a.

Imprinted genes allelic expression can be absolute, partial, temporal or tissue-specific with some genes such as *Growth Factor Receptor Bound Protein 10 (Grb10)* displaying both maternal and paternal-exclusive expression depending on tissue type (Blagitko *et al.*, 2000, Dent and Isles, 2014). Thus recently the definition of an imprinted gene has more accurately been described as one that shows differential expression of parental alleles in one or several tissue types (Moore, 2001). The predicted danger of monoallelic expression of genes is that the protection offered from single point mutations in biallelic expression is forfeited (Wilkins and Haig, 2003). Similarly, due to the dosage-related function of most imprinted genes, loss of silencing can also result in phenotypic changes (John and Hemberger, 2012). This is the case Silver Russell Syndrome (SRS) (Yoshihashi *et al.*, 2000), Prader-Willi

Syndrome (PWS) (McNamara and Isles, 2013) and cancer (Rainier *et al.*, 1993). In BWS, SRS (human chromosomal region 11p15) and PWS (human chromosome 15) both genetic and epigenetic abnormalities on these chromosomes have been linked to distinct phenotypes associated with these diseases (Davies *et al.*, 2015, Kent *et al.*, 2008). Specifically BWS and SRS are thought to be reciprocal growth disorders that result as a consequence of loss and gain in expression of paternally and maternally expressed genes, for example *IGF2* and *CDKN1C* (Shmela and Gicquel, 2013). It is now commonly accepted that dysregulation of imprinted gene expression either through deletion, inappropriate silencing or loss of imprinting can lead to or be the causal factor in sporadic, inherited and environmentally induced growth disorders (Butler, 2009, Miozzo and Simoni, 2002).

1.9.1 IMPRINTING AND BEHAVIOUR

The importance of imprinted genes to physiological systems has been widely accepted and demonstrated through the use of gene knockout studies and those parthenogenetic and androgenetic studies previously described (Falls *et al.*, 1999, Isles and Wilkinson, 2011a,b, Jelinic and Shaw, 2007). Several of the imprinted genes that have been described in the literature have been shown to be involved with several neurological and psychological traits found in humans (Arima *et al.*, 2005, Buettner *et al.*, 2005, Diaz-Meyer, 2003).

These disorders are most commonly investigated through mouse models, as these offer the most tractable genetic analogs. Perhaps one of the most relevant studies into this area is the study by Li *et al.* (1999) that investigated the phenotypic effect that knocking out the paternally expressed *Paternally Expressed Gene 3 (Peg3)* gene had in female mice. Loss of function of *Peg3* resulted in LBW and the *Peg3+/-* mothers exhibited aberrant maternal behaviour (Li *et al.*, 1999). These findings were later corroborated through a study that demonstrated a marked reduction in

oxytocin-producing neurons and impaired maternal behaviour (Curley *et al.*, 2004). These mice have been shown to have a marked delay in reaching puberty (Broad *et al.*, 2009). *Peg1/Mest* a closely related gene has also been studied by Li *et al.* (2002), where they found an association between embryonic growth and maternal behaviour. Vitally these genes are analogous to *Paternally Expressed Gene 1 (PEG1)* and *PEG3* in humans on chromosome 7q32. The link between imprinted genes and maternal behaviour through the direct alteration of specific neurons in the maternal brain, highlights the potential for further analysis of this area to help understand the co-occurrence between LBW and maternal mood disorders.

1.10 The Evolution of Imprinting

Genomic imprinting is also found to occur in plants, more specifically imprinted genes are expressed during the development of the endosperm (Kinoshita *et al.*, 1999, Vinkenoog *et al.*, 2003). The evolution of imprinting in animals and plants evolved independently, despite there being similar mechanisms used for gene regulation between the two (Feil and Berger, 2007).

The fact that genomic imprinting represents the monoallelic expression of genes over the biallelic expression of certain genes is fiercely questioned as the protection offered from single point mutations in biallelic expression is forfeited (Wilkins and Haig, 2003). Specifically, the reason why imprinting evolved in mammals at all is hotly debated (Patten *et al.*, 2014). There are a few potential theories that offer explanations why imprinting may have evolved in mammals.

The first, known as the "Adaptation of Host Defence" theory cites the idea that the human genome is made up of a considerable amount of "junk" DNA, potentially over 40% (Smit, 1999). The fundamental theory that at least some of this "junk" is actually transposable elements that have been inserted over time, with the majority

of them no longer active, holds true (Palazzo and Gregory, 2014). These transposable elements could pose a threat to the host organism if incorporated into endogenous genes and ultimately the effect this has upon the gene product. The mammalian genome inactivates these elements via methylation and chromatin modifications (Slotkin and Martienssen, 2007). These mechanisms are the same ones that control the expression of imprinted genes (Li *et al.*, 1993). Therefore it follows that some have theorised that due to this common usage of control mechanisms, genomic imprinting has evolved as a result of these host defence mechanisms (Ono *et al.*, 2001, Tunster, 2009).

The second theory, which has widely been disbanded is the "Ovarian Time Bomb Hypothesis". The theory purports that imprinting arose as a way to prevent unfertilised oocytes implanting and causing malignant trophoblast disease (Varmuza and Mann, 1994). The theory by Varmuza and Mann (1994) proposes that these "tumors" become less invasive as a direct result of imprinting silencing genes that promote trophoblast invasion. The authors pointed to the phenotypic characterisation of complete hydatidiform molar pregnancies (when there is the fertilisation of an enucleated egg and duplication of the paternal genome), which can be highly invasive (Kajii and Ohama, 1977). Critically this theory does not explain the imprinting of genes that are not involved in trophoblast development or indeed the presence of imprinting in the male genome. Meaning that it has been almost entirely rejected (Haig, 1994).

Thirdly and perhaps the most popular theory is "The Parental Conflict Hypothesis" described by Moore and Haig (1991). This theory postulates that paternal genes enhance, and maternal genes suppress, fetal growth. This is done through the control of the distribution and availability of resources by these imprinted genes. The paternal genome maximises the resources that can be extracted from the mother during development, whilst the maternal genome acts to limit the nutrients available to individual offspring and the offspring as a whole. The evidence that this theory is based upon was the discovery of the reciprocally imprinted *Igf2* and *Insulin-like Growth Factor 2 Receptor (Igf2r)* genes, which are described in more detail in the

next section.

1.10.1 Imprinting and the Placenta

Imprinting is more prevalent in eutherian mammals than marsupials (John and Surani, 2000). This suggest the possibility that imprinting is driven by the extended *in utero* development, as seen in Eutherians, adding additional selective pressure. While both marsupials and eutherian mammals possess a placenta, and diverged from monotremes 170 million years ago (**Figure** 1.6). The marsupials have less extensive fetal development and less extensive imprinting and a less elaborate noninvasive choriovitelline placenta supports this development. This is in contrast to the more extensive imprinting that occurred in eutherian mammals alongside a more invasive placenta and longer gestational times (John and Surani, 2000). This leads onto the interesting fact that imprinted genes are only sparsely expressed in the adult mammal, whilst they show comparatively high and prolific expression in the placenta (Coan *et al.*, 2005, Frost and Moore, 2010, John and Hemberger, 2012). In total there are thought to be around 100 protein coding imprinted genes almost all of which are expressed in the placenta and yolk sac (Bressan *et al.*, 2009, Fowden *et al.*, 2011, Tunster *et al.*, 2013). Importantly, a significant number of mouse studies demonstrate that imprinted genes often act to regulate placental development (Tunster *et al.*, 2013). Imprinted genes are expressed in the brain and are essential in brain development as originally suggested by Keverne (1997) when there was non-random distribution of cells within the androgenetic and gynogenetic genomes in chimeric brains. Androgenetic cells tended to contribute to the hypothalamus and areas associated with primary motivated behaviour, while gynogenetic cells accumulate in the neocortex and regions linked to more complex behaviours (Keverne, 1997).

The first two protein encoding imprinted genes to be discovered were *Igf2* and its

receptor *Igf2r* (Barlow *et al.*, 1991, DeChiara *et al.*, 1991). Both of which confer reciprocal functions in placental and fetal growth (DeChiara *et al.*, 1990). *Igf2* encodes a growth hormone (Chao and D'Amore, 2008). Its loss of function resulted in embryonic growth restriction phenotype following paternal transmission of a specific deletion (Constância *et al.*, 2002, DeChiara *et al.*, 1990). This was initially put down to haploinsuffiency, but was later ruled out after *Igf2* expression levels were noted to be lower than a 50% reduction. It followed that DeChiara *et al.* (1991) went on to show that the locus of the gene was paternally imprinted. In contrast *Igf2r* has been shown to be maternally expressed, and mediates the growth regulating properties of *Igf2* by attenuating the signalling of Igf2 by clearing Igf2 from the cell surface. These phenotypes identified in mice were what ultimately gave rise to the parental conflict hypothesis proposed by Moore and Haig (1991), and the seminal experiments demonstrated that there were the tools necessary to answer the unanswered questions about genomic imprinting (Barlow *et al.*, 1991). A select few of these genes are pivotal in the regulation of the endocrine lineage of the mouse placenta (John, 2013), something that has led to a new focus suggesting a link between imprinted genes, endocrine placental signalling and maternal behavioural adaptations.

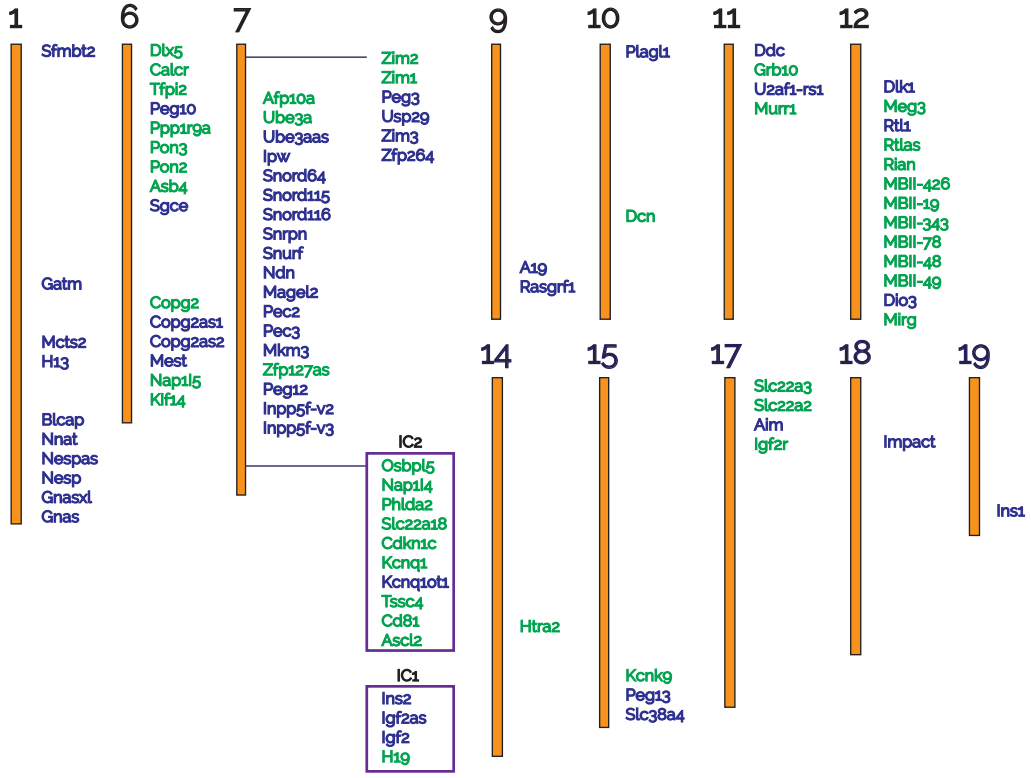
1.11 Chromosome 7 and *PHLDA2/Phlda2*

1.11.1 Chromosome 7

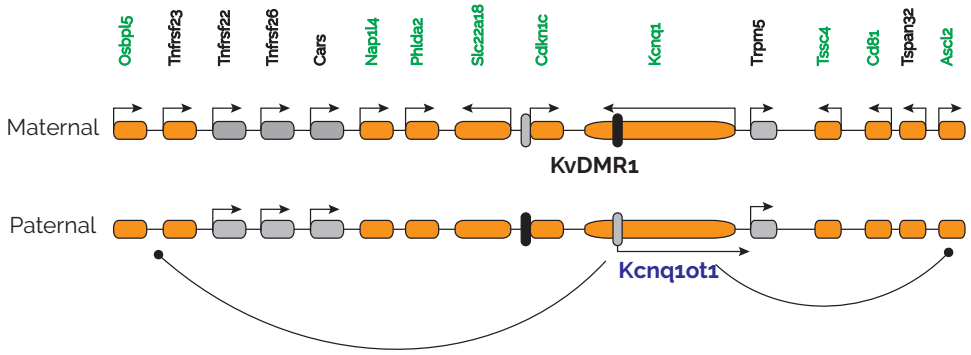
Imprinted genes are commonly located within domains which can encompass several megabases of DNA with both paternally and maternally expressed transcripts (Bartolomei and Ferguson-Smith, 2011). One well studied domain is the human chromosome 11p15/mouse distal chromosome 7 domain (Burton, 2011, Shmela and Gicquel, 2013). In mice this domain is divided into two mechanistically distinct domains, these being Imprinting Centre 1 (IC1) and Imprinting Centre 2 (IC2). IC2

contains several genes that are imprinted and expressed in the placenta. This domain has been shown to contain genes important for placental and fetal growth. Two studies in particular, one by Fitzpatrick *et al.* (2002) and another by Mancini-Dinardo *et al.* (2006) showed that through either the targeted deletion of the imprinting centre or the removal of the long non-coding strand, *Kcnq1ot1*, respectively (**Figure** 1.7), it is possible to restrict placental and fetal growth. Three key maternally expressed genes from this region are *Achaete-Scute Family bHLH Transcription Factor 2 (Ascl2)* (formerly *Mash2*), *Cyclin Dependent Kinase Inhibitor 1C (Cdkn1c)* and *Pleckstrin Homology Like Domain family A member 2 (Phlda2)*, have been targeted in mice with additional over expression data for *Phlda2* (Tunster *et al.*, 2013). More recently the consequence of over expressing *Ascl2* have been reported (Tunster *et al.*, 2016).

In humans, the domain is linked to a number of rare imprinting disorders including BWS, SRS and IMAGe syndrome (Arboleda *et al.*, 2012, Butler, 2009, Soejima and Higashimoto, 2013). While *ASCL2* is not imprinted in the human placenta (Miyamoto *et al.*, 2002), the gene sequences are well conserved which supports role for these genes in the growth phenotypes reported in these syndromes (Shmela and Gicquel, 2013). Imprinted genes found in domains are regulated by genomic regions known as imprinting centres (ICs), but are also referred to as Imprint Control Elements (ICEs) and Imprinting Control Regions (ICRs) (Bartolomei and Ferguson-Smith, 2011). These ICs are differentially methylated in the germline, marks which are maintained after fertilisation. Importantly, they have been functionally defined through careful engineering of targeted deletions in mice (Chamberlain and Brannan, 2001, Williamson *et al.*, 2006). If a deletion is inherited through one of the parental germ lines, it releases every gene that is located in the domain from their imprinted status causing a Loss of Imprinting (LOI).



(a) Imprinting Map in Mice



(b) Imprinting at IC2 domain in mice

Figure 1.7: Imprinting. **(a)** An imprinting map of the mouse (*Mus musculus*) showing the paternally expressed genes (blue) and maternally expressed genes (green) **(b)**The imprinting centre 2 (IC2) of the mouse (*Mus musculus*) showing the paternally expressed genes (blue) and maternally expressed genes (green). Adapted from Tunster *et al.* (2013).

1.11.2 *PHLDA2/Phlda2*

Phlda2 is the imprinted gene and the focus of this study. It is a maternally expressed imprinted gene that has been known by several other names since its first discovery, namely in humans *BRW1C*, *HLDA2*, *IPL* and *TSSC3* (Salas *et al.*, 2004). Studies from the Tycko laboratory and our research group have defined a role for this gene in the development of the placenta (Frank *et al.*, 2002, Salas *et al.*, 2004, Tunster *et al.*, 2015, 2010). Importantly, loss of expression and increased *Phlda2* dosage has a reciprocal effect on the junctional zone of the mouse placenta (Frank *et al.*, 2002, Tunster *et al.*, 2015, 2010, 2014), described in more detail below.

Phlda2 encodes a small, cytoplasmic PH domain only protein whose function is not yet known (Qian *et al.*, 1997). Although the function of *Phlda2* has not been assessed, a closely related protein, *Pleckstrin Homology Like Domain family A member 3 (Phlda3)* has been identified in having a role in the inhibition of Akt-regulated processes (Kawase *et al.*, 2009). *Phlda2* is preferentially expressed in the extraembryonic tissue, more explicitly the ectoplacental cone and extraembryonic membranes (the visceral endoderm of the yolk sac). It then becomes restricted to the labyrinth zone by E10.5 by which point the mature placenta has formed (Frank *et al.*, 1999). Past this point, E10.5, *Phlda2* is solely expressed in the forming type II trophoblast and at a lower level in type III trophoblast cells. It is after E14.5 that *Phlda2* expression declines.

Our most recent studies by Tunster *et al.* (2015) demonstrated that *Phlda2* specifically and exclusively regulates the size of the SpT lineage of the junctional zone. The SpT is a key source of placental prolactins and Placental Specific Glycoproteins (Psgs). Consequently *Phlda2*, indirectly, negatively regulates the production of placental hormones. The RNA expression analysis (see Chapter 3) identified the SpT transcriptome (Tunster *et al.*, 2015).

The SpT expresses a number of *Prls* and *Psgs*, displayed in **Table** 1.6 (Simmons *et al.*, 2008, Tunster *et al.*, 2015). In the published model of *Phlda2* $^{-/+}$ (KO) placenta express a number of *prolactin* family members at higher levels than wildtype (WT) (Tunster *et al.*, 2015). Conversely, *Phlda2* $^{+/+\ \mathrm{BACx1}}$ (TG) placenta (which carry an extra copy of the *Phlda2* gene on a Bacterial Artificial Chromosome (BAC), Chapter 2) express lower levels of these same hormones. This work led to the hypothesis that the expression of imprinted genes in the placenta could influence maternal adaptations to pregnancy (John, 2013). Importantly, these well characterised mouse models provided a systems to test this hypothesis in a physiological relevant context.

Table 1.6: Placental Lactogens and Glycoproteins found in SpT

PRL Family Name	Official Symbol
Prolactin Family 3, subfamily b, member 1	*Prl3b1*
Prolactin Family 8, subfamily a, member 8	*Prl8a8*
Prolactin Family 8, subfamily a, member 9	*Prl8a9*
Prolactin Family 7, subfamily a, member 2	*Prl7a2*
Prolactin Family 3, subfamily c, member 1	*Prl3c1*
Prolactin Family 3, subfamily a, member 1	*Prl3a1*
Prolactin Family 2, subfamily c	*Prl2c*
Pregnancy Specific Glycoproteins	*Psgs*

1.12 PROJECT OVERVIEW

Prolactin and the Prls are known to play an indirect role in maternal behaviour. In this project we used the imprinted gene *Phlda2* to manipulate the levels of the size of the SpT lineage of the mouse placenta, and thus the subsequent expression levels of the *Prls* and *Psgs* (**Figure** 1.8). Embryos with zero, one and two-fold expression of *Phlda2* have 200%, 100% and 50% the levels of expression of SpT-specific hormones. Using a process called RET, preimplantation embryos can be transferred into WT recipient females to test the functional relevance of these different dosage of *Phlda2* and subsequent placental hormone levels. This technique will allow the manipulation of the *Prls* without having to delete the *prolactin* locus.

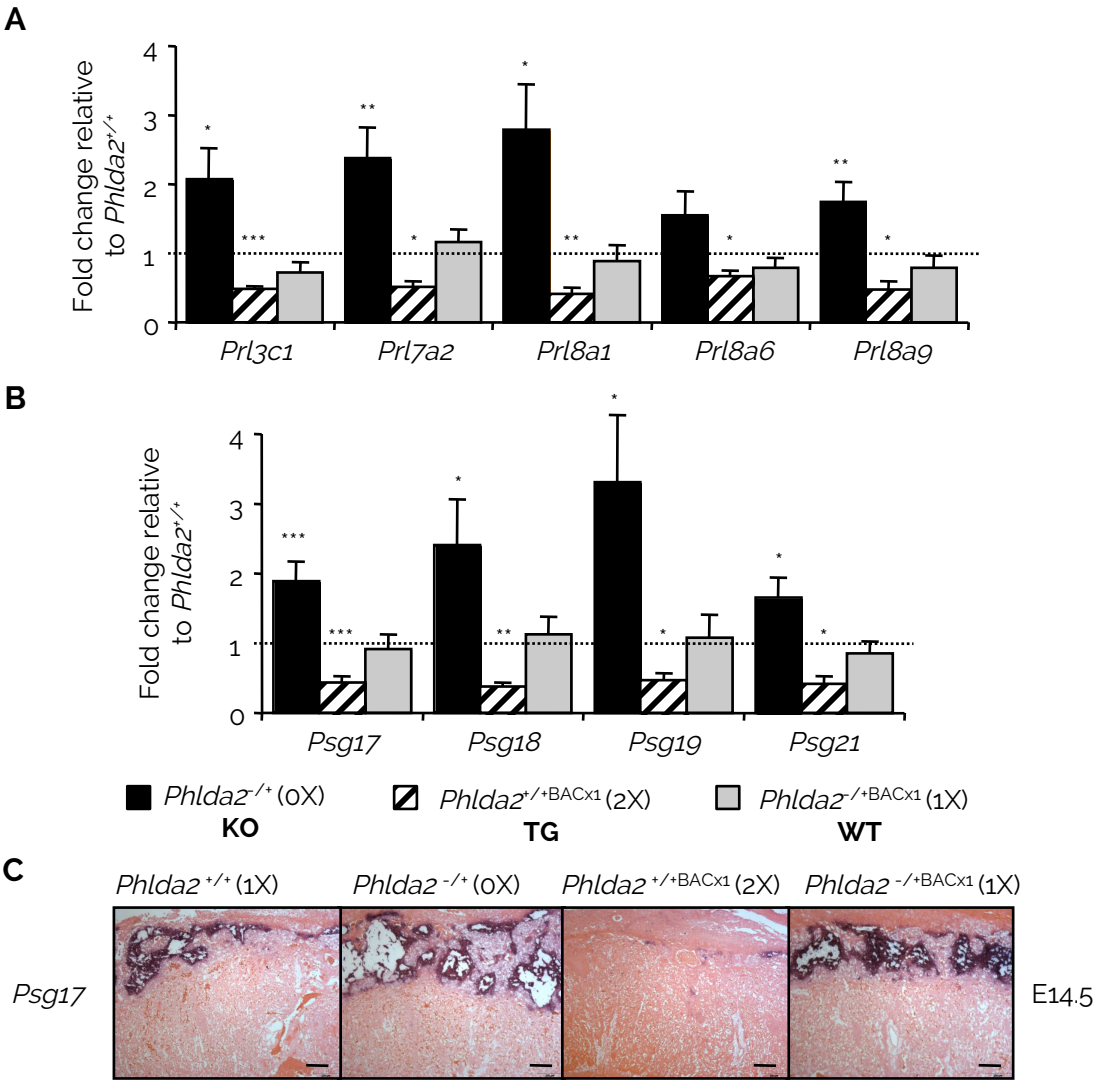


Figure 1.8: Average fold changes for specific genes shown to have differential expression in the placenta of dams at E14.5. *Phlda2* indirectly suppresses the expression of key placental hormones. **A.** qPCR comparison of *Prls* at E14.5 between *Phlda2* $^{+/+}$ (WT, 1X), *Phlda2* $^{-/+}$ (maternal KO; 0X), *Phlda2* $^{+/+\ \mathrm{BACx1}}$ (2X) and *Phlda2* $^{-/+\ \mathrm{BACx1}}$ (rescue; 1X) 129 placentae. **B.** qPCR comparison of *Psgs* at E14.5. **C.** *In situ* hybridisation of midline sections E14.5 placentae using *Psg17* to label the spongiotrophoblast (scale bar = 200 µm). For the qPCR analysis, n = 4 placenta per genotype (2 vs 2 from 2 independent litters). Error bars represent SEM. Statistical significance: $*p < 0.05$, $**p < 0.01$ and $***p < 0.005$. Data generated by Simon Tunster.

The behavioural outcomes of these mothers carrying different placenta will be assessed, and then a basic biomolecular characterisation performed. This will be done at an earlier time point than when the behavioural tests were carried out, in order to determine whether there are any molecular changes that predate the behavioural phenotype and therefore offer an explanation on the mechanism that may cause any behavioural changes that are observed.

Finally the offspring of the low placental signalling (*Phlda2* TG) model dams will be assessed in order to determine whether there is any lasting effect on the behavioural and biomolecular phenotypes of the offspring. This model will also help determine whether these effects are due to the *in utero* environment or post natal maternal care.

1.13 Hypotheses

1. Imprinted genes influence maternal care behaviours via the placental endocrine compartment.

2. Placental endocrine dysfunction can impact offspring outcomes either via fetal growth restriction or poor maternal care.

1.14 Aims

The aims below are outlining the major focusses for each experimental chapter and are by no means extensive.

1. To use microarray analysis to identify the SpT transcriptome in the placental lineages of the different *Phlda2* gene dosage mouse models.

2. To assess maternal behaviour changes as a consequence to abnormally high or low placental signalling. To do this three embryo types were transferred into three cohorts of wild type female mice:

    - *Phlda2*-transgenic embryos (TG). These are a low signalling model and contain an extra copy of *Phlda2*.
    - *Phlda2*-knockout embryos (KO). These are a high signalling model, one copy of *Phlda2* has been ablated causing high placental signalling.
    - Wild type embryos (WT). These are the control with normal dosage of *Phlda2* and levels of signalling.

    Several key maternal behaviours will be assessed including:

    - Maternal anxiety in response to the three models using the elevated plus maze test.
    - Pup retrieval and Nest building in a specially designed home cage capable of 24 hour monitoring.
    - Specific nurturing behaviours scored manually using the 24 hour surveillance of mothers during the nest building behavioural assay.
    - Fetal weights and pup weights will also be recorded as indicators of maternal nurturing behaviour effectiveness.

3. Perform a basic biomolecular characterisation of each of the three maternal cohorts. Microarray on brain regions and analyse data to identify genes and changes in gene expression in the maternal brain as a result of altered placental signalling will be used to generate hypotheses about specific genes of interest. These mothers will also have their neurogenesis levels evaluated using immunohistochemistry.

4. Using the maternal care model for low placental signalling (*Phlda2* TG) the offspring outcomes will be characterised for both a behavioural phenotype and a biomolecular phenotype.

Methods 2

2.1 Mouse Model

The function of *Phlda2* in pregnancy was studied using two mouse models, a *Phlda2* knockout (KO) mouse model (Frank *et al.*, 2002) and a *Phlda2* over expression (TG) mouse model (Tunster *et al.*, 2010). The KO mice were originally generated by targeted ablation of both coding exons of the *Phlda2* gene as well as half the upstream CpG island (Frank *et al.*, 2002). Male *Phlda2* heterozygotes (absence of paternal allele, regarded as phenotypically wild type (WT)) were crossed to WT 129S2/SvHsd (129) females for $>$ 12 generations and maintained by the John Lab. To generate embryos without expression from the maternal allele (termed *Phlda2* $^{-/+}$ maternal KO; 0X), female *Phlda2* homozygotes were mated with wild type 129 males (**Figure** 2.2).

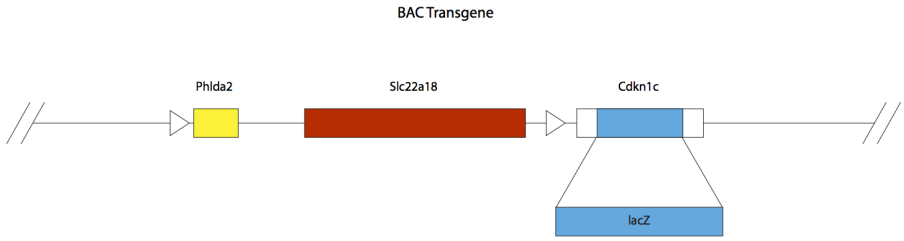


Figure 2.1: BAC Transgene. Basic schematic illustrating the BAC transgene used in our mouse model. The BAC spanned *Phlda2* (yellow) and two other genes, *Slc22a18* (red) and *Cdkn1c* (blue). *Cdkn1c* expression from the transgene was replaced by a beta-galatosidase cassette (lacZ), and the promotor ablated.

In order to mimic the effect of the loss of imprinting, a BAC transgenic model (10-10) was utilised. These mice possess a single copy of the BAC transgene (**Figure** 2.1) resulting in expression at twice the endogenous level in the placenta (Tunster *et al.*, 2010). The transgene is 85kb in length and doesn't undergo imprinting meaning that *Phlda2* is expressed on either the paternal or maternal allele; *Phlda2* $^{+/+\ \mathrm{BACx1}}$ (single copy *Phlda2* transgene, TG; 2X). All the models were maintained on a pure 129 genetic background. The TG 10-10 line was maintained through mating 10-10 TG females with 10-10 TG males. Homozygous transmission of the TG results in animals that are non-viable thus the line was kept as hemizygous.

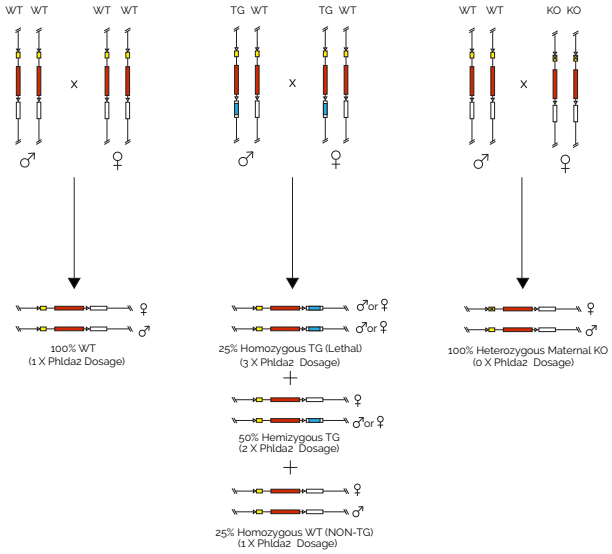


Figure 2.2: Crosses/Matings performed. A schematic outlining the various crosses/matings performed in order to generate the embryos utilised for the recipient embryo transfer procedure. *Phlda2* is maternally imprinted, therefore the maternal heterozygous KO results in no expression of *Phlda2*. The transgene (TG) is not imprinted and can be transmitted on either the maternal or paternal allele.

All animal studies and breeding was approved by the University of Cardiff ethical committee and performed under a UK Home Office project license (RMJ, 30/3134). Mice were housed on a 12 hour light/dark cycle with lights coming on at 07.00 hours with a temperature range of 21°C ± 2 with free access to tap water and standard chow unless otherwise stated. All procedures performed upon the mice were carried out exclusively by Personal Licence Holders.

2.2 Recipient Embryo Transfer (RET)

In order to isolate the affects of *Phlda2* on the phenotype of the mothers in this study, it was crucial to maintain the dams WT status. Therefore the RET procedure was required. RET was carried out in the Cardiff Transgenic Production Facility (TPF) by Bridget Allen. Through controlled matings pre-implantation embryos that were either WT (*Phlda2* $^{+/+}$), TG (*Phlda2* $^{+/+\ \mathrm{BACx1}}$) or (*Phlda2* $^{-/+}$) KO (**Figure** 2.2) were generated. Pseudopregnant WT females are then produced through the controlled mating of virgin females with vasectomized males. Embryos that were harvested at E1.5 were then implanted via bilateral oviduct transfer into pseudopregnant WT females at E0.5 (**Figure** 2.3).

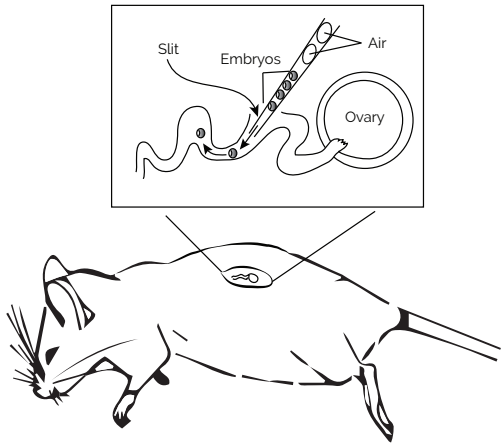


Figure 2.3: Recipient Embryo Transfer Schematic. A schematic demonstrating the RET procedure performed, in order to maintain the WT status of the dams.

On the fifth day of pregnancy these females were moved from a barrier unit at Cardiff University to the Science Research Infrastructure Fund (SRIF) housing, where they were monitored and weighed at predetermined intervals, so as to check general health and for signs of pregnancy. The females that were viewed as being pregnant were culled at E16.5 and dissected, or allowed to give birth and used for behavioural testing (**Figure** 2.4).

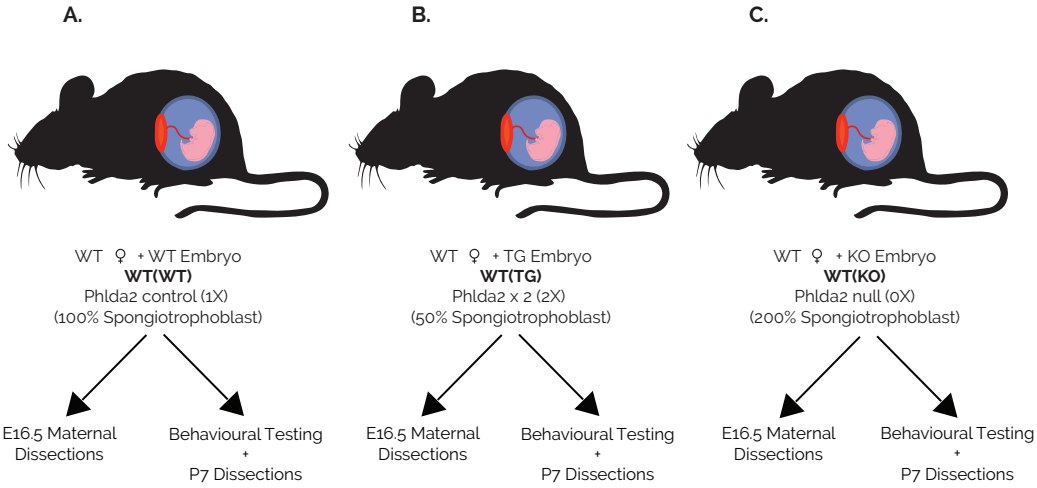


Figure 2.4: The Three Cohorts for Behavioural and Biomolecular Analysis. Simplified diagram explaining the basis of the mouse model generated through RET. Around 14-16 embryos carrying varying doses of *Phlda2* were implanted into 3 cohorts of 20 WT females. These WT females then carried either **A.** WT or **B.** *Phlda2* - TG or **C.** *Phlda2* - KO fetuses. 12 dams were allowed to give birth and then tested for behavioural changes before dissection. The other 8 dams were dissected at E16.5 for bimolecular analysis.

2.3 Biomolecular Procedures

2.3.1 Genotyping

Polymerase Chain Reaction (PCR) was utilised to identify the genotype of the donor females, stock animals and experimental animals born from recipient females. The yolk sacs or ear biopsies from pups taken at weaning were digested in lysis buffer (50 mM Tris (pH 8.0), 100 mM EDTA (pH 8.0), 100 mM NaCl and 0.5% SDS in water) containing 400 µl/ml Proteinase K (PK) at 55°C overnight.

Samples were shaken vigorously and allowed to cool at room temperature for 40 minutes. Lysates were diluted either 1:10 for ears or 1:20 for tails and yolk sacs in 10 mM Tris (pH 8) and taken up to 95°C for 30 minutes to inactivate PK. Dilutions were vortexed to mix, allowed to cool for 30 minutes and 1 µl used as a template in

a reaction volume of 15 µl as in **Table** 2.1.

Table 2.1: PCR constituents

Reagents	Volume per reaction (µl)
H_2O	10.3
10X Buffer	1.5
25 mM $MgCl_2$	1.2
dNTPs	0.3
Primers	0.6
Taq	0.1
Template	1.0

Primers were designed using Primer3 (**Table** 2.4) and purchased directly from Sigma Genosys. They were then diluted to 100 µM in 10 mM Tris (pH 8). Both the forward and the reverse primers were combined at a 1:4 dilution in 10 mM Tris (pH 8) and 0.6 µl of appropriate primer mix used in each PCR reaction.

The thermocycler was set at 94°C for 15 minutes, followed by 35 cycles of 94°C for 30 seconds, 60°C. for 30 seconds, 72°C for 30 seconds and then 72°C for 3 minutes for each PCR. The reactions were loaded in 1% 1X Tris-acetate EDTA (TAE) agrose gel containing 0.5 µg/ml of SafeView (NBS Biologicals) and run against a 100 bp PCR Ranger ladder (Gene Flow) and visualised using a transilluminator (BioRad).

2.3.2 Dissection of Pregnant Females at E16.5 and P7

The mice were weighed and then sacrificed according to schedule 1 (through cervical dislocation and death confirmed) at either E16.5 or P7. The uterus was removed and weighed and the location of the pups in the uterine horn was noted before the uterus containing the individual pups and placentae were placed into ice cold PBS. E16.5 and P7 dams were decapitated and the maternal brain was removed from the skull as described in section 2.3.9 and specific regions dissected. The areas dissected from the brain were the olfactory bulbs, hippocampus, frontal cortex and the hypothalamus,

which were placed in labelled 0.5 ml eppendorfs before being flash frozen on dry ice and stored at -80°C.

2.3.3 Serum Collection

After cervical dislocation and before decapitation the thoracic cavity was exposed to reveal the heart. A 19G needle connected to a 1 ml syringe was used to puncture the left ventricle and then allowed to remain at negative pressure in order to collect the blood from the animal. Any excess blood present in the thoracic cavity was also collected. The blood was then injected into microtainer tubes (BD, Oxford, UK) and left to coagulate before being centrifuged at max speed (13,000 rpm) for 5 minutes whilst further dissections took place. After the 5 minutes serum was flash frozen on dry ice and stored at -80°C.

2.3.4 Preparation of RNA

RNA Extraction - Method 1

Hippocampal tissue was homogenised in 1 ml of RNA-Bee (Ams Biotechnology) with an RNA-Free pestle (VWR International) in a 1.5 ml microfuge tube. 200 µl of chloroform was then added, homogenates mixed and incubated on ice for 5 minutes. Homogenates were centrifuged at 13,000 rpm for 15 minutes at 4°C. The aqueous phase was removed and added to 0.5 ml of isopropanol, mixed, incubate at room temperature for 10 minutes and centrifuged at 13,000 rpm for 10 minutes. Pelleted RNA was washed in 1 ml of 75% Ethanol (EtOH) centrifuged at 13,000 rpm for 10 minutes, left to air dry for 30-60 seconds and re-suspended in an appropriate volume of 10 mM Tris (pH 8). RNA concentration and integrity was evaluated using a NanoDrop Spectrophotometer, with RNA concentration adjusted to approximately

1 µg/µl using 10 mM Tris (pH 8).

Genomic DNA DNase treatment was performed. Approximately 1-3 µg of RNA was treated using RQ DNase1 (Promega) in a 1.5 ml microfuge tube at 37°C for 45 minutes. 1 µl of RQ DNase1 was used per µg of RNA. The volume of the solution was made up to 500 µl by adding Tris (10 mM). Then 500 µl of phenol was added and mixed well by inverting. This mixing was repeated every few minutes for 10 minutes. A new set of 1.5 ml eppendorfs was labelled and 500 µl of chloroform added to each, 45 µl of 3 M NaOAc (pH 6) was added to a second set of eppendorfs. The DNA/phenol mix was spun at 12 000 rpm for 10 minutes at room temperature. On completion the upper aqueous layer was removed and added to chloroform and mixed. A further spin at 12 000 rpm for 10 minutes was then performed before the top layer was removed once more before being added to the NaOAc for precipitation. 1 ml of 100% EtOH was mixed with this solution and then left to precipitate the RNA overnight at -20°C. The next day this is spun at 4°C and the EtOH poured off. 1 ml of 70% EtOH is then added for a final wash and spun at 12 000 rpm a final time for 10 minutes before removing the remaining EtOH and re-suspending the pellet in 20 µl of 10 mM Tris.

RNA extraction - Method 2

RNA was extracted from neural tissue using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, UK). The samples were homogenised in 500 µl of Lysis Buffer containing 5 µl 2-Mercaptoethanol using an RNA-Free pestle (VWR International) in a 1.5 ml microfuge tube. Lysate was transferred into the GenElute filtration column and then centrifuged at 12 000 rpm for 2 minutes. Then 500 µl 70% EtOH was added to the filtered lysate and mixed by pipetting. Once mixed 500 µl of the mixture was added to the GenElute binding column at a time before being centrifuged for 15 seconds at maximum speed. The remaining 500 µl of the lysate/ethanol mix was run through the GenElute binding column before the flow

through was discarded. Wash solution 1 was run through the binding column at 250µl for 15 seconds at maximum speed. At this point 10 µl of DNase 1 was combined with 70 µl of DNase Digest Buffer for each preparation and mixed gently by inversion in order to prevent the denaturation of the enzyme. 80µl was then added directly onto the binding column and left at room temperature for 15 minutes. After 15 minutes another wash was performed using 250µl of wash solution 1 and a 15 second spin at max speed. The column was then transferred to a clean collection tube where it was washed with two washes of wash solution 2, 500µl each time, for 15 seconds then 2 minutes respectively. Once the flow through was disposed of an extra 1 minute spin step was added so as to make sure the column was completely dry before placing the column in a clearly labelled 1.5 ml RNase free eppendorf and adding 50µl of Elution Solution to the column before centrifuging for 1 minute at maximum speed. The RNA was then quantified using the Nanodrop and stored at -80°C until needed.

2.3.5 Preparation of cDNA - Reverse Transcription

To generate cDNA, total RNA was reverse transcribed (RT). For each sample, two reactions were set up: RT+ and RT-. Reactions were set up in strip tubes. Then for each reaction components in **Table** 2.2 were included.

Table 2.2: Reverse Transcription Components

Reagents	**Volume per reaction (µl)**
DNAse Treated RNA	5 (µg)
0.5 (µg/µl) random hexamers	1 (µl)
RNAse free H_2O	Upto 11 (µl)

Tubes were incubated at 70°C for 10 minutes whilst a master mix was made as per **Table** 2.3. Tubes were removed from incubator and placed immediately on ice. 8 µl of master mix was added to each tube. After 2 minutes at 37°C 1 µl of MMULV was added to the RT+ reactions only. The tubes were incubated for 2 hours at 37°C

before a 15 minute heat shock at 70°C. 5 µl was taken from each reaction and diluted in 100 µl of 10 mM Tris.

Table 2.3: Master Mix reagents for each RT reaction

Reagents	Volume per reaction (µl)
5X first strand synthesis buffer	4 (µl)
10 mM dNTPs	1 (µl)
RNAse free H_2O	3 (µl)

2.3.6 cDNA Quality Control

After preparation of cDNA, PCR reactions were performed to confirm that the samples were free of genomic DNA contamination and that cDNA had been produced correctly and in sufficient quantities. A PCR was performed on the RT+ and RT- dilutions using primers *Gapdh* (149/150) and *β-Actin* (151/152) (**Table** 2.4) in a volume of 20 µl as described in section 2.3.1. cDNA quality was demonstrated by the presence of a band in the RT+ samples and the absence of a band on the RT- samples.

2.3.7 Microarray

This microarray data analysis was performed using the Bioconductor platform that was an open source software project based on the R programming language. A brief outline of the major steps involved from annotation to normalisation, diagnostic plots and pathway analysis is presented in the following sections. A more detailed description of the techniques used can be found in the chapter written by Zhang *et al.* (2009).

RNA Extraction for Microarray

RNA was extracted from whole hypothalamic and hippocampal brain regions using the methods described in section 2.3.4. The RNA was then sent to Central Biotechnology Services (CBS) for RNA quality testing and processing followed by microarray. The system used was the Affymetrix GeneChip Mouse Gene 2.0 ST Array.

Software

The data set was analysed using the R Statistical Package and all additional packages required were downloaded from the Bioconductor website, installed and then consequently loaded at the start of each new R session.

Sample Preparation

Sample information was manually into a .txt file containing the relevant comma-seperated-value (.csv) information regarding the genotype, sample name and tissue type (**Figure** 2.5).

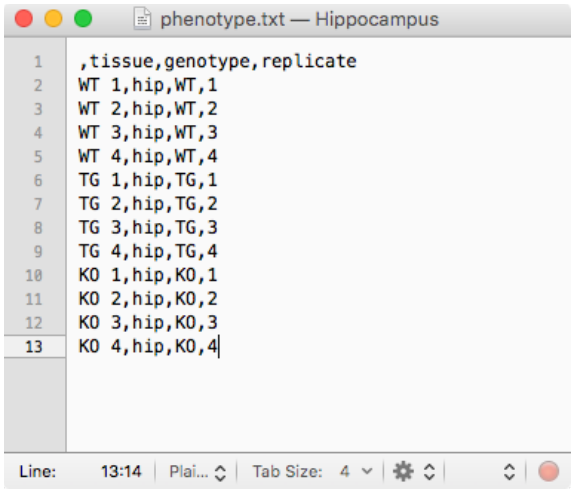


Figure 2.5: Phenotype Data File. Phenotype data input file .csv

Annotation and Normalisation

CEL files (.cel) were imported into R and normalised using the rma package. The phenotype data was then added to the normalised data set and checked before annotation. The probe set was annotated in R and a random selection of probes listed to check annotations were successful (probes: 10060-10070). At this point it was also possible to check the data graphically after it had been normalised, an example of these can be seen in the boxplot and histogram below (**Figure** 2.6 and **Figure** 2.7).

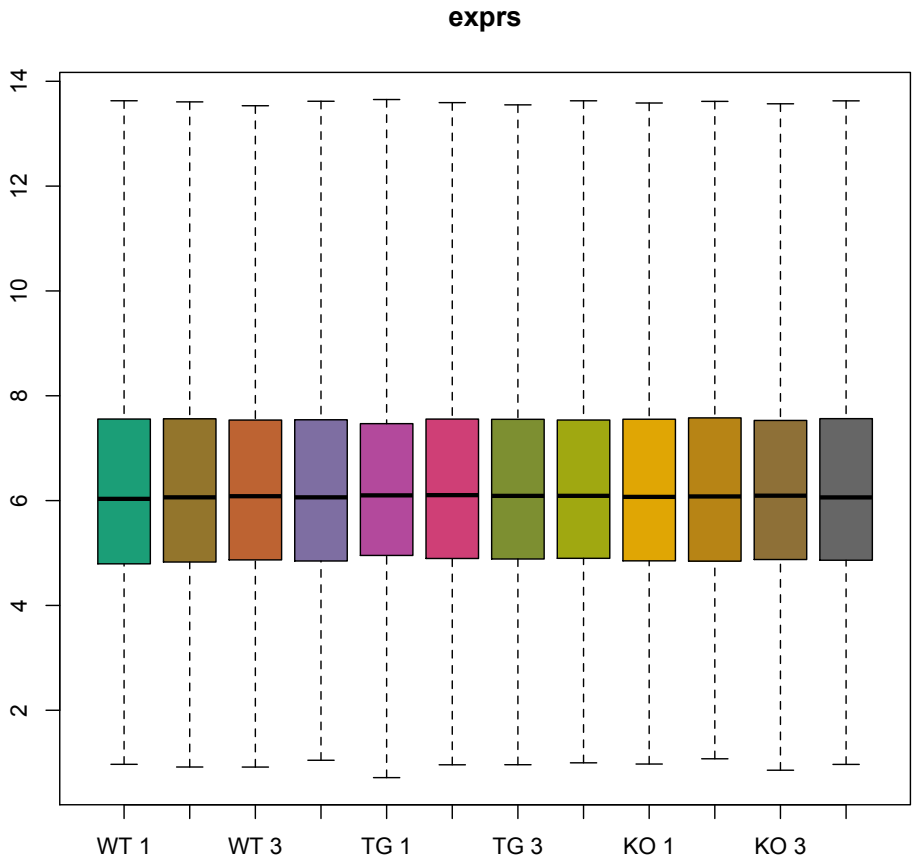


Figure 2.6: Boxplot of Normalised Data. Microarray data after normalisation.

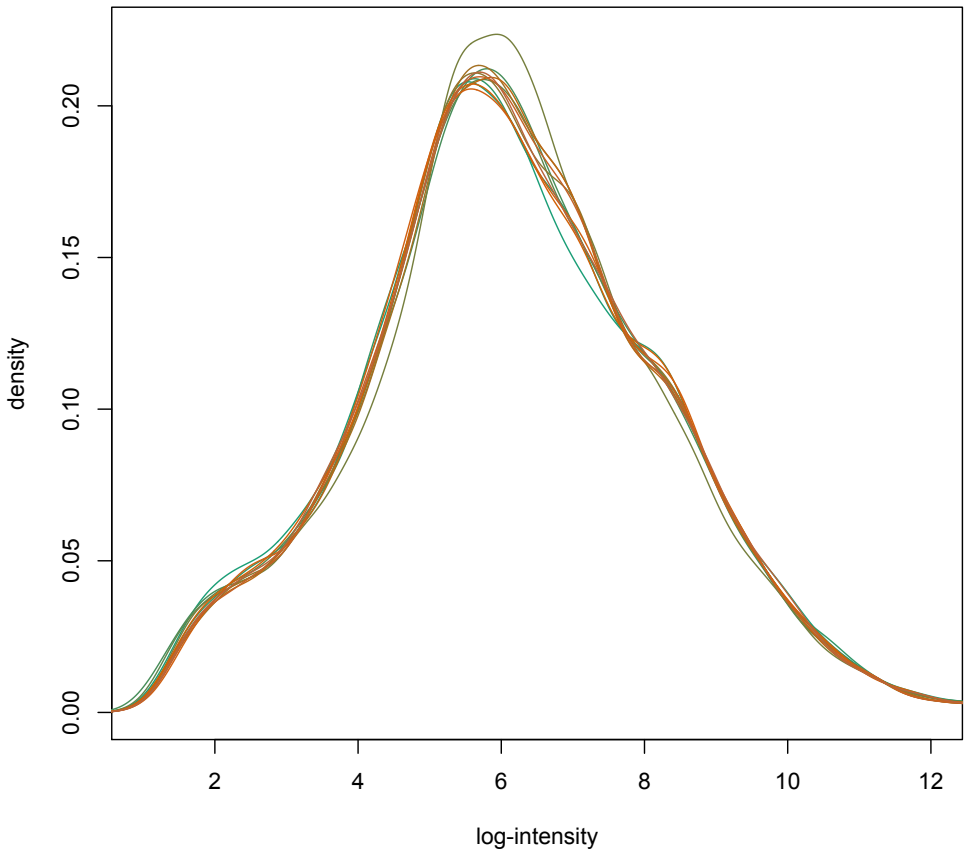


Figure 2.7: Normalised Histogram. Microarray data after normalisation.

Analysis using LIMMA

Once the data was annotated and normalised it was then possible to choose and use the model for analysis. The complexity of this experiment and the number of RNA targets meant that LIMMA was the perfect choice. LIMMA stands for a "Linear Model for Microarray data Analysis", it was designed to identify differentially expressed genes between all pair-wise comparisons of the samples groups.

LIMMA is run in R using a Bioconductor package. In order to perform LIMMA two matrices were constructed. These matrices consist of the design matrix, which provides a representation of the different sample groups that have been analysed,

and the contrast matrix. The contrast matrix allows the coefficients defined by the design matrix to be joined into contrasts of interest. Therefore each contrast corresponds to a pairwise comparison between specified sample groups. LIMMA is an ideal choice for microarray analysis because microarray experiments usually only utilise a small number of replicates for each experimental condition, making gene expression level estimation difficult (Smyth, 2005). LIMMA employs a system that leverages the large number of observations in a microarray experiment so as to moderate the variation estimates based on data dependent information (Zhang *et al.*, 2009).

The results of this analysis were then plotted into a series of venn diagrams selecting for varying significance levels and showing the numbers of differentially expressed genes between the various pair wise comparisons and across all the samples.

These venn diagrams were then used to generate lists of significant gene changes that were similar in expression between all three genotypes and between specific genotypes compared to a single genotype. These gene names and labels could then be used for further cluster and signalling pathway analysis.

Pathway Analysis

In order to see whether the gene expression changes detected from the microarray analysis coincided with any particular signalling pathways and thus biological processes, the gene ENSEMBL labels were extracted from each list and inputted into the online platform DAVID (https://david.ncifcrf.gov/) as described by Huang *et al.* (2009). DAVID performed analysis *in silico* that generated a list of potentially affected signalling pathways.

2.3.8 Gene Expression Analysis by qPCR

qPCR - Method 1

The genes of interest were assessed for expression in the maternal hippocampus at E16.5 using quantitative PCR (qPCR). Primers were designed using Primer3 program before being purchased from Sigma Genosys (**Table** 2.4). Each primer was designed to work optimally at an annealing temperature of 60°C, and spanning at least 1 intron producing a product size of less than 200 bp. Before qPCR the newly designed primers were rigorously checked using standard PCR on whole brain cDNA in order to confirm the amplification of the expected band size and absence of primer dimers using the original cDNA from the earlier 20 µl RT reaction.

The RT+ cDNA was diluted further for use in qPCR, up to a total volume of 400 µl by adding 380 µl 10 mM Tris to these dilutions. The primer mixes were made for each of the four genes of interest and the two designated reference genes. This was done by adding 25 µl of primer to 50 µl of 10 mM Tris. Then 5 µl of cDNA was aliquoted into the appropriate wells on a 96 well plate and a master mix made for the 96 reactions using the constituents seen in **Table** 2.5.

Eight 1.5 ml alpha tubes were labelled and 9.6 µl of each required primer mix was pipetted into the designated tube. Then 179 µl of master mix was added to each tube and mixed briefly by spinning. At this point 14.9 µl of each master mix is aliquoted into the appropriate wells on the 96 well plates before being run on the qPCR machine using program "60deg with 75deg anti dimer step".

Table 2.4: Primer Sequences

Gene	Forward Primer	Reverse Primer	Product Size (bp)
Dat/Slc6a3	GGCTGAGATGAGGAACGAAG	CTATCCAAACCCAGCGTGAT	204
Ucn3	ATATGCACAGGGGAGTGGAG	CCTAGGAGCAGCAGAAGTGG	246
Kcnh2	GGATGGGGAGAAAAGTGACA	AGGGCTAGACAAGGGGATGT	164
Th	AGGAGAGGGATGGAAATGCT	GCGCACAAAGTACTCCAGGT	78
Tph2	CTGCTGTGCCAGAAGATCATCA	TGCTGCTCTCTGTGGTGTCG	129
Endod1	GCACGTGAGGATCTGTCAAC	CCCAGATTGTCCACTGAGGT	223
Htr2/5-HT2	CCATCTGCTTCACCTACTGC	GGTGGTCAGACGCCTACTGT	157
Oxtr	TGCTGGACGCCTTTCTTCTT	GCCCGTGAAGAGCATGTAGA	172
Olfr-45	TCTGTGAGATTCCCCCACTC	AGAAAAGGCCTTCCTCTTGC	185
Olfr-59	ATTGTCCTGGCCATTAGCAC	GATTGTCCTGCTTTGGGTGT	144
Olfr-347	TGGCTCGCCTAACTCACTTT	CTTGTTGGTGGTGGTCTG	101
Olfr-481	ATCTGCTCACCCCTGCTCTA	ATTCATGCATCCACCCAAAT	90
Olfr-615	TCTTCTTCCATGGGTTCAGG	GGTGACTCGTGAGTTCGTCA	108
Olfr-686	GGATATCCCTTCCCTTCTGC	GGGAGAGGACCAGATCAACA	147
Olfr-945	TGGAGGACATGACATCAGGA	CTGGAGTTCTGGCTCCTCTG	80
Olfr-1239	ATCATTGCCCTCACTCTGCT	TGGGAATGCAGGTTGTTGTA	106
Olfr-1410	TCACTCCAGTGTCCTTGCAG	AAGTACATGGGGGAGTGCAG	172
Olfr-1491	TGTCCTTGCAGATCATTGCC	ATTGTCCTGAGGTTGTTGTA	98
H2-M2	GTGCCTTGGATGGAACAGAT	TCATCCTTTGGATGGTGTGA	151
H2-M9	GGCTCTGCTTATGGGATCAG	AGGCTTTTTTCCTGCTGATGA	199
H2-Q10	AGATTGGGGAAGAGCAAGGT	TGAAGTCAACACACCCCAACAA	93
Oprk1	GGGCAATTCTCTGGTCATG	AACCAAAGCATCTGCCAAAG	246
Oprm1	CACCATCATGGCCCTCTATT	TGGTGGCAGTCTTCATTTTG	146
Oprd1	GAGGATAAGTGGGGGATGGT	AGCCTCAGCCTCCACTATGA	101
Drd1	GGACACCGAGGATGACAACT	TGGCTACGGGGGATGTAAAAG	89
Drd2	CTGGTGTGCATGGCTGTATC	TAGACGACCCAGGGCATAAC	119
Gapdh (149/150)	CACAGTCAAGGCCGAGAATG	TCTCGTGGTTCACACCCATC	242
β-Actin (151/152)	CCTGTATGCCTCTGGTCGTA	CCATCTCCTGCTCGAAGTCT	260
Hprt	ATGATCAGTCAACGGGGGA	GAGAGGTCCTTTTCACCAG	189
BAC Transgene (10-10)	CACATACGTTCCGCCATTCC	CCACTTCAACGTAACACCGC	314
Phlda2 KO	GCTTCAAGCAATGGGTAAGG	TCCAGTGATGGAGGTTGTCA	187

Table 2.5: qPCR Reagents

Reagents	Volume per reaction (µl)
H_2O	1048
10X Buffer	192
25 mM $MgCl_2$	154
dNTPs	39
Sybr Green	15.4
Taq	12.3
Template	4

qPCR - Method 2

Each primer designed and used for this method was made up to an initial concentration of 100µM, to be used as stock. Then aliquots of 3 concentrations for each primer were made up: 50, 300 and 700nM. These different concentrations of primers were then used in 9 combinations and tested on pooled cDNA in order to work out the optimum concentration graphically for the forward and reverse primers for a given primer set (**Table** 2.4).

Once primers were optimised a 96 well disk was set up using a Corbett Robotics CAS-1200™ (Qiagen) machine to contain triplicates of each sample and NTC, along with the master mix seen in **Table** 2.6. The ring contained genes of interest and two housekeeping genes run for 40+ qPCR cycles in a Rotor-Gene 6000 (Qiagen).

Table 2.6: qPCR Reagents

Reagents	Volume per reaction (µl)
H_2O	up to 20
2X SensiMix SYBR No-Rox (Bioline,UK)	10
Forward Primer	1.4
Reverse Primer	1.4
Template	5

2.3.9 BrdU Injection

The cohorts of WT(WT), WT(TG) and WT(KO) dams were weighed in the morning of E16.5 and injected intra-peritoneally at 20 minute intervals with BrdU Cell

Proliferation Reagent (GE Life Sciences), 1 µl/g. Each dam was carefully monitored in their home cage for 5-10 minutes after being injected before being left for 3 hours to allow for the BrdU to be incorporated.

Anaesthesia and Surgery

The animal was weighed and the appropriate dose of euthatol administered intraperitoneally. Once the mouse was unresponsive to these stimuli the mouse was placed securely onto a grid above a collection dish for the excess fixative and buffer as well as other bodily fluids. The procedure itself was performed inside a chemical fume cupboard. An incision was made through the skin of the mouse and the entire ribcage and thoracic region was exposed the diaphragm was separated from the chest wall at the base of the ribcage. At this point two separate lateral cuts were performed dorsally creating a ribcage flap. This flap was then opened up to expose the heart and secured.

The right atrium was cut and then left ventricle was punctured carefully using a blunted 24G needle (**Figure** 2.8). The animal was perfused, initially with PBS (20-35 ml) followed by 10% formalin fixative (20-35 ml).

Brain Dissection

The mouse was then decapitated at the base of the skull. Using a series of cuts the dorsal skull was removed from the surface of the brain. The head was then inverted and the brain was gently teased out, after excess connective tissue was cut (**Figure** 2.9). The brain was transferred into a vial of 10% PFA before being stored at 4°C. After 24 hours the brain was washed twice in 1 x PBS before being sent for sectioning.

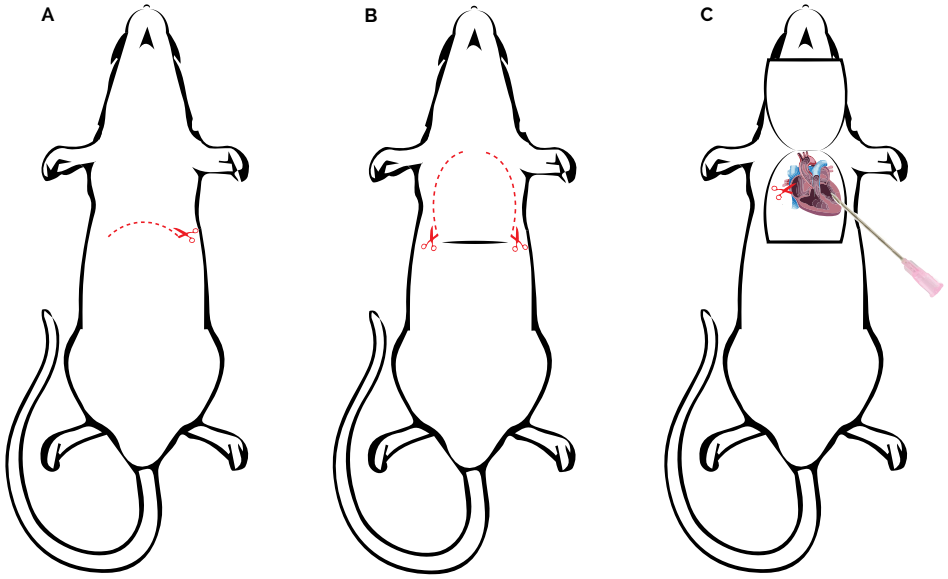


Figure 2.8: Perfusion Fixation Procedure. A basic schematic depicting how to perform trans-cardiac perfusion fixation on a terminally anaesthetised mouse.

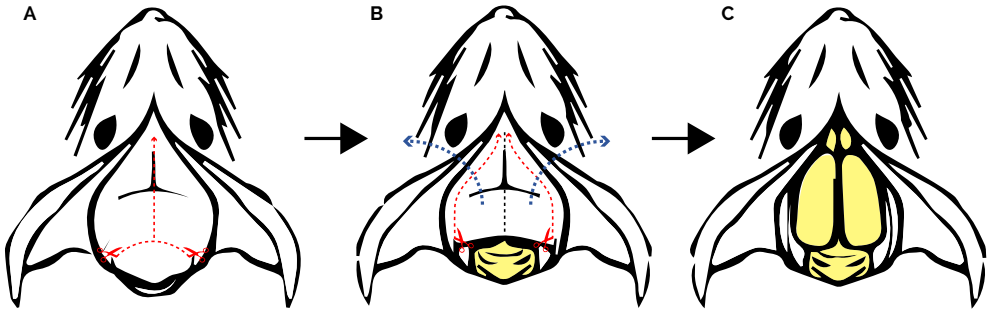


Figure 2.9: Brain Dissection. A basic schematic depicting the process of removing an intact adult brain from a mouse skull.

2.3.10 Immunohistochemistry

Sections were produced by Cardiff University's School of Biosciences Histology facility. Sections were sliced 10 µm thick coronally. 100 sections were taken for each brain around the regions of interest (Bregma to -2). 3 sections mounted per slide. All slides were processed in the same way over a three day protocol.

Slides were de-waxed by carrying out 2 x 10 minute washes in 100% xylene. The slides were then rehydrated by washing them in decreasing concentrations of ethanol

Table 2.7.

Table 2.7: Ethanol Washes

Ethanol Concentration	Number and time of each Wash
100%	2 x 5 mins
95%	1 x 5 mins
70%	1 x 5 mins

The slides were then washed in distilled water before antigen retrieval and unmasking. Diluted 10 mM citrate buffer was preheated in a 90 - 95°C water bath. The slides were then placed in the preheated solution for 20 minutes before being allowed to cool for 30 minutes at room temperature in the solution. The slides were then cycled through three washes of 1 x PBS/T for 5 minutes each.

To prevent endogenous staining slides were blocked for 20 minutes with Envision+ peroxidase block (200 µl per slide) and placed in a dish contain a damp tissue to prevent the slides from drying out. Slides were then washed in 1 x PBS/T wash cycle of 3 x 5 minutes before being blocked in 1% BSA diluted in 1 x PBS/T (200 µl per slide) at room temperature.

At this stage excess solution is shaken off before the primary antibody is added. The antibodies used were either a 1:150 dilution of mouse anti-BrdU (BD, cat no: 347580) or a 1:1000 Nestin antibody (Abcam, cat no: ab6142) or a 1:1000 DCX antibody (Abcam, cat no: ab18723) diluted in 1% BSA in PBS/T overnight at 4°C.

The following morning the slides were washed in PBS/T 3 times before the secondary antibody is added (DAKO Envision+ anti-mouse-HRP-polymer secondary for BrdU and DCX or DAKO Envision+ anti-rabbit-HRP-polymer secondary for Nestin) for 1 hour at RT and then washed with PBS/T 3 more times.

DAB is made up and applied to each of the slides. The DAB reagents were made up by mixing one drop of chromagen per 1 ml of substrate buffer (200 µl per slide, i.e. 1 ml will do 5 slides). Once applied to each slide, slides were monitored for the development of colour (between 5-10 mins). The final PBS/T series of washes is

then carried out before slides were transferred to distilled water.

After 5 minutes the water was poured off and the slides counterstained using Haematoxylin for 45 seconds and then washed under a running tap until the water runs clear. The final stage of the protocol requires the dehydration of the slides in ethanol by a series of washes listed in **Table** 2.8 followed by 2 x 2 minute washes in xylene.

Table 2.8: Ethanol Washes

Ethanol Concentration	Number and time of each Wash
70%	1 x 30 secs
95%	1 x 30 secs
100%	2 x 30 secs

The slides were then mounted in DPX and left to dry overnight.

2.3.11 SLIDE SCANNING AND CELL COUNTING

Slides were scanned at high resolution and stored in a digital format using the Carl Zeiss Axio Scan.Z1. Blind cell counts were performed on Zen Blue Software (Carl Zeiss). The equipment was calibrated using manual focussing for the coarse focus and fine focus settings before automating the process (**Figure** 2.10).

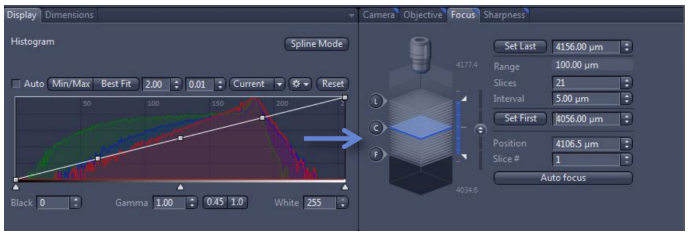


Figure 2.10: Axioscan Focus Settings. Screenshot indicating the basic focus settings used to scan the slides through the Zeiss Axio Scan.Z1.

Images were converted from .czi files into .tiff files using either the Zen Blue Software (Carl Zeiss) or CZItoTIFF Convertor open source software (`http://hcbi.fas.harvard.edu/resourcessoftware`). Total cell counts were produced manually using Fiji imaging software.

2.3.12 High Performance Liquid Chromatography (HPLC)

Tissue from the hypothalamus, hippocampus, olfactory bulb and frontal cortex was rapidly frozen using dry ice after being dissected from the brains of the dams at E16.5. This tissue was then homogenised in 200 µl of perchloric acid and an ultrasonic cell distributor (Microson, UK) and then sent to the Psychology Analytical Laboratory (Cambridge University, UK), for processing.

2.3.13 ELISA

Prolactin - abcam®, UK (ab100736)

All materials and reagents were equilibrated to RT. Then 100 µl of each standard and sample was added into wells on the plate in duplicate. Wells were covered and incubated for 2.5hrs at RT. The solution in the wells was discarded and washed 4 times with 300 µl of 1X Wash Solution. Care was taken to completely remove all of the liquid before 100µl of Biotinylated Prolactin Selection Antibody was added to each well and incubated for 1hr at RT with gentle shaking. Following this the solution was once again discarded and wash with 300 µl of 1X Wash Solution again. Then 100 µl of the provided 1X HRP-Streptavidin solution was added to the individual wells before being incubated for 45mins at RT with gentle shaking. After another wash step, as previously described, 100 µl of TMB One-Step Substrate Reagent was put into each well then after another incubation, this time for 30 minutes at RT in the dark with gentle shaking; 50 µl of stop solution was added to each reaction. The plate was then read immediately at 450nm and the results analysed.

Placental Lactogens - NovateinBio

All reagents were prepared as per manufacturer's instructions and then brought to RT along with the other components and samples. Sample diluent was added at 50 µl to the standard wells in duplicate in order to act as the zero standard before the pre-prepared standards were gently shaken and added in order (S1-S6) to corresponding wells. Then 50 µl of samples were added to the sample wells. The conjugate was then added to each well at 100 µl and mixed thoroughly. The plate was then covered and incubated at 37°C for 1 hr. Once the incubation period was complete the plate was manually washed for a total of five washes, taking extreme care to thoroughly dry the the plate after the washes. 50 µl of Chromagen Solution A and 50 µl of Chromagen Solution B was pipetted into each well and then the plate was covered and incubated for 15 minutes in the dark before 50 µl of stop solution was added to each well and care taken to mix thoroughly. The plates optical density (OD) was then immediately read at 450 nm on a microtiter plate reader.

Prolactin-8A8 - Wuhan

Constituent reagents provided were stored as per manufacturer's instructions while not is use. Materials not supplied but that were required were prepared whilst reagents and samples were brought to RT. When at RT reagents that needed further preparation were sorted. This included taking care to ensure that any crystals formed in the wash buffer concentrate were fully dissolved before the 30 ml wash buffer concentrate was diluted up to a 750 ml with distilled water. Standard was reconstituted with 1 ml of sample diluent forming a concentration of 5000 pg/ml. The standard sat for 15 minutes before serial dilutions were made using 500 µl each time. Detection reagents A and B were diluted using Assay Diluent A and B at 1:100 respectively.

Firstly 50 µl of standards, blanks and samples were added to the appropriate wells. Then immediately 50 µl of detection A was added to each well before being sealed and mixed. At this point the first incubation was performed at 37°C for 1 hour. After the first incubation the wells were aspirated 3 times using 400 µl of the wash solution leaving for two minutes then removing the liquid. The second stage involved adding 100 µl of detection reagent B before also being sealed and incubated for a further 45 minutes at 37°C. After 45 minutes the second wash was performed as previously explained. In the third phase 90 µl of substrate solution was added to the wells before sealing and mixing. Then the last incubation of between 15-30 minutes was carried out in the dark at 37°C. Then 50 µl of solution was added to each well and mixed thoroughly before being read straight away at 450 nm to obtain the optical densities of each well.

2.3.14 Statistical Analysis

qPCR data was run in triplicate and the fold change of each gene was calculated using the $2-^{\Delta\Delta C_T}$ method described by (Schmittgen and Livak, 2008). Each qPCR assay had its mean C_T calculated then the ΔC_T also determined by subtracting the mean C_T for the reference genes from the mean C_T of each gene of interest. Each of the ΔC_T values had a Shapiro-Wilk test for normality performed upon them. Following this $\Delta\Delta C_T$ for each cohort was calculated by subtracting the ΔC_T of the WTs from the transgenic lines. Finally the $2-^{\Delta\Delta C_T}$ was calculated as follows:

$$2-^{[C_T \ x \ \text{GOI} \ - \ C_T \ x \ \beta-Actin] \ - \ [C_T \ WT \ GOI \ - \ C_T \ WT \ \beta-Actin]}$$

N.B $\beta - Actin$ is replaced with each reference gene and $2-^{\Delta\Delta C_T}$ calculated as well.

$\Delta\Delta C_T$ and $2-^{\Delta\Delta C_T}$ was used to visualise the data the ΔC_T was the actual data after each GOI was normalised to the housekeeping genes.

Where data was normally distributed the statistical analysis was performed using the two tailed students t-test. In the cases where data was not normally distributed the non-parametric Mann Whitney U-test was used in order to gauge statistical significance. To correct for multiple comparisons a Bonferroni corrected α was used.

HPLC and IHC data was analysed using a repeated measures ANOVA for specific brain regions, where MOLECULE or CELL COUNT was the within subject variable and GENOTYPE was the between-subjects variable. Similarly to qPCR data Bonferroni corrections were utilised and the adjusted F and values displayed.

Microarray statistical analysis was performed in R as described in section 2.3.7 using LIMMA and subsequent linear models fitted for every gene making it more appropriate for microarray data compared to ordinary ANOVA. A more in depth description of how LIMMA analysis works can be found (Smyth, 2005).

2.4 Behavioural Testing

2.4.1 Elevated Plus Maze (EPM)

The EPM is an anxiety assay that is commonly used to assess a rodent's aversion to open space and height (Komada *et al.*, 2008). The EPM was constructed of several cut perspex pieces each uniformly wrapped in white tape so that the tracking software could effectively and precisely track the dark coloured mice. The EPM used (Figure 2.11) has four arms, two were enclosed on either side with an open roof (190 x 80 x 150 mm, length x width x height), the other two arms are totally exposed on every side (175 x 78 mm, length x width). This section was the elevated by 940 mm above the floor before being evenly lit by a 60 w fluorescent bulb.

The subjects were each placed centrally on the EPM, on separate trials, facing

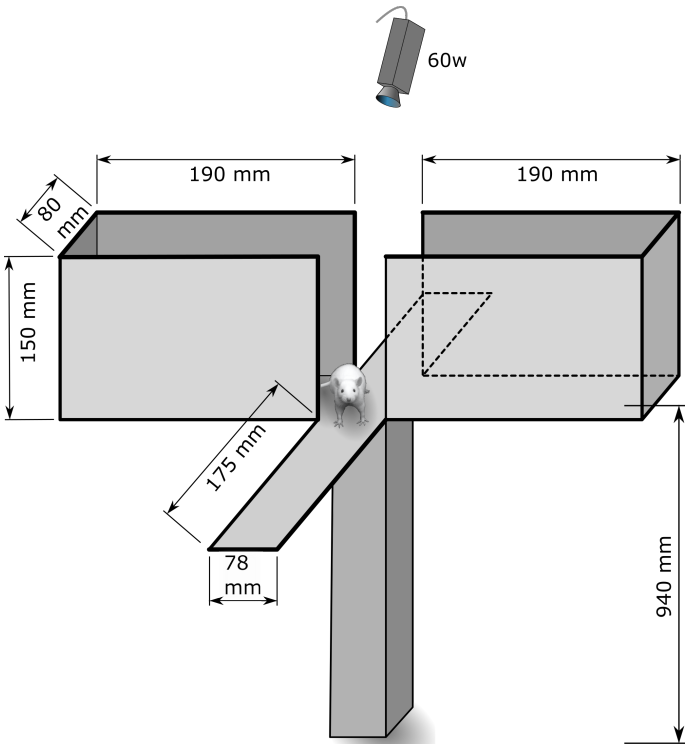


Figure 2.11: The Elevated Plus Maze. The EPM used as a simple anxiety assay for this study.

an exposed/open arm. The new dam was then left for five minutes while being recorded and tracked using Ethovision tracking software. Additional behaviours were recorded alongside the zone transitions and durations recorded by the software. These additional behaviours were: number of "Stretch Attend Postures" (SAPs) and "Head Dips" that occurred over the edge of the open arms. Zones were defined in the Ethovision software as both arms combined, thus the two open arms were defined as the "Open Zone" and the two enclosed arms are known as the "Closed Zone". Each dam was placed in the EPM on post gestational day 2 (P2) between 0900 hours and 1700 hours and given an additional 20 minutes before testing began in order to habituate to the lower light levels. Offspring were tested in a similar manner at between 6 and 8 weeks of age.

2.4.2 Open Field

Another anxiety related behavioural assay is the Open Field (OF) test (Bailey and Crawley, 2009). OF apparatus consisted of a perspex box without a lid (750 x 750 mm). The perspex used for the walls was opaque and the arena it enclosed was evenly illuminated using a 60w bulb. The arena itself was divided into two inner and outer virtual zones. The zones marked the central area of the arena (450 x 450 mm) and the external edge/periphery (300 mm). Animal subjects were released into the arena and left to explore for 20 minutes (1200 seconds) whilst there movements were tracked remotely using Ethovision tracking software (Noldus, UK). Movements were subsequently interpreted in relation to distance moved, number of entries made into each zone and total time spent in each zone. The zones consisted of the outer, middle and inner zones. The outer zone is the least anxiogenic whilst the inner is the most anxiogenic. The usual behaviour for mice is to show thigmotaxis and stay in the outer zone.

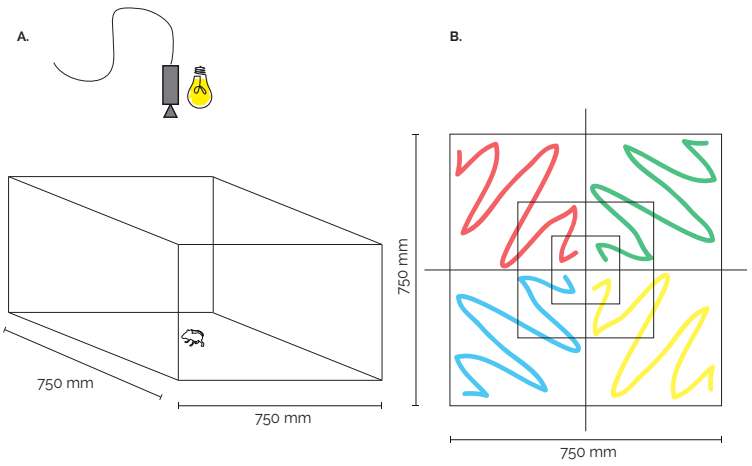


Figure 2.12: The Open Field Arena. The Open Field (OF) assay is a proxy measure of anxiety phenotypes that may be expressed by an animal (in this case mice). **A.** Is the OF arena where the mouse is placed in the centre and left to explore the area for 20 minutes or 1200 seconds. **B.** Basic zones used by the Ethovision tracking software to determine the location of the mouse and the distance travelled. The outer, middle and inner zones.

2.4.3 Phenotyper Boxes

To allow for the continuous monitoring of the dam and her pups each dam was relocated on P2 from their home cages to a specific unit, the Phenotyper Cage (Noldus). Each Phenotyper was stocked with clean fresh water and food, like any normal home cage. However the major difference was the special top unit. The specifically designed top unit contained a camera that allows for the continuous recording of each dam and her behaviour with her pups over a set period. These videos could then later be retrospectively analysed using the Ethovision XT tracking software package to assess various parameters. There were four Phenotyper boxes in total, each measuring 30 x 30 cm containing an exercise wheel and sawdust (**Figure** 2.13). Once the dams had been moved in, their weights, the pup weights and also the food and water weights were recorded. After an initial 24 hour habituation period to their new homes, which had identical 12 hour light and dark cycles to their previous homes (between 0800 and 2000 hours) with controlled temperature and humidity.

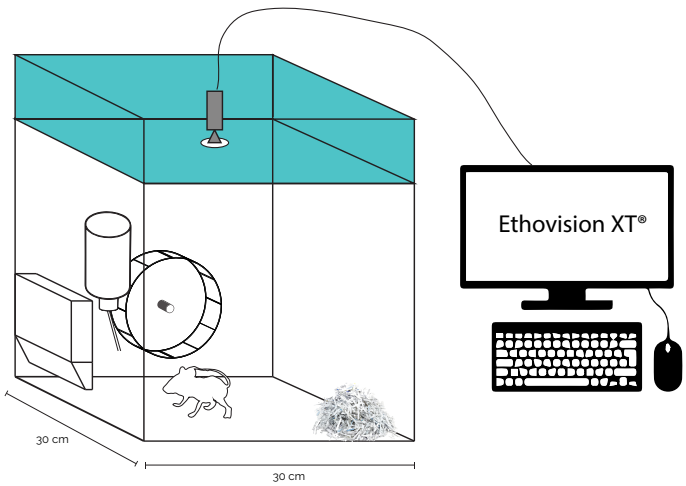


Figure 2.13: Phenotyper Box. A diagram showing one of the phenotyper boxes that was used in conjunction with ethovision tracking software to monitor the dams over a 3 day period up to 24 hours at a time.

2.4.4 Pup Retrieval

After the 24 hour habituation period in the Phenotypers the first behavioural assay was performed on P3. The pup retrieval test is a simple assay that measures the latency it takes for a dam to collect her pups when they are removed from the nest and scattered either in a set location or randomly around the cage/arena. In order to carry out this test on the dams, they were each temporarily removed from the Phenotypers and placed back into their original home cages. Then the pups were evenly scattered along the opposite side of the cage to the nest in the Phenotyper.

The video recorder was then started and the dams re-introduced. The pups were checked every half an hour and returned to the nest after one hour if not collected by this time. At the end of the test period in the Phenotyper of 24 hours the video recording was removed and analysed for each dam. The latency to sniff the pups and the latency to collect the first pup and return it to the nest was recorded (**Figure** 2.14).

2.4.5 Ethovision - 1 hr and 23 Hour Monitoring

Immediately following the nest building assay, the video recorder was set to record for 23 hours continuously until the following morning before the nest building assay was performed. The videos themselves were analysed *post hoc* in a methodical manner using pre-determined and pre-tested parameters, however in order to check the reliability of the software, a random selection of videos were manually analysed.

The programme was set up in the following way:

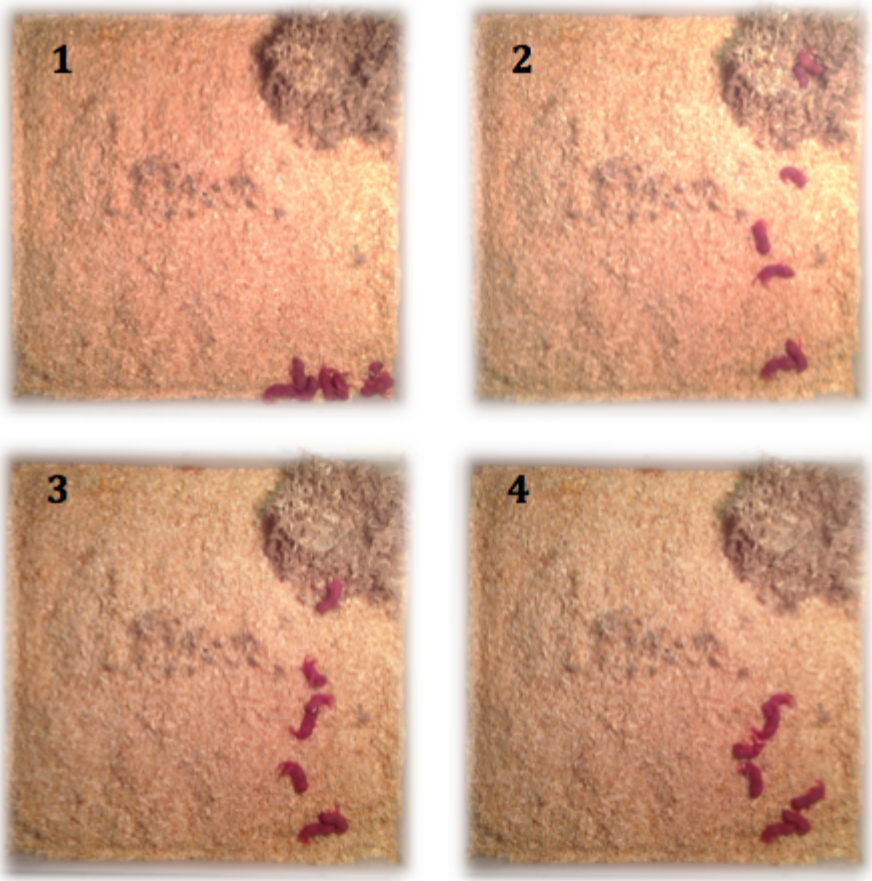


Figure 2.14: Pup Retrieval Set Up. 1. Schematic illustrating how the pups are arranged during the pup retrieval test **2, 3 and 4**. The varying stages of retrieval, **2.** show pups having been retrieved, although the time is stopped after just one pup is placed inside the nest. In **3.** it can be seen that the pup has been taken to the edge of the nest but not into the centre of it yet, this does not count as having been retrieved. Picture **4.** gives an example of a failed retrieval by the dam after the designated time period.

Arena settings

These were defined to match the Phenotyper cage's size and dimensions. Once this was completed it was possible to "grab" different zones of specific shapes and also define quadrant zones using various tools provided in the GUI environment. At this point it was necessary to calibrate the scale by measuring the size of the arena or zone areas. Once the arena settings are validated it is possible to move on to setting up the trial control instructions.

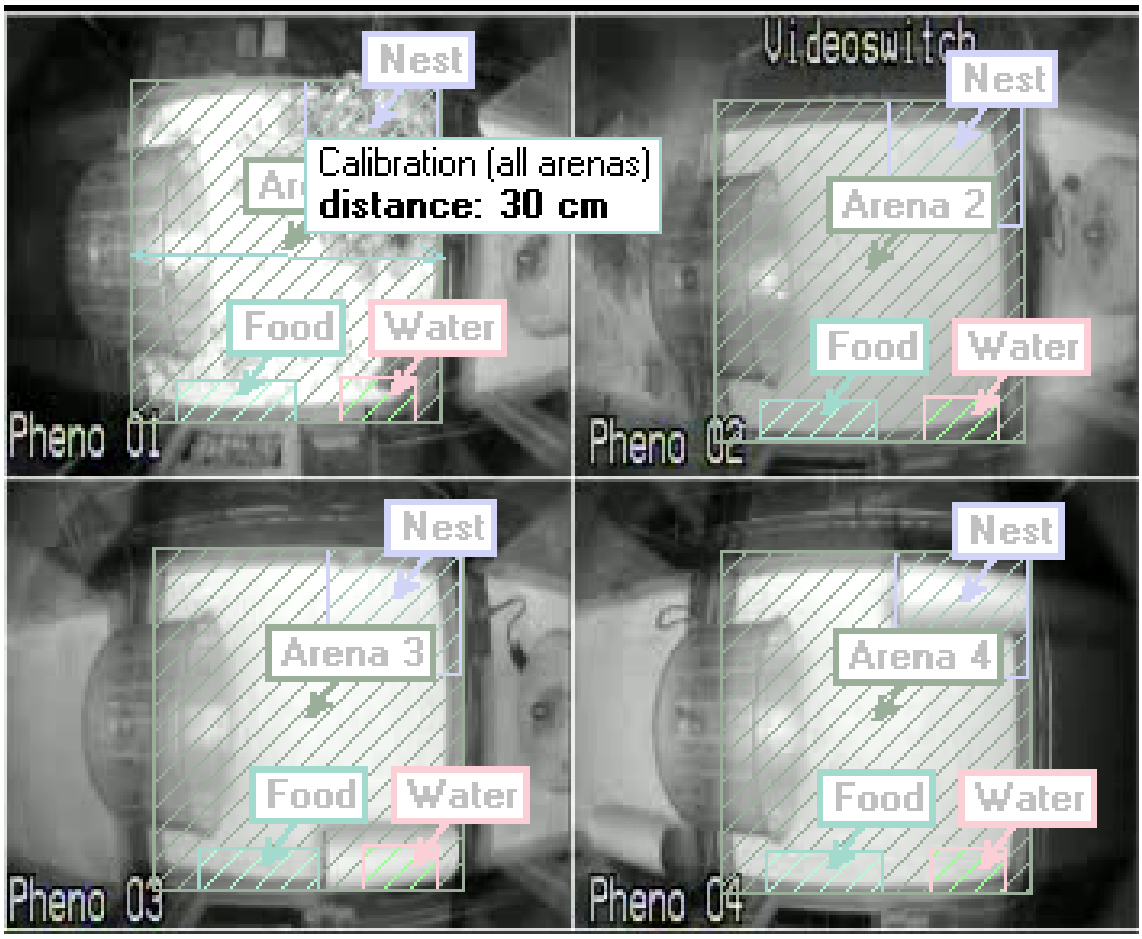


Figure 2.15: Ethovision Arena Layout and Settings. A screen grab of the areas defined in the Ethovision analysis software program for each arena (Phenotyper Cage). These areas were calibrated using a scale, then used to help track the movements of the subjects throughout the testing period.

Trial control settings

These are typically useful for controlling external hardware. However on a more fundamental level it is critical for defining when a trial starts and when a trial ends. In this sense it was possible for conditions to be made that told the programme when to start recording/tracking and when to stop.

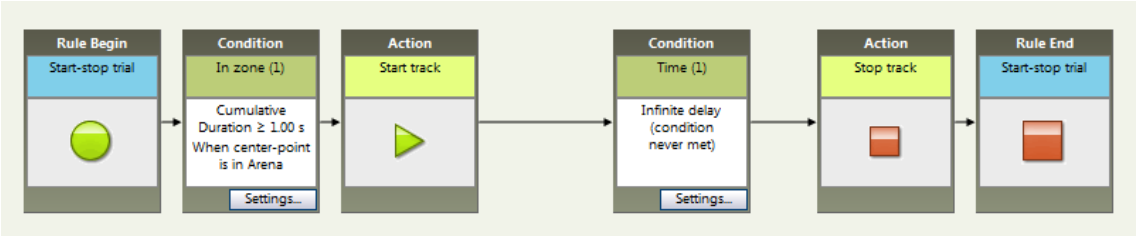


Figure 2.16: Ethovision Trial Control Settings. This demonstrates the typical control flow of a trial. It defines the start as when movement takes place for longer than 1 second and the end, which is the duration of the trial.

Detection settings

The critical settings for analysis as defining the correct parameters in this section are important in order refine the method by which the animal will be tracked. On occasion this would need to be tweaked for each individual animal and/or trial in order to ensure a good and reliable track. The standard parameters used can be seen in **Table** 2.9.

Table 2.9: Detection Parameters

Parameter Name	Definition
Method	Differencing/Dynamic Subtraction
Sample Rate	12.5 per second
Subject	Darker (than background)
Dark Contrast	40-255
Subject Size	200-12500
Contour Erosion	Yes (1 pixel)
Contour Dilation	Yes (1 pixel)

Hidden Zones

In order to combat the issue of poor tracking in certain areas of the Phenotyper cage, like underneath the exercise wheel or within the nest, hidden zones were created. This is were an animal/subject enters a zone in which its detectable body point is no longer detected. During tracking if the animal is no longer detected it is assumed to have entered this hidden zone. When there is more than one hidden zone in an arena then entry an exit zones can be created. These were used so as to reduce the risk of an overestimation of entrances into and exits from hidden zones and the inevitable issues such a result would have on the other readings.

2.4.6 Nest Building

The protocol used in this study to assess nest building behaviour was a simple rudimental scale of: 0 (no nest built), 1 (nest built but pups not placed in the nest) or 2 (nest built and pups placed inside the nest). On P4 after 23 hours into the tracked Phenotyper activity, the recording was paused and the dams removed and replaced into their respective home cages once more. The nest was then removed from the Phenotyper cage and replaced with a cardboard tube filled with a 30 x 30 cm strip of tissue paper. The pups were placed next to this nesting material and the dams were then put back into each cage and recording resumed for 1 hour. After the hour the mice were returned permanently to their home cages and the weights of the dams and pups recorded (**Figure** 2.17).

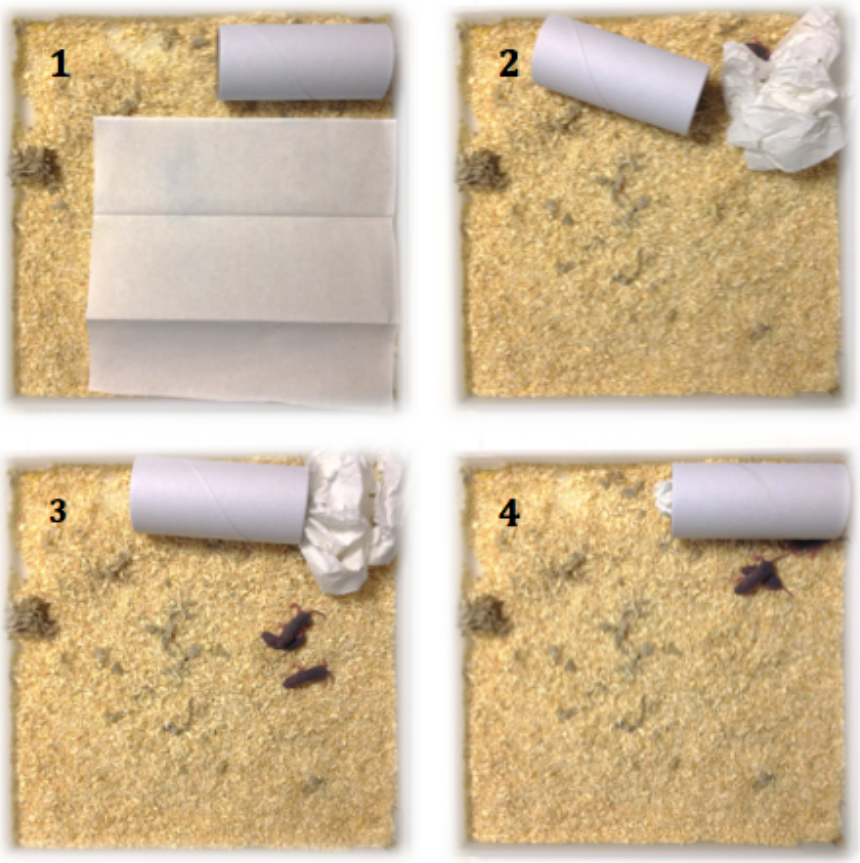


Figure 2.17: Nest Building Set Up. 1. Apparatus used for the nest building test including a cardboard tube and a piece of tissue paper. **2.** Scenario 1: In this picture you can see that the dam has built a nest and moved her pups inside the nest, this was scored as "2" **3.** Scenario 2: Dam has built a nest but pups not placed in the nest, scored as "1". **4.** Scenario 3: Dam doesn't build a nest within the given time frame, scored as "0".

Alongside the nest building task itself and the Ethovision tracking of the dams throughout this assay, manual scoring of a select number of key grooming and nurturing behaviours were recorded (**Table** 2.10). These were recorded manually as at the time the tracking software was not able to do this accurately. The number or frequency of occurrences of each behaviour was recorded and the total duration of time spent performing the behaviour in question was recorded.

Table 2.10: Manually Scored Nest Building/Grooming Behaviours

Behaviour	Behaviour Description
Crouched Nursing	Dam is positioned over the pups to permit sucking or thermoregulation with a low to moderate arch in her back.
Arched Nursing	Dam is positioned over the pups with a high arch in her back to permit sucking and pup movement.
Passive nursing	Dam is lying on her side with her ventrum exposed to the sucking pups.
Licking/grooming Pups	Dam is licking pups (anogenital or body region).
Nest-building	Dam is picking up pieces of bedding and retrieving these to the nest or moving bedding in the nest with her snout.
Self-grooming	Dam is licking herself (often occurs during bouts of pup licking).

2.4.7 Locomotor Activity

Locomotor Activity (LMA) behaviour of the offspring was assessed using 12 perspex chambers (210 x 360 x 200 mm), these chambers each contained two infra red beams that crossed the cages 30 mm from each end and 10 mm from the floor of the chamber boxes. The number of beam breaks was indicative of varying degrees of activity. The number of breaks was recorded by customised software BBC Basic V6 programming and the ARACHNID interface (Cambridge Cognition Ltd, UK). LMA was carried out over a 2 hour period each day over the course of three days, the 2 hour period was consequently reduced to 5 minute bins (**Figure** 2.18). The final measure of activity was the total number of beam breaks over the whole test period and within each bin and gave an indication of the animals ability to habituate to a novel environment.

2.4.8 Acoustic Startle and Pre-pulse Inhibition

Acoustic Startle Response (ASR) and Pre-Pulse Inhibition (PPI) were monitored using equipment re-designed for mice by SR-Labs. Both tests occurred in the same

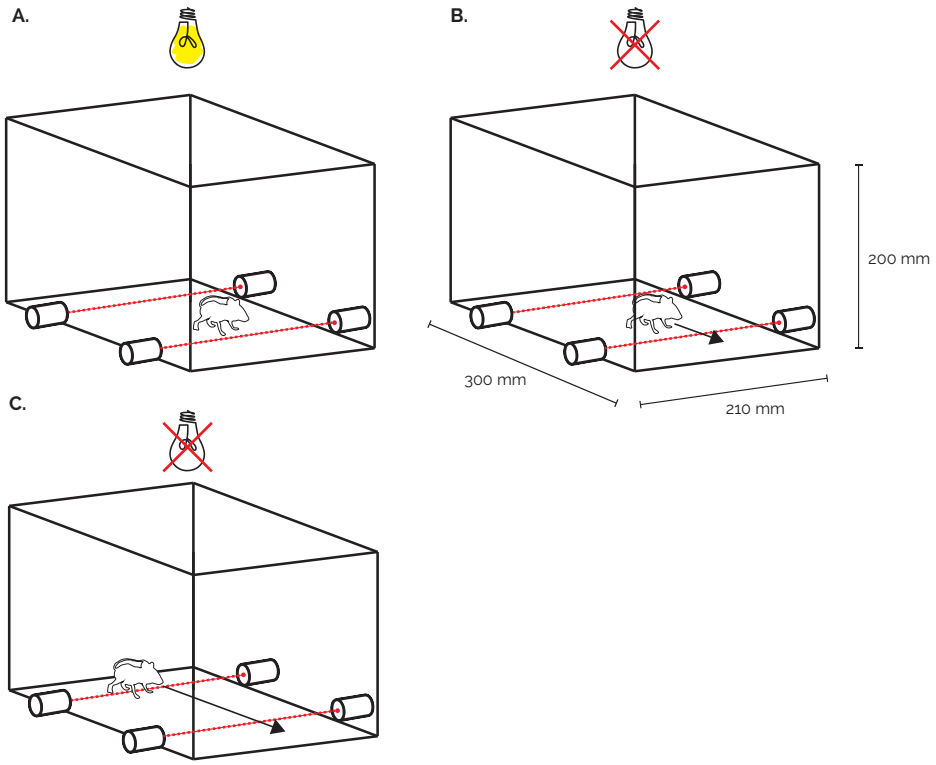


Figure 2.18: Locomotor Activity Boxes. Schematic diagram showing the experimental set up for the locomotor activity boxes. **A.** Shows the mouse being placed into one of the perspex locomotor activity boxes with the lights on. **B.** Shows the mouse demonstrating a "break", this is when the mouse only breaks one of the beams dissecting the box. **C.** Shows what is classified as a "run" this is when the mouse breaks both beams one after the other quickly. The test runs for 120 mins or 2 hrs and is carried out in the dark.

sound attenuating chamber with a speaker fitted to the roof 120 mm above the animal. A perspex tube with a diameter of 35 mm housed each animal at the start of the test, mounted on a perspex plinth (**Figure** 2.19).

The ASR assay was performed after 5 minutes of habituation for each animal in their new surroundings, then the test began. The test consisted of 3 blocks of differing amplitude startles, each set to a background white noise of 70 db. The first block had an amplitude of 120 db followed by 105 db in the second and finally 80 - 120 db in the third.

The procedure for the PPI test was to carry out pulse alone trials and pre-pulse trials on each of the animals. The pulse alone trials consisted of a 40 ms startle noise

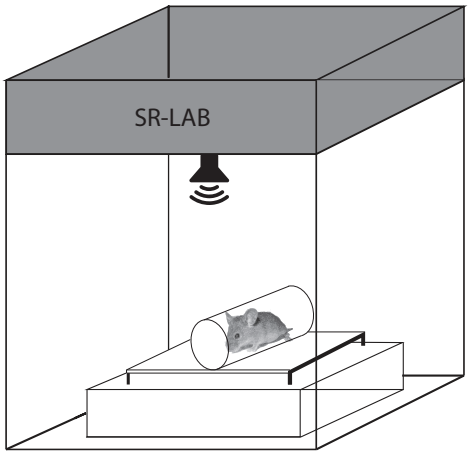


Figure 2.19: Pre-pulse Inhibition and Acoustic Startle Response Apparatus. Schematic diagram showing the ASR and PPI equipment set-up

of 120 db. In contrast the PPI tests started with a short 20 ms acoustic pulse of 4, 8 and 16 db above the baseline background noise (70 db) followed by a 70 ms break and then the usual 40 ms startle stimulus of 120 db.

Each block consisted of 5 single pulse trials, followed by 1 no stimulus trial and the 6 pre-pulse trials, two for each of the various pre-determined pre-pulse startles (4,6 and 8 db more than the baseline). The stimuli themselves were presented pseudorandomly throughout every block.

ASR was quantified using a piezoelectric transducer setup linked to a computer. It was recorded from the pulse alone trials and the inhibition to responding to pre-pulse noise in a 65 ms window after each startle stimuli. Similarly PPI was deduced by calculating the percentage reduction in the startle response between the pre-pulse and pulse alone trials.

2.4.9 Lick Cluster Analysis

The Lick Cluster Analysis (LCA) experimental apparatus was custom made and set up in an isolated room. The apparatus consisted of 16 automated drinking chambers (Med Associated, St Albans, VT, USA) measuring (32 x 15 x 12 cm, L x W x H), each with a steel mesh floor and white acrylic sides (**Figure** 2.20). Mice were food restricted for 16 hours overnight before each test (17:00 to 09:00 hrs), care was taken to weigh each mouse before and after food restriction weight loss greater than 15% meant the mouse was excluded from the test. After the food restriction subjects were placed into the test cages and left for 20 minutes to drink the available sucrose solution through stainless steel drinking spouts connected to 50 ml falcon tubes.

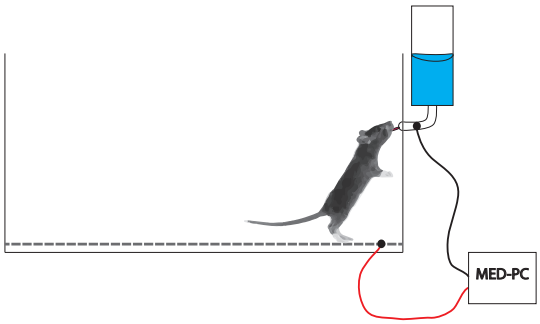


Figure 2.20: Lick Cluster Analysis Equipment. Schematic diagram showing the Lick Cluster Analysis equipment set-up

The number of licks and the time of each lick was recorded using a contact sensitive lick-o-meter attached to a microcomputer running MED-PC software (Med Associates) that registered the time of each lick to the nearest 0.01 second. Lick clusters were defined as a group of licks with lick intervals didn't exceed 500 ms. Thus licks that occurred after a 500 ms period were recorded as a new cluster. Mice were trained over two solutions (8% and 12% sucrose) before being tested. This was to ensure a sufficient baseline level of licks were being performed and to check the equipment was working correctly. The two differing concentrations of sucrose solution used for the test period were 4% and 16% made up with deionised water.

A second task was performed for one week following the sucrose test, in which saccharin was used to create a 0.1% solution. This test was carried out for one week only in order to discern whether there was any possibility of calorie seeking behaviour in the mice undergoing the testing.

2.4.10 Statistical Analysis

The experimental data for the all the behavioural tests were analysed using SPSS Version 23 (SPSS, USA) unless otherwise stated. All behavioural data was presented as mean values with the standard error of the mean (± SEM) also displayed. Each behavioural test was analysed separately according to genotype, followed by any appropriate comparisons between genotypes or across genotypes. This was done using separate ANOVAs for various between-subject factor of genotype and within subject-factors.

Maternal and Offspring Behavioural Tests

All data analysed using repeated measures ANOVA's was tested using Mauchly's test of sphericity in order to check that the variances of the differences between all combinations of related groups are equal. In any case where sphericity was violated, indicating significant differences between variances within related groups, the degrees of freedom was adjusted appropriately using the Greenhouse-Geisser adjustment procedure.

EPM and OF assays where analysed with one-way ANOVAs in order to determine the effect of genotype on various behaviours or factors during the test. The factors were: Time spent in open/closed/middle zones of the EPM and Time spent in outer/middle/inner/central zones of the OF arena. Several more ANOVAs were also performed to analyse differences between factors including distance moved in each

zone and overall for the EPM and OF tasks. Furthermore preference for the specific zone (closed and outer) in both the EPM and OF tasks, respectively, that are known to be the least anxiogenic were determined through the use of a paired sample t-test.

Specific Offspring Behavioural Tests

LMA habituation trial data was assessed using a number of repeated measures ANOVAs for each session. DAY or BIN was the within-subjects factor and Genotype was the between session factor.

Similarly ASR and PPI data was analysed using repeated measures ANOVAs with either TRIAL or INTENSITY as the within-subject factor and Genotype as the between subjects factor.

LCA analysis was normalised then analysed through a series of General Linear Models (GLMs) for within-subjects factors: Total Consumption, Total Licks, Licks per Bout, Average Inter-Lick Interval and Volume. These factors were then further screened using a number of ANOVAs to look at between-subject effects of Genotype upon the previously listed within-subject factors.

Significant tests were presented at an alpha level of 0.05. The results that were found to be significant through the main ANOVA were then further analysed using post-hoc tests and pair wise comparisons and these findings reported. Bonferroni corrections were used for multiple comparisons when needed and Greenhouse-Geisser degrees of freedom corrections also applied to repeated measure factors.

Placental Endocrine Lineage Analysis

3

3.1 Overview

Loss of function of several maternally expressed imprinted genes results in an expansion of the SpT lineage (John, 2013). This pattern suggests that the paternal genome has selectively silenced genes that limit SpT specific functions. There are a number of mouse mutants in which alterations in the SpT lineage have been reported, however, these defects often occur in parallel to alterations in other placental lineages. In particular, the glycogen cell lineage and four of the six distinct TGC lineages, all of which share a common progenitor with the SpT (John, 2013) confounding their functional assessment (Chapter 1).

As previously discussed, the loss-of-function of *Phlda2* results in an enlarged placenta, an expanded junctional zone and more placental glycogen but without affecting fetal growth (Frank *et al.*, 2002). When the expression is elevated to two-fold the endogenous level, the effect is placental stunting, with the loss of the SpT lineage and significantly reduced placental glycogen accumulation. The latter occurs without any changes in the representation of the glycogen cell lineage or the parietal TGCs which line the maternal decidua (Salas *et al.*, 2004, Tunster *et al.*, 2010). The main observation regarding elevated *Phlda2*, was that it drives a late, asymmetric fetal growth restriction (Tunster *et al.*, 2014). When compiled the data suggested that *Phlda2* acts indirectly to restrict fetal growth through the limitation of the SpT lineage's expansion, which is essential in order to stimulate glycogen accumulation.

The effects of increasing *Phlda2* gene dosage on all the TGC lineages has not been reported. Moreover, a characterisation of the placental lineages in the context of loss-of-function has not been performed.

This chapter sought to investigate the role of *Phlda2* in regulating the placental endocrine lineages. The data entitled "The imprinted gene *Phlda2* gene modulates a major endocrine compartment of the placenta to regulate placental demands for maternal resources" was published in *Developmental Biology* by Tunster, Creeth and John (2015). In the paper we also looked at the role *Phlda2* has in glycogen accumulation and fetal growth.

The experiment focussed on performing an in depth examination of the placental lineages in the different *Phlda2* gene dosage mouse models. qPCR and DNA Microarray was used to assess the expression levels of the key genes that code for the endocrine proteins/hormones that are known to be expressed in the various lineages of the placenta.

3.1.1 Placental qPCR

The initial qPCR was performed by Simon Tunster on E14.5 placenta, it indicated that there were several Prolactin-like Proteins (*Prls*) that were elevated in the KO placenta and reduced in the TG placenta (**Figure** 1.8). The SpT is also known to be a source of Placental specific glycoproteins (*Psgs*). *PSGS* in humans function as immunomodulators by inducing secretion of IL-10, IL-6 and TGF-β 1 by monocytes (Snyder *et al.*, 2001). They have similarly been linked by several studies in having immunoregulatory, pro-angiogenic, and anti-platelet functions (Moore and Dveksler, 2014, Wu *et al.*, 2008a).

The most abundant *Psgs* expressed during gestation are: *Psg17*, *Psg18*, *Psg19* and *Psg21* all of which were tested using qPCR and showed distinct increases in

expression levels for the KO placenta and reduced expression levels in the TG placenta (**Figure** 1.8). This combined with *in situ* hybridisation data of *Psg17* (**Figure** 1.8) revealed a reciprocal relationship between *Phlda2* and the *Psgs*. The work done by Simon Tunster also looked at the double transgenic placenta that carried both the BAC transgene and also the maternal *Phlda2* allele (*Phlda2* $^{-/+\ \mathrm{BACx1}}$ (rescue; 1X)), meant that these changes could be solely attributed to *Phlda2*. This data was then substantiated through the use of microarray to look at the changes in gene expression profiles at E16.5.

Finally due to the different sizes of the SpT, a key endocrine lineage, across the three cohorts and the published data regarding qPCR gene expression levels of the *Prls*. It was hypothesised that the levels of the Prls and prolactin in the blood of the dams will show differing concentration levels. To test this ELISAs were performed upon the maternal blood serum of a newly generated cohort of dams in order to determine levels of specific Prls and prolactin in their system.

3.2 METHODS

RNA was extracted from whole placenta at E14.5 for qPCR and E16.5 for microarray using the extraction method described in Chapter 2 and section 2.3.4. The RNA for microarray were all sent to Cardiff Biotechnology Service (CBS) for quality control before finally undergoing microarray. RNA was only used if it had a RNA Integrity Number (RIN) value equal to or higher than 7 (**Table** 3.1).

3.2.1 MICROARRAY

RNA was hybridised to Affymetrix Mouse Gene 2.0 ST chips. The data was analysed essentially as described in chapter 2 and section 2.3.7 and also outlined by

Zhang *et al.* (2009). Additionally the use of Partek® Genomics Suite™ Version 6.6 (Partek Incorporated, Missouri, USA) was employed in order to generate a principle components analysis graph and validate the gene lists generated.

Finally, genes found to be either significantly up in *Phlda2* $^{-/+}$ (maternal KO; 0X) and/or significantly down in *Phlda2* $^{+/+\ BACx1}$ (single copy *Phlda2* transgene, TG; 2X) E16.5 whole placenta were tested for enrichment of gene ontology molecular function and biological process using the Database for Annotation, Visualisation and Integrated Discovery (DAVID).

3.3 RESULTS

Females were culled on E16.5 and whole placenta was taken. The yolk sacs for the fetuses were genotyped before RNA was extracted and sent for microarray. The total N = 11 with each cohort containing an n = 4. The samples were only used if they had a RIN value equal to or higher than 7 (**Table** 3.1).

Table 3.1: RNA Values for E16.5 Microarray of Placenta

Sample ID	Tissue	RNA ng/µl	RIN Value
WT_PL1	Placenta	433.0	8.4
WT_PL2	Placenta	419.0	8.3
WT_PL3	Placenta	566.0	8.8
WT_PL4	Placenta	390.0	8.3
KO_PL1	Placenta	361.0	8.3
KO_PL2	Placenta	336.0	9.3
KO_PL3	Placenta	347.0	7.5
KO_PL4	Placenta	310.0	8.3
TG_PL1	Placenta	704.0	8.7
TG_PL2	Placenta	118.0	8.0
TG_PL3	Placenta	213.0	7.0
TG_PL4	Placenta	465.0	n/a

3.3.1 Microarray

Microarray analysis was performed on E16.5 placenta to give a more objective analysis of the gene expression at this time point. A heat map was produced to indicate visually any differences in gene expression levels between the three groups using Partek™across the gene chips (**Figure** 3.1). A separate 3D principle components analysis plot clustering placental gene expression across the three cohorts for the differing placenta was also generated in a similar manner to get a better idea about the arrangement of these differences and help identify if there were any outliers (**Figure** 3.2).

The bulk of the analysis was performed at the genome wide level from the .CEL files generated from the microarray using the Bioconductor package LIMMA and custom scripts (see Chapter 5) to generate comprehensive gene lists highlighting the differentially expressed genes between all pair-wise comparisons of the groups. A venn diagram indicating the total number of differently altered genes, either up or down, between the differing KO and TG placenta at the $p\text{-}value > 0.05$ confidence level (**Figure** 3.3).

Pathway analysis was performed using the Database for Annotation, Visualisation and Integrated Discovery (DAVID). DAVID allows the user to generate molecular function and biological processes pathway data on the entire dataset highlighted significant KEGG pathway changes in genes involved in cell cycle, cytokine-cytokine interactions and sphingolipid metabolism (**Table** 3.2). The Venn diagram identifies the genes that are both significantly up in the KO and significantly down in the TG whole placenta, it is likely that these represent the SpT transcriptome. When this dataset was similarly analysed by DAVID the output highlighted 10 distinct gene clusters including: cytoskeleton pathways, cell division pathways and importantly the protein superfamily prolactin/lactogen/growth hormone cluster (Cluster 3 Enrichment Score: 4.25, **Table** 3.3).

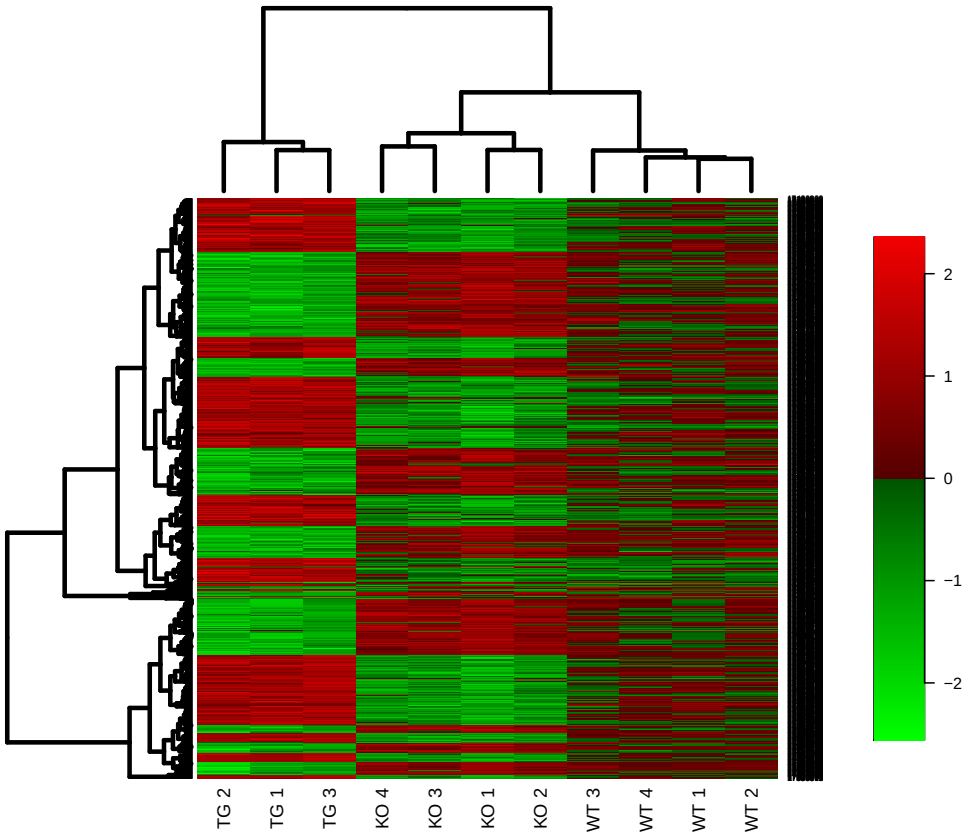


Figure 3.1: Heat map indicating expression levels of genes in the Placenta. A three way heat map showing the differing gene expression levels from the microarray across the three cohorts in the placenta. Green indicates genes that were down regulated and red represents genes that were up regulated relative to baseline.

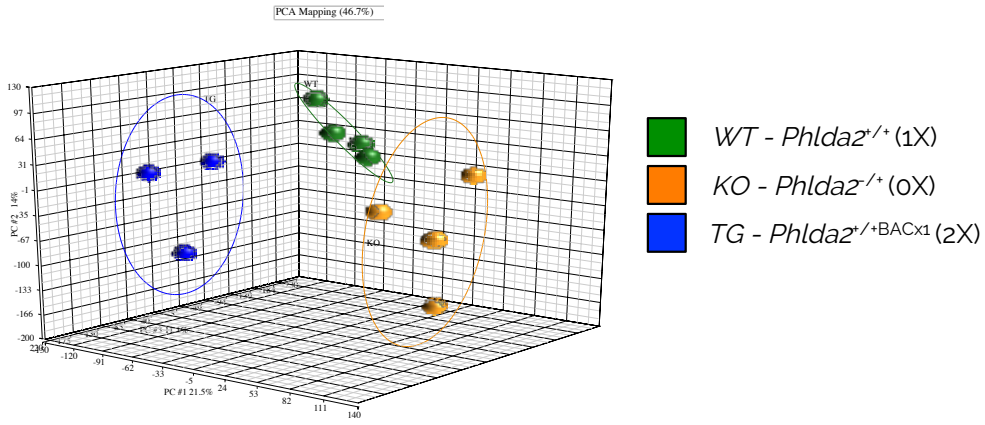


Figure 3.2: Principal Component Analysis of the Placenta. Three-dimensional principle components analysis plot clustering placental gene expression across the three cohorts for the differing placenta.

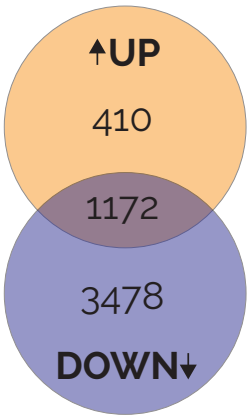


Figure 3.3: Venn Diagram for Genes that were Significantly Up or Down Regulated in the Placenta. The venn diagrams from the LIMMA analysis showing the genes that are significantly up or down between the KO and TG placenta.

Table 3.2: Placenta Signalling Pathways

Term	Count	%	*P-Value*	Genes List	Gene	Total	Pop Hits	Pop Total	Fold Enrichment	Bonferroni	Benjamini	FDR
Cell cycle	12	3.59	≤ 0.001	ENSMUSG00000030867	Plk1	97	128	5738	5.545747422680413	8.26786740754426E-4	8.26786740754426E-4	0.008755491664069748
				ENSMUSG00000032218	Ccnb2							
				ENSMUSG00000027379	Bub1							
				ENSMUSG00000005410	Mcm5							
				ENSMUSG00000026355	Mcm6							
				ENSMUSG00000019942	Cdk1							
				ENSMUSG00000020415	Pttg1							
				ENSMUSG00000059552	Trp53							
				ENSMUSG00000041859	Mcm3							
				ENSMUSG00000027715	Ccna2							
				ENSMUSG00000040084	Bub1b							
				ENSMUSG00000022285	Ywhaz							
Oocyte meiosis	9	2.70	≤ 0.001	ENSMUSG00000030867	Plk1	97	115	5738	4.6294935006723446	0.06392347569087509	0.03248952237759983	0.6968424671623286
				ENSMUSG00000032218	Ccnb1							
				ENSMUSG00000040385	Ppp1ca							
				ENSMUSG00000019773	Fbxo5							
				ENSMUSG00000027379	Bub1							
				ENSMUSG00000019942	Cdk1							
				ENSMUSG00000020415	Pttg1							
				ENSMUSG00000057897	Camk2b							
				ENSMUSG00000022285	Ywhaz							
DNA replication	5	1.50	0.003	ENSMUSG00000020471	Pold2	97	35	5738	8.450662739322533	0.24029136931372375	0.08753610974242165	2.867301122002619
				ENSMUSG00000005410	Mcm5							
				ENSMUSG00000026355	Mcm6							
				ENSMUSG00000041859	Mcm3							
				ENSMUSG00000025395	Prim1							
p53 signaling pathway	5	1.50	0.03	ENSMUSG00000022385	Gtse1	97	69	5738	4.286568056178096	0.9497057653873082	0.5264350506466848	27.130726789922477
				ENSMUSG00000032218	Ccnb1							
				ENSMUSG00000040152	Thbs1							
				ENSMUSG00000019942	Cdk1							
				ENSMUSG00000059552	Trp53							
Cytokine-cytokine receptor interaction	9	2.70	0.05	ENSMUSG00000025929	Il17a	97	244	5738	2.1819334122021297	0.9959681825435983	0.668028980517003	44.21447884480347
				ENSMUSG00000018914	Il3							
				ENSMUSG00000022971	Ifnar2							
				ENSMUSG00000073889	Il11ra1							
				ENSMUSG00000055170	Ifng							
				ENSMUSG00000027720	Il2							
				ENSMUSG00000020122	Egfr							
				ENSMUSG00000029371	Cxcl5							
				ENSMUSG00000044052	Ccr10							
Progesterone-mediated oocyte maturation	5	1.50	0.05	ENSMUSG00000030867	Plk1	97	85	5738	3.4796846573681015	0.9970074965434431	0.6203887188783928	45.9474267108672
				ENSMUSG00000032218	Ccnb2							
				ENSMUSG00000027379	Bub1							
				ENSMUSG00000019942	Cdk1							
				ENSMUSG00000027715	Ccna2							

Table 3.3: Cluster Analysis of E16.5 Placenta: Cluster 3

Gene Name	KO Fold change	KO *p-value*	TG Fold change	TG *p-value*
prolactin family 2, subfamily c, member 1	↑ 1.36	7.1x10-4	↓ 1.91	9.2x10-7
prolactin family 2, subfamily a, member 1	↑ 1.13	1.9x10-2	↓ 1.16	1.9x10-2
prolactin family 3, subfamily c, member 1	↑ 1.20	1.3x10-2	↓ 1.96	9.1x10-3
prolactin family 5, subfamily a, member 1	↑ 1.56	1.5x10-2	↓ 2.82	9.7x10-5
prolactin family 7, subfamily b, member 1	↑ 1.32	2.0x10-3	↓ 1.53	2.3x10-3
prolactin family 8, subfamily a, member 6	↑ 1.10	2.9x10-2	↓ 1.69	2.6x10-3
inhibin beta-B	↑ 1.17	3.9x10-2	↓ 1.48	2.4x10-4
interleukin 3	↑ 1.16	7.4x10-3	↓ 1.26	2.2x10-3
oncostatin M	↑ 1.10	4.8x10-2	↓ 1.10	4.8x10-2
secretin	↑ 1.17	2.4x10-2	↓ 1.35	4.3x10-4

Cluster 3 represented a large proportion of *placental prolactins \lactogens* known to be expressed within the SpT (Simmons *et al.*, 2008) and *secretin*, also highly expressed within the SpT (Knox *et al.*, 2011). When these data is combined with the qPCR data it was confirmed that *Phlda2* acts as a major rheostat for placental hormone gene expression.

3.3.2 ELISA

qPCR and microarray demonstrated changes in the expression of key placental hormone genes at both E14.5 and E16.5. In order to determine whether this translated to altered serum levels of these hormones in the blood of the dams, ELISA was used. No study had reported the use of an ELISA kit to measure specific mouse Prls but there were commercially available kits for Prl8a8 (Wuhan, China), Prl3b1 (NovateinBio, USA) and prolactin (abcam®, UK).

Placental Lactogen - (Prl8a8)

The maternal serum from three newly generated independent cohorts of WT dams carrying different fetuses with the different placentas (described in Chapter 2 section 2.2, **Figure** 2.4) was collected and then analysed in duplicate to determine serum levels of Prl8a8 using a specific Prl8a8 ELISA (Wuhan, China) (Chapter 2). There were n = 15 WT(WT), n = 8 WT(TG) and n = 9 WT(KO) serum samples. While there was an increase of around 2 ng/ml of Prl8a8 in the WT(KO) maternal serum compared to WT(WT) dams levels (**Table** 3.4 and **Figure** 3.4), the observation was not found to be statistically significant. WT(TG) maternal serum compared to WT(WT) maternal serum was not greatly different indicating that Prl8a8 levels were similar between these groups. Importantly, there was a large variation in both WT(WT) and WT(KO) samples and the absolute levels were 100 times lower than anticipated. This could be explained either due to a lack of specificity or serum quality or potentially because *Prl8a8*, despite being very highly expressed in the placenta, is not being released into the maternal circulation at E16.5.

Table 3.4: Prl8a8 ELISA

Genotype	Concentration (ng/ml)	SEM
WT(WT)	6.55	± 1.79
WT(TG)	7.61	± 0.63
WT(KO)	8.19	± 1.58

Placental Lactogen - (Prl3b1)

The serum was run on a kit specific for Prl3b1 (NovateinBio, USA). This kit failed to work and generated no useful data.

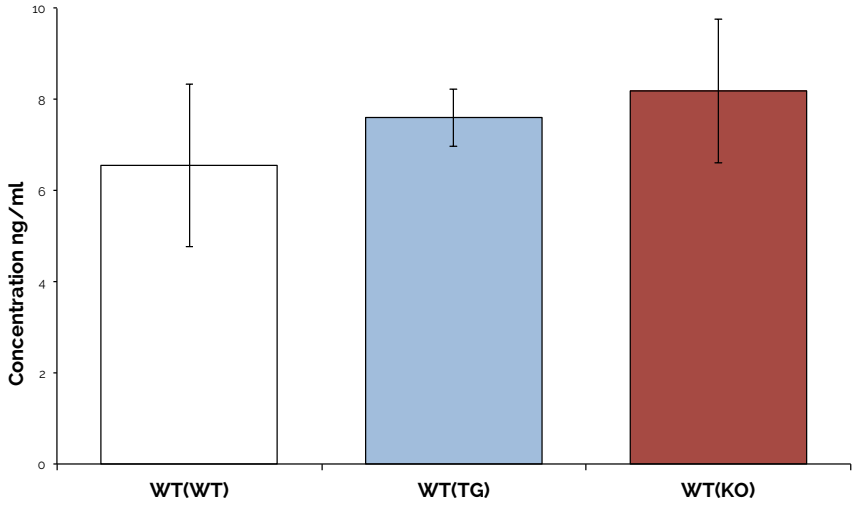


Figure 3.4: Prl8a8 Levels in Blood Serum. Average levels of Prl8a8 in the blood serum of WT(WT), WT(TG) and WT(KO) dams. Error bars represent SEM.

Prolactin

To establish whether there was an issue with serum quality, prolactin levels were analysed in the maternal serum of the original three cohorts that were generated for the biomolecular characterisation (Chapter 5) using a validated prolactin ELISA (abcam, UK). For these groups there was n = 8 WT(WT), n = 8 WT(TG) and n = 12 WT(KO) serum samples. The results can be seen in **Figure** 3.5.

Normally the pregnancy-induced spike in prolactin levels resolves by mid-pregancy (Soares, 2004) so the prediction would be similar levels across the three cohorts. WT(KO) dams had on average a higher concentration of prolactin in their blood serum at E16.5 compared to both WT(WT) and WT(TG) dams. In **Table** 3.5 it can be seen that WT(KO) dams had an average of 2715.39 pg/ml of prolactin in their serum, this was nearly 3 times the levels seen in the WT(WT) dams which only had 939.90 pg/ml. There was a large amount of variation however between samples in WT(KO) dams which meant these findings did not reach statistical significance when comparing across the three cohorts using a one-way ANOVA ($p\text{-}value > 0.05$). The fact that the ELISA kit worked with the serum samples suggested that the

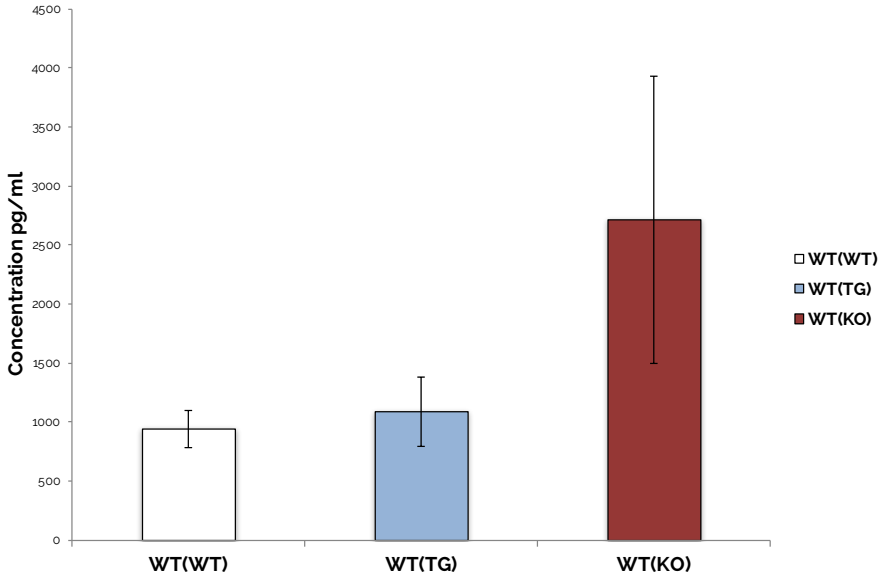


Figure 3.5: Prolactin and Prolactin-Like Protein Levels in Blood Serum. Average levels of Prolactin and Prolactin-Like hormones in the blood serum of WT(WT), WT(TG) and WT(KO) dams. Error bars represent SEM.

Prl8a8 experiment results were due either the absence of Prl8a8 in circulation or a failure of the ELISA reactivity.

Table 3.5: Generic Prolactin/Prolactin-like ELISA

Genotype	Concentration (pg/ml)	SEM
WT(WT)	939.90	± 156.59
WT(TG)	1088.37	± 291.95
WT(KO)	2715.39	± 1218.60

3.4 DISCUSSION

This work showed that the imprinted gene *Phlda2* acts exclusively to constrain the expansion of the SpT compartment of the mature mouse placenta, without significantly altering the cellular composition of other trophoblast lineages which share a common progenitor. In this way *Phlda2* becomes the first gene that has been described that has this specific function. Taking what is already known about *Phlda2*, the data suggests that it acts in placental development after the lineage

decisions are made, but before *Phlda2* expression subsides (**Figure** 3.6). This means that it is now possible to assess *in vivo* the specific function of the SpT lineage.

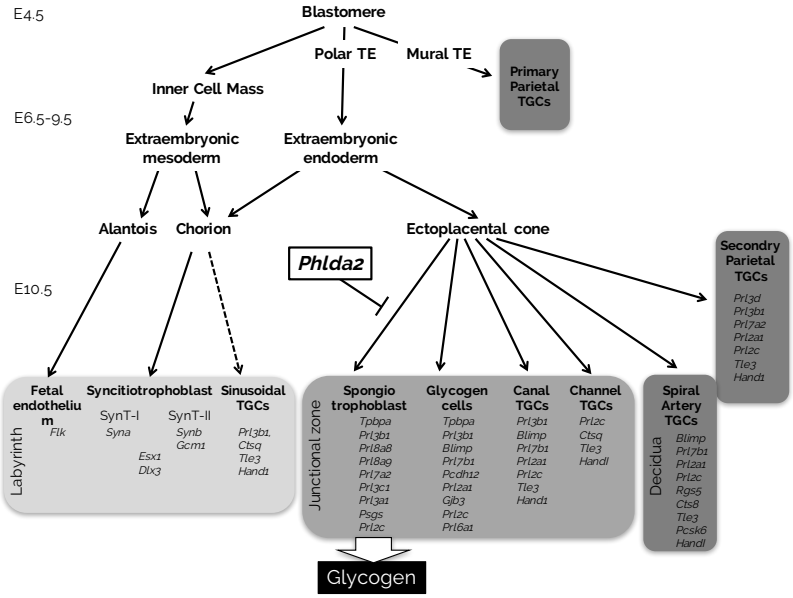


Figure 3.6: *Phlda2* Functions. Schematic summarising the cell autonomous and non-cell autonomous functions of *Phlda2*. Taken from Rai and Cross (2014).

Our other major discovery that is presented in this chapter, alongside the specific action *Phlda2* has upon the SpT lineage, is that through the use of loss-of-function and gain-in-expression models, we now know that *Phlda2* negatively regulates the expression of key placental hormones. This could indicate a novel signalling function of the SpT during placental and fetal development. The work by Tunster *et al.* (2015) published in *Developmental Biology* also highlighted that there was a link between having an expanded SpT and excessive storage of placental glycogen in both the TG and NON-TG placenta that shared the same uterine environment. The implications of this finding is that the SpT is likely to produce a signal that acts both locally, resulting in a direct effect, and at a distance causing an indirect effect upon glycogen accumulation. The knowledge that the SpT produces a vast array of Prls means that these are prime candidates for causing this nutrient accumulation. Placental hormones have a documented role in ensuring a successful pregnancy, through the channelling of maternal resources to the fetus (Bhattacharyya *et al.*, 2002, Samaan *et al.*, 1966, Wu *et al.*, 2008b).

Shingo (2003) showed that maternal neurogenesis is stimulated by prolactin during pregnancy. The discovery that the SpT compartment is a key source of numerous Prls, which are far more highly expressed during late gestation than prolactin (Soares *et al.*, 2007) and some of which bind to the Prlr (Freeman *et al.*, 2000), has led us to the hypothesis that these hormones are key regulators of the development of the maternal instinct. The fact that *Phlda2* negatively regulates the size of the SpT provided a unique model to test this hypothesis in a mouse model. The ELISA results although not statistically significant gives weight to the model. Specifically in the WT(KO) dams which had up to 3 times the level of prolactin and a rise in the Prl8a8 proteins. There is the distinct possibility that the prolactin ELISA was detecting certain Prls that were shown to be more highly expressed through the qPCR and microarray in this chapter. Either way the fact that the lactogenic hormone profiles are disrupted make this a novel and exciting way to directly assess the effects of having different placentas has upon the behavioural outcomes of the dams and the offspring.

NOTE: Further to the identification of *Phlda2*'s role in regulating the expansion of the SpT lineage, we published a paper entitled "Increased dosage of the imprinted *Ascl2* gene restrains two key endocrine lineages of the mouse Placenta.", that was also published in *Developmental Biology* by Tunster, MacNamara, Creeth and John (2016), further supporting the original hypothesis that imprinted genes play a key roe in regulating placental hormones (John, 2013). *Ascl2* plays a vital role in placental and intestinal development. Its loss-of-function results in the expansion of the parietal TGC lineage and an almost complete loss of *Tpbpa* positive cells in the ectoplacental cone alongside embryonic failure at E10.5. In this paper Tunster *et al.* (2016) showed by using a genetic cross that *Ascl2* requires the activity of *Phlda2* in order to limit the increased size of the SpT. Confirming that genomic imprinting is a critical element to placental development, and that it can be hypothesised that imprinting plays a vital role in placental signalling. Placental signalling that ultimately helps adapt the mother and fetus to pregnancy and the outside world.

Caveats

It must be noted that the use of the specific BAC transgene in this experiment means that two other genes are added into the mice, *Cdkn1c* and *Slc22a18*. *Cdkn1c* has been implicated in many disorders over the years, however its presence here is unlikely to affect the phenotype of the mice as the promotor is absent on the BAC transgene, thus *Cdkn1c* is not expressed. *Slc22a18* is a solute carrier that has a role in the kidney (Imai *et al.*, 2014) and in cancer (Chu *et al.*, 2011), it has not been linked to placenta growth or function. The story of *Phlda2* began when Frank *et al.* (2002) discovered placental overgrowth in mice lacking *Ipl* (*Phlda2*). Then Salas *et al.* (2004) showed that LOI of *Phlda2* (and *Slc22a18* using a BAC transgene model lacking the promotor to *Cdkn1c*) resulted in placental retardation. Then to isolate the role of *Phlda2* a series of crosses between *Phlda2*$^{+/-}$ females and *Kvdmr1* $^{+/-}$ males the role of *Phlda2* was isolated. This has since been genetically verified by (Tunster *et al.*, 2010) by use of a BAC transgene to create a double transgenic (*Phlda2* $^{-/+\,\mathrm{xBAC1}}$, 1X âĂŞ like wildtype) that rescued the placental and IUGR phenotype seen in the *Phlda2* $^{+/+\mathrm{xBAC1}}$ transgenic (2X). This conclusively excluded the role of *Slc22a18* in placental growth restriction/IUGR. Despite this evidence indicating that *Slc22a18* is not directly involved in the placental phenotype, it is plausible that it is having wider affects on the phenotypes of the mice that may confound further findings.

Summary of Findings

- *Phlda2* acts exclusively to constrain the expansion of the SpT compartment of the mature mouse placenta.
- *Phlda2* negatively regulates the expression of a number of key placental hormones, including the *Prls*.

- ELISA analysis of Prl8a8 levels and prolactin levels showed an increase in blood serum concentration of prolactin in the WT(KO) dams serum but no difference in Prl8a8.

- Thes role of *Phlda2* on the expression of key endocrine genes from the major endocrine compartment of the placenta provides the ability to test the hypothesis that changes in expression levels of Prls from the SpT are vital in initiating the maternal instinct.

Maternal Behavioural Characterisation

4

4.1 Overview

A number of indirect experiments suggest that placental endocrine function could contribute to the programming of maternal care behaviours in rodents but this has not been tested directly. This chapter describes the use of unique animal models in which placental endocrine function was genetically manipulated to explore the consequences for maternal behaviour.

In this study, the WT genetic status of all the dams was maintained using RET (Chapter 2, section 2.2). This was to isolate the direct impact of the placental phenotype on maternal behaviour without the confounder of the dams genotype. The mouse models used were chosen after previous work in our lab demonstrated that altering levels of the imprinted gene *Phlda2* causes a significant change to a key endocrine lineage situated within the junctional zone of the placenta called the SpT (Tunster *et al.*, 2015) (Chapter 3). This lineage expresses a number of prolactin like proteins (*Prls*) (Simmons *et al.*, 2008) and placental specific glycoproteins (*Psgs*) (Coan *et al.*, 2006). Loss-of-function of *Phlda2* results in an enlargement of the SpT compartment by approximately 200% (Tunster *et al.*, 2015). Conversely, two-fold expression of *Phlda2* from a trangene results in a reduction of the SpT compartment to 50% normal (Tunster *et al.*, 2010). By combining RET with these two genetic models, the consequences of manipulating the SpT compartment on maternal behaviour could be studied.

4.1.1 Behavioural Assays

A number of behavioural assays were used to investigate the maternal phenotypes of three cohorts of primiparous dams exposed to different placenta. Assessing maternal behaviour in laboratory rodents involves a number of classic assays primarily examining behaviour towards pups (Franks *et al.*, 2011). The tests that were chosen were pup retrieval, nest building and 23 hour monitoring. All the tests occurred between Postnatal Day (P)2-4 as it is widely reported that this is when maternal behaviours are most prevalent in rodents. After P 6 these behaviours show a decline (Champagne *et al.*, 2007, 2003).

The Elevated Plus Maze (EPM) is an established test for testing anxiety in rodents (Komada *et al.*, 2008). it exploits the natural aversion of rodents to open and elevated spaces, plus their ability to spontaneously explore novel environments. It has been used numerous times on male and female mice to determine anxiety phenotypes. It is not commonly used on primiparous lactating female mice, although has been recently used in a mouse model of pre-natal trauma (Golub *et al.*, 2016).

Pup retrieval is a maternal behavioural test as it measures the dams responsiveness. Responsiveness to pups is a critical behaviour in mice in order to keep pups safe. There are natural variations across mouse strains in this behaviour (Champagne *et al.*, 2007), but nonetheless, the behaviour persists making it a viable test for maternal responsiveness to offspring.

Rodents build nests to keep warm (26°C), protect themselves and to protect and nurture their young. The nest acts as a shelter and is important in reproductive behaviour (Bond *et al.*, 2002). There is research suggesting that hormones are needed for the elicitation of nest building (Lisk *et al.*, 1969). There are several methods in the literature on how to measure nest building, the most common method is to score the nest on an arbitrary scale, in this chapter an adapted version of this technique is

used that also takes into account the dams responsiveness to her pups by scoring whether nest building also elicits the maternal instinct to cover and nurture the pups inside the nest once/if built within the time scale (Bond *et al.*, 2002, Lisk *et al.*, 1969).

Pup retrieval and nest building took place in specially designed home cages known as the Phenotypers (Noldus, UK). The Phenotypers allowed for the continuous tracking of the animals throughout the tests. This meant that a complete behavioural 23 hour profile could be created using the software to perform retrospective computer and manual scoring of a subset of maternal and standard behaviours. This type of analysis can uncover patterns of behaviour during the "light" and "dark" phases of a day that looked beyond just nest building activity as previously described by Shoji and Kato (2006). Behaviours scored manually in the Phenotyper were specific to those behaviours outlined in chapter 13 Mood and Anxiety-related Phenotypes in Mice: Characterisation Using Behavioural Tests by Pietropaolo (2010). Key techniques in measuring variations in maternal behaviour are described including specific home cage behaviours (**Table** 2.10) that are known to have key behavioural outcomes for offspring (Coutellier *et al.*, 2009a). These behaviours are therefore seen to be a reliable assessment of maternal nurturing behaviour.

4.2 RESULTS

The three experimental cohorts were generated as described (Chapter 2). Briefly, female mice of the appropriate genotype were superovulated, mated with the appropriate male and then embryos were harvested from plugged females two days after a visible plug was observed (E1.5). The next day, WT 129 virgin females between 8-10 weeks of age were set up with vasectomised males to generate pseudopregnant females. After a short period of incubation in M2 media (SIGMA, cat no: M7167), 14-16 newly fertilised E1.5 embryos were transferred into E0.5 pseudopregnant WT

recipients (**Table** 4.1).

For the behavioural assessment 14 WT(WT), 11 WT(TG) and 13 WT(KO) pregnant females were generated. Females were monitored through out pregnancy at set intervals by carrying out regular weight and health checks to determine pregnancy and well being. The day of birth was recorded as day P 0.5, with a note made of litter size at time of birth and length of gestation. Litter size was also recorded at P7 (**Table** 4.2). Females with litter sizes between 6 and 12 were used for the behavioural assessments.

Table 4.1: Matings for RET

Matings	Embryos Generated
Phlda2 KO ♀ x WT 129 ♂	100% KO (no *Phlda2*)
10-10 TG ♀ x 10-10 TG ♂	25% Homozygous TG ** / 50% heterozygous TG / 25% WT
WT 129 ♀ x WT 129 ♂	100% WT

Note: ** No homozygous offspring as they are embryologically lethal.

4.2.1 LITTER SIZE

The average litter size across the three cohorts of WT dams carrying different genotype pups was not significantly different (**Figure** 4.1 and **Table** 4.2; $F_{2, 36} = 0.83$, *p-value* = 0.45). Pups generated from 10-10 TG and KO dams were all genotyped in order to determine the number of pups in a litter that were either *Phlda2* $^{+/-}$ (wildtype, WT; 1X), *Phlda2* $^{-/+}$(maternal KO; 0X) and *Phlda2* $^{+/+\ BACx1}$ (single copy *Phlda2* transgene, TG; 2X) at least 50% of the litter needed to be of each genotype to be included. The WT(WT) cohort was likewise genotyped to ensure that the litter was fully WT. In the WT(TG) model, this was particularly important as we were unable to breed the transgene to homozygosity. Embryos were generated by crossing het males with het females which resulted in litters with >60% pups carrying the transgene (**Table** 4.1).

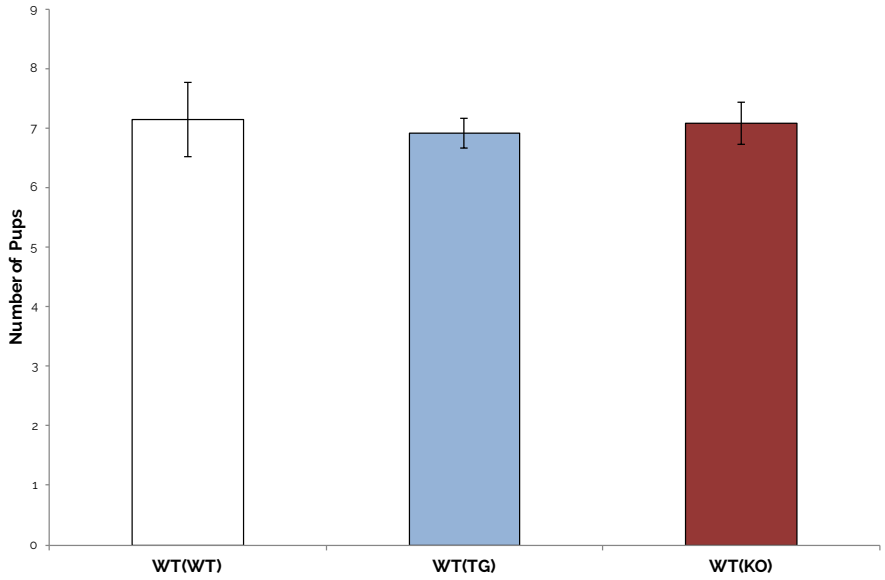


Figure 4.1: Average Litter Size across cohorts. Basic Bar-Chart illustrating that there is no significant difference in litter size across the three cohorts ($F_{2,\ 36} = 0.83$, *p-value* $= 0.45$). Error bars represent SEM.

Table 4.2: Litter Sizes

Pup Genotype	Litter Size (SEM)
WT	7.14 (± 0.63)
TG	6.91 (± 0.25)
KO	7.08 (± 0.35)

4.2.2 FETAL AND PUP WEIGHTS

The average weight of the fetal and pups (n = 256) from WT(WT), WT(TG) and WT(KO) dams were recorded at E16.5 and P7 in order to determine whether there was any difference between the offsprings weights. The results showed that there was no significant difference in weights between the three cohorts of mice at P7 (**Figure** 4.3). There was fetal growth restriction at E16.5 in line with previous reports linking IUGR to *Phlda2* TG (Tunster *et al.*, 2014) and data here demonstrated rapid postnatal catch-up growth (**Figure** 4.2).

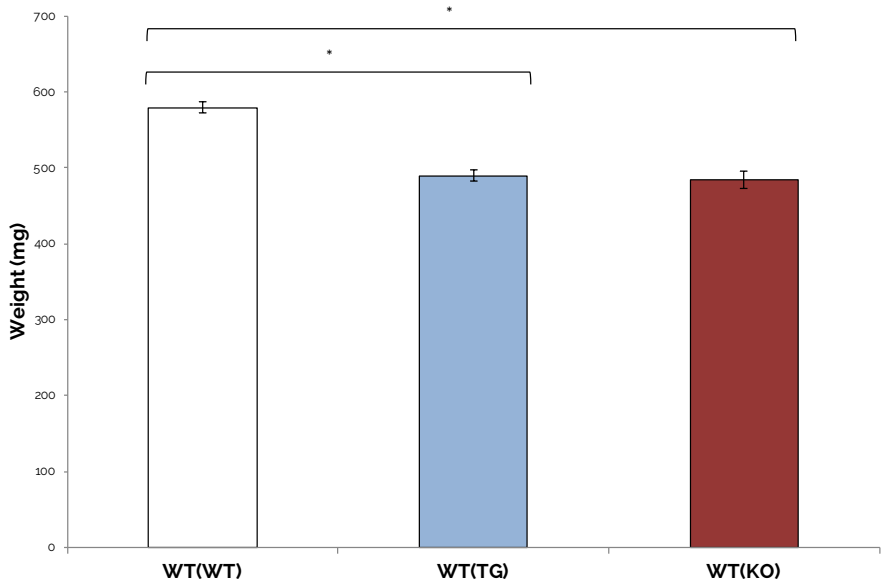


Figure 4.2: Average Pup Weight at E16.5. Basic Bar-Chart illustrating that there is a significant difference in fetal weight at E16.57 between the three cohorts litters ($F_{2,\ 254} = 4.7$, *p-value* $= 0.045$). x-axis represents pup genotype, not mothers genotype (which were all WT). Error bars represent SEM. Statistical significance: $^*p < 0.05$.

4.2.3 Elevated Plus Maze (EPM)

The EPM was carried out on P2 on each of the primiparous dams across the three cohorts n = 16 WT(WT), n = 10 WT(TG) and n = 12 WT(KO). Each new dam was given 10-15 minutes to habituate to the test room before embarking on the 5 minute test away from her pups.

The duration of time spent across the zones did not differ between cohorts ($F_{2,\ 36} = 1.44$, *p-value* $= 0.25$). There was no significant change in time spent in the most anxiogenic open zone (($F_{2,\ 36} = 0.16$, *p-value* $= 0.85$, **Figure** 4.4). However, all three cohorts did not behave as expected in the middle zone.

Although the velocity by which the mice moved around the zones of the EPM was not different overall ($F_{2,\ 36} = 2.87$, *p-value* $= 0.069$), the mean velocity of the WT(KO) dams in the open zone was over twice that of WT(WT) mice (*p-value* $= 0.077$; **Figure** 4.4).

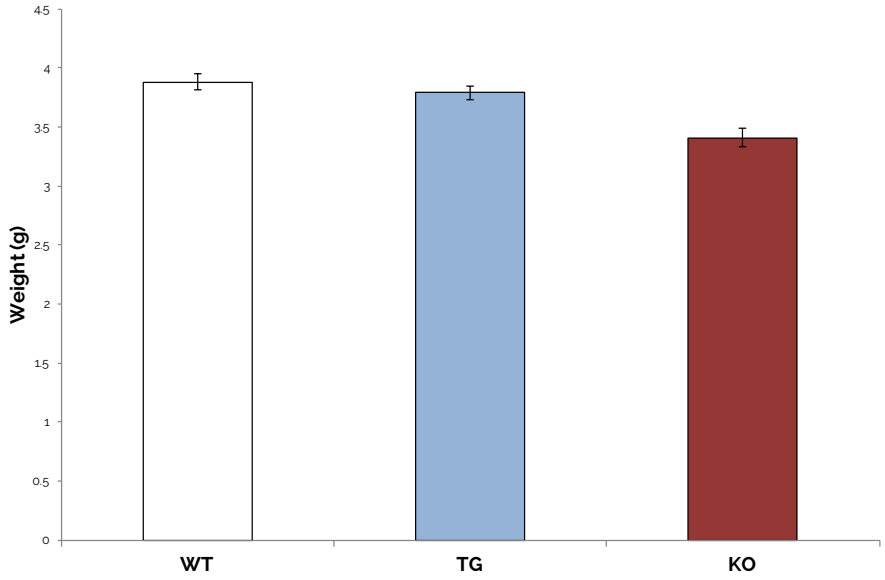


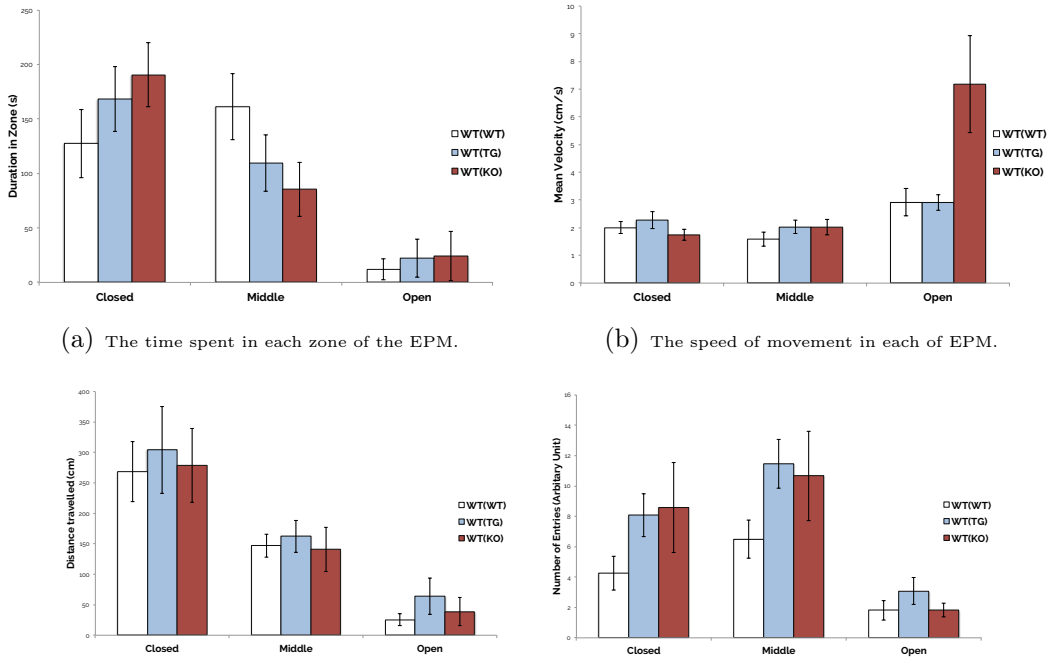
Figure 4.3: Average Pup Weight at P7. Basic Bar-Chart illustrating that there is no significant difference in pup weight at P7 between the three cohorts litters ($F_{2,\ 254} = 0.76$, *p-value* $= 0.53$). x-axis represents pup genotype, not mothers genotype (which were all WT). Error bars represent SEM.

The increased speed of WT(KO) dams was not matched in the distance travelled by them when compared to the WT(WT) and WT(TG) mice (**Figure** 4.4,$F_{2,\ 36} = 1.58$, *p-value* $= 0.22$) or the frequency of entries into the different zones of the EPM (**Figure** 4.4, $F_{2,\ 36} = 1.7$, *p-value* $= 0.19$).

Average stretch and dip frequency was recorded manually in order to help quantify explorative and anxious behaviour in the different zones of the EPM. No significant differences between genotypes across the cohorts were found for stretch and dip frequency ($F_{2,\ 36} = 1.0$, *p-value* $= 0.395$ and $F_{2,\ 36} = 2.4$, *p-value* $= 0.076$, respectively).

4.2.4 PUP RETRIEVAL

The pup retrieval test demonstrated that there was no significant difference (*p-value* > 0.05) across the three cohorts in the time taken for dams to sniff their pups, with WT(WT) and WT(TG) mice taking 49-53 seconds and WT(KO) dams taking 67



(a) The time spent in each zone of the EPM.

(b) The speed of movement in each of EPM.

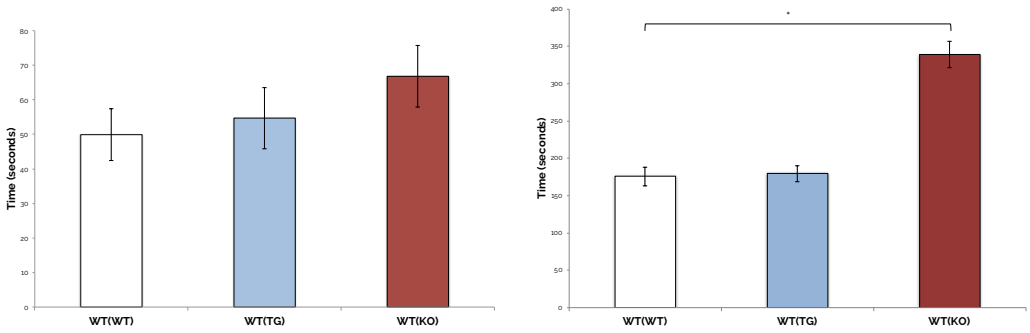
(c) The distance moved in each zone of the EPM.

(d) The frequency of entries into each zone of the EPM.

Figure 4.4: The Elevated Plus Maze. (a) A barchart showing the percentage time spent in each zone of the EPM. There was no significant difference in time spent in each zone across the three cohorts ($F_{2,\ 36} = 1.44$, *p-value* $= 0.25$). **(b)** A barchart showing the speed/velocity in each zone of the EPM. Statistical analysis indicated that there was no significant difference between the speed of the genotypes across the different zones ($F_{2,\ 36} = 2.87$, *p-value* $= 0.069$) although WT(KO) dams did show a trend of moving faster in the open zone compared to WT(WT) dams. **(c)** A barchart showing the distance travelled in each zone of the EPM ($F_{2,\ 36} = 1.58$, *p-value* $=$ 0.22). **(d)** A barchart showing the frequency of entries into each zone of the EPM ($F_{2,\ 36} = 1.7$, *p-value* $= 0.19$). Error bars represent SEM.

seconds on average (**Figure** 4.5) ($F_{2,\ 36} = 1.1$, *p-value* $= 0.33$). This can be taken to suggest that there were no issues regarding either the WT(TG) or WT(KO) dams ability to recognise the pups.

There was a significant difference in the latency to retrieve the first pup in the pup retrieval task ($F_{2,\ 36} = 4.8$, *p-value* $= 0.015$). This was driven by WT(KO) dams taking longer to retrieve their first pup compared to WT(WT) dams (*p-value* $= 0.01$) and compared to the WT(TG) dams (**Figure** 4.5). WT(WT) and WT(TG) dams took between 109 - 139 seconds to collect their first pup. Wt(KO) dams took three times as long to retrieve their first pups with an average time of 339 seconds.



(a) Latency to Sniff Pups across cohorts. (b) Latency to Retrieve Pups across cohorts.

Figure 4.5: Pup Retrieval Task. (a) A barchart showing the differences in the time taken to sniff pups and the time taken to retrieve pups across the 3 cohorts. The time taken to retrieve pups was significantly different between WT(KO) and WT(WT) ($F_{2,\ 36} = 1.1$, *p-value* $= 0.33$). **(b)** A barchart showing the differences in the time taken to sniff pups and the time taken to retrieve pups across the 3 cohorts. The time taken to retrieve pups was significantly different between WT(KO) and WT(WT) ($F_{2,\ 36} = 4.8$, *p-value* $= 0.015$). Error bars represent SEM. Statistical significance: $^*p < 0.05$.

Within the pup retrieval paradigm the time taken to retrieve the first pup is a measure of maternal responsiveness to pups. The overarching finding that WT(KO) dams took longer to retrieve their pups but not sniff them in comparison to both WT(WT) and WT(TG) dams implies that these dams have an impaired maternal response that is independent of the basic recognition of the pups new location outside of the nest, in the home cage. This phenotype was not anticipated as these dams were exposed to placenta with a larger endocrine compartment and were predicted to have enhanced maternal behaviours. However, there are different interpretations of these data, as will be discussed later.

4.2.5 ETHOVISION - MONITORING OF PUP RETRIEVAL

Using the Phenotyper set up allowed for the continuous monitoring of the dams' behaviour over the duration of the pup retrieval assay. This analysis was done retrospectively so as to be able to ensure accurate tracking and control for any software issues that if performed during the task may have been problematic.

The Ethovision software was calibrated to track the dams movement during the duration of the task. All the dams had collected the first of their pups within 20 minutes of the assay and the analysis continued for a further 40 minutes in order to monitor behaviours associated with the collection of the remaining pups. An example of the tracking can be seen in **Figure** 4.6 where the yellow signifies the dam and the red the central axis of the tracker.

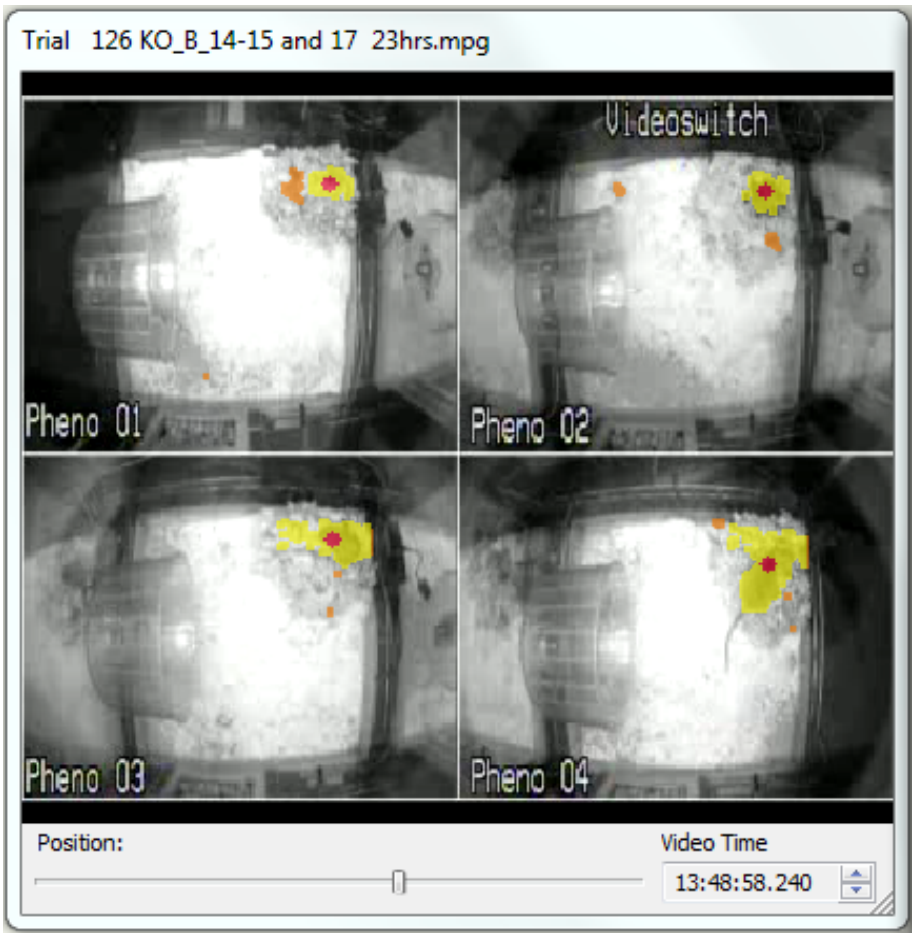
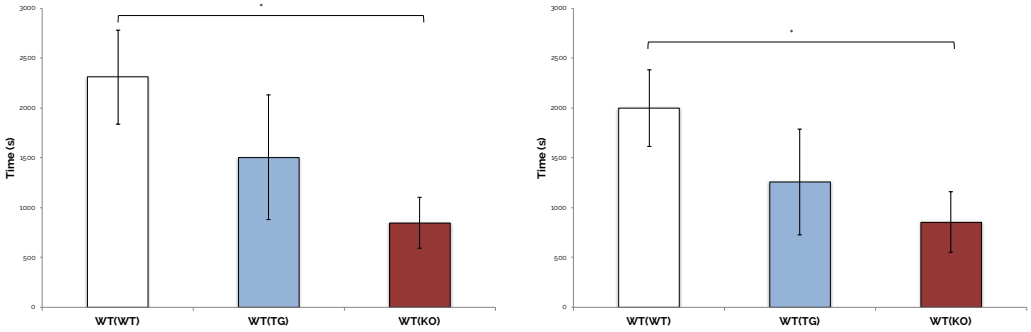


Figure 4.6: Ethovision Tracking. A screenshot of the tracking software and how it locates the dams within the home cage (yellow) and then monitors their movement in the predefined home cage and sub locations within the home cage.

.

Ethovision video tracking analysis from the 60 minute period of the pup retrieval assay demonstrated a tendency for WT(KO) dams to visit the food zone significantly over twice as quick WT(WT) dams (df = 1, p-*value* = 0.029), and twice as quick as WT(TG) dams, although this was not significant (**Figure** 4.7). The WT(KO)

dams were also significantly (df = 1, *p-value* = 0.046) quicker to visit the water zone during this test period compared to WT(WT) dams (**Figure** 4.7).



(a) Time taken to visit food zone during pup retrieval. (b) Time taken to visit water zone during pup retrieval.

Figure 4.7: Ethovision Analysis of Pup Retrieval. (a) A barchart showing the time taken for the dams across the 3 cohorts to first visit the food zone during the pup retrieval task. WT(KO) dams visited significantly sooner than WT(WT) dams (df = 1, *p-value* = 0.029). **(b)** A barchart showing the time taken for the dams across the 3 cohorts to first visit the water zone during the pup retrieval task. WT(KO) dams visited the water zone significantly sooner compared to WT(WT) dams (df = 1, *p-value* = 0.046). Error bars represent SEM. Statistical significance: $*p < 0.05$.

There are several interpretations of what these marked differences in behaviour displayed by the WT(KO) dams mean in relation to maternal care and nurturing behaviour. The use of other maternal behavioural assays like the nest building assay aided the interpretation. Although some of the findings were challenging to interpret in the absence of reliable data from the EPM test, as will be discussed later.

4.2.6 NEST BUILDING

The results of the nest building, similarly to the pup retrieval task, indicated a significant phenotype present in the WT(KO) dams behavioural response to the task. The WT(KO) model dams exhibited poorer nest building behaviour in comparison to both the WT(WT) and WT(TG) models (**Figure** 4.8). These WT(KO) dams failed to build a nest 60% of the time, in contrast to WT(WT) and WT(TG) dams whom built a nest 70-85% of the time. Intriguingly WT(TG) dams performed better than the WT(WT) dams in this test, using the chosen method of scoring the nests.

The WT(TG) dams were twice as successful at building nests and moving the pups into the nest than WT(WT) control mice (*p-value* = 0.027).

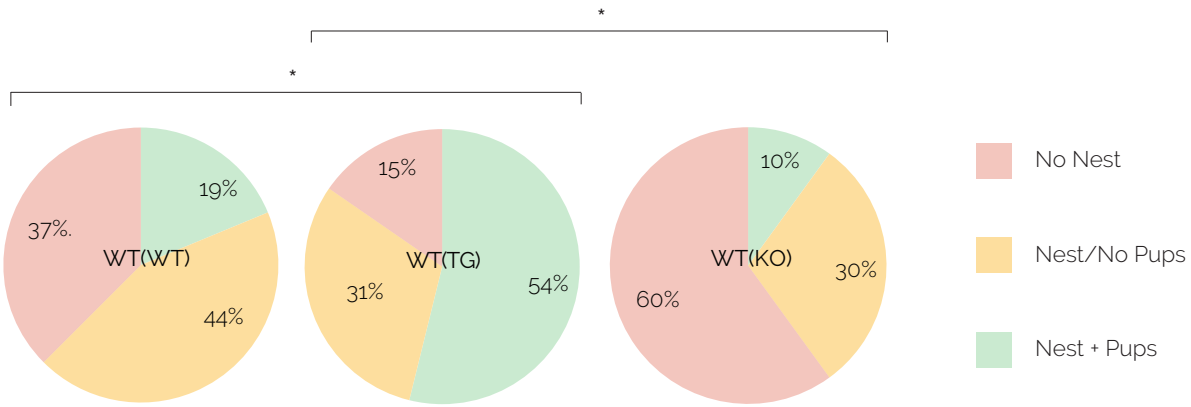


Figure 4.8: Nest Building: A series of pie charts highlighting the differences in nest building behaviour across the 3 cohorts ($F_{2,\ 36} = 4.3$, *p-value* = 0.022). WT(KO) significantly differed from WT(TG) (*p-value* = 0.019). WT(TG) dams differed significantly from WT(WT) dams (*p-value* = 0.027). Statistical significance: $*p < 0.05$.

4.2.7 ETHOVISION - MONITORING OF NEST BUILDING

Ethovision video tracking analysis from the 60 minute period of this test highlighted significant differences in the distance travelled across the three cohorts ($F_{2,\ 36} = 7.12$, *p-value* = 0.001), with the WT(KO) dams travelling considerably further than both WT(WT) (*p-value* < 0.001) and WT(TG) (*p-value* = 0.001), driving this difference (**Figure** 4.10). This was mirrored in the frequency of visits to the food zone across the cohorts ($F_{2,\ 36} = 8.4$, *p-value* = 0.001) where WT(KO) dams visited more frequently than both WT(WT) (p-value = 0.001) and WT(TG) groups (*p-value* = 0.01, **Figure** 4.10).

Using the video recordings for each of the 38 dams from the nest building task it was possible to unobtrusively score other key behaviours. The behaviours that were scored can be seen in table 2.10. Manual scoring these behaviours was performed blind for duration and frequency by two separate investigators to exclude bias. The results were compared across the genotypes and suggested that there was a significant

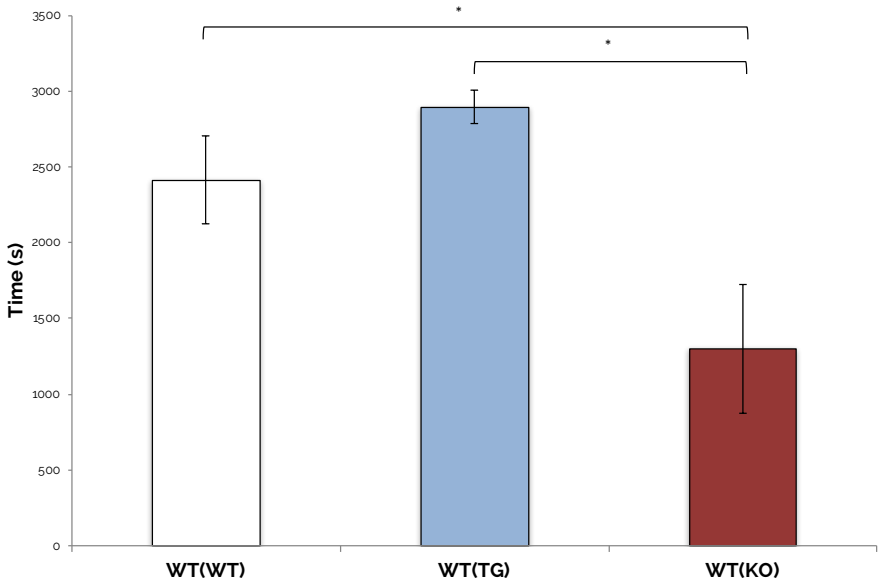
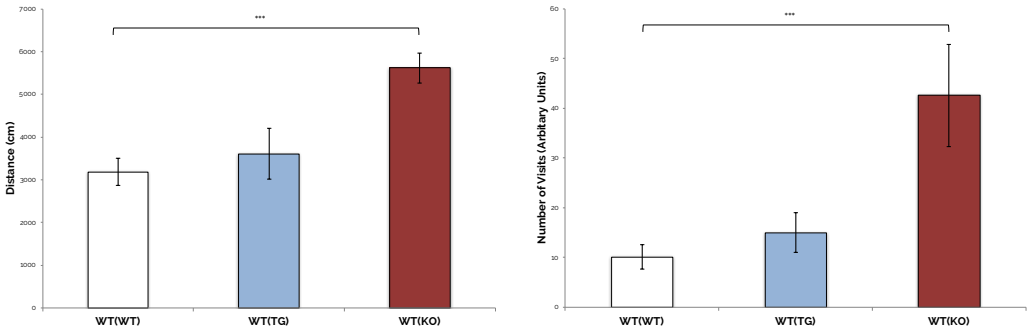


Figure 4.9: Duration of time spent Nest Building. Graph indicating the duration of time spent building a nest across the cohorts during the nest building task ($F_{2,\ 36} = 6.967$, *p-value* = 0.003). In particular WT(KO) spent significantly less time building nest compared to both WT(WT) and WT(TG) mice, *p-value* = 0.029 and *p-value* = 0.002, respectively. Error bars represent SEM. Statistical significance: $*p < 0.05$, $**p < 0.01$, and $***p < 0.005$.



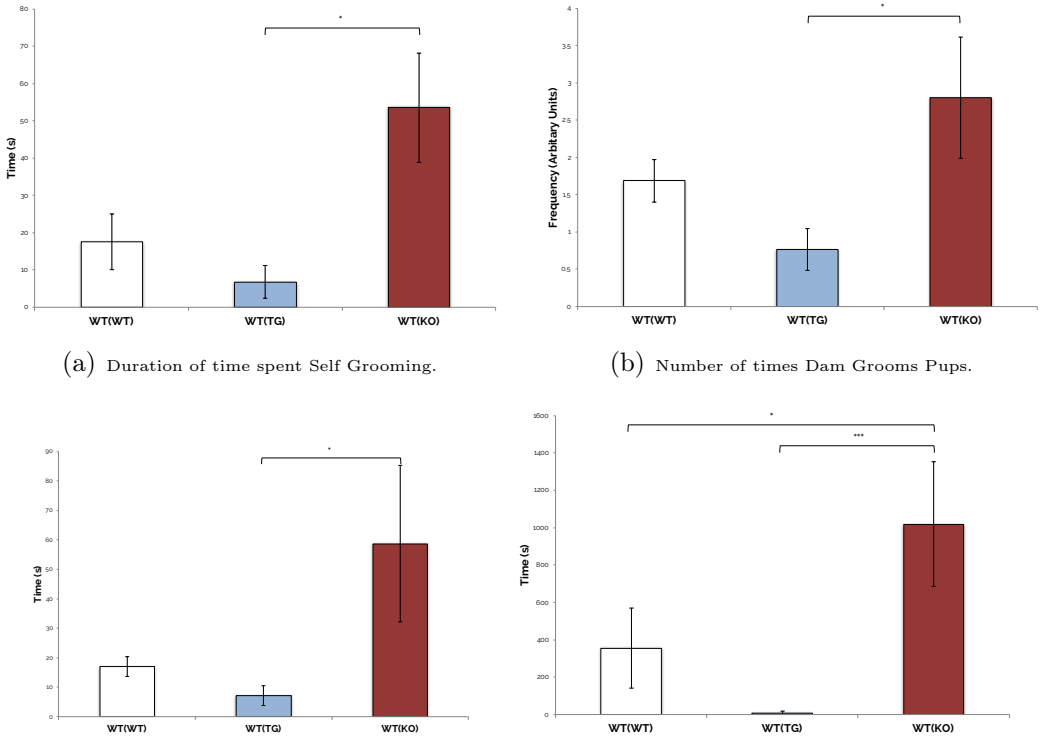
(a) Distance travelled by mothers during nest building. (b) Number of visits to the food zone during nest building.

Figure 4.10: Ethovision Analysis of Nest Building. **(a)** A barchart showing the total distance travelled by the dams across the 3 cohorts during the nest building test ($F_{2,\ 36} = 8.7$, *p-value* = 0.001). **(b)** A barchart showing the number of visits to the food zone during the nest building test across the 3 cohorts of dams. The WT(KO) signalling model visited the zone significantly more than WT(WT) dams ($F_{2,\ 36} = 8.4$, *p-value* = 0.001). Error bars represent SEM. Statistical significance: $*p < 0.05$, $**p < 0.01$, and $***p < 0.005$.

difference in the duration of time spent self grooming between the genotypes ($F_{2, 36}$ = 6.61, *p-value* = 0.004, **Figure** 4.11). Specifically, the most significant difference was between WT(KO) and WT(TG) (p-value = 0.009) which showed WT(KO) dams spent five times longer self grooming than WT(TG) dams and two times longer than WT(WT) dams. This was also found to be the case with the frequency that dams undertook pup grooming during the nest building task (**Figure** 4.11, $F_{2, 36}$ = 4.59, *p-value* = 0.017). In particular this difference was evident between WT(TG) and WT(KO) dams (*p-value* = 0.012). These two cohorts also showed a significant difference in the duration of time spent grooming their pups, despite taking into account the large variation between individual dams (**Figure** 4.11, $F_{2, 36}$ = 4.39, *p-value* = 0.02). It was also evident that WT(KO) mice spent significantly less time building nests compared to WT(WT) (p-value = 0.029) and WT(TG) (p-value = 0.002) mice (**Figure** 4.9), independently corroborating the results displayed in the specific nest building test.

Crouched nursing frequency ($F_{2, 36}$ = 6.54, *p-value* = 0.004) showed over a two fold increase in the WT(KO) (*p-value* = 0.024) dams compared to WT(WT) and was four times less in WT(TG) dams (*p-value* = 0.003) (**Figure** 4.11). The difference crouched nursing duration was also significantly different across the groups (**Figure** 4.11, $F_{2, 36}$ = 4.97, *p-value* = 0.012); with the behaviour most significantly altered between WT(TG) and WT(KO) (*p-value* = 0.009). Arched nursing, however, was not found to be significantly altered in either duration or frequency (p-values $>$ 0.05) and neither was passive nursing (p-value $>$ 0.05).

The *post-hoc* tracking of each cohort of dams allowed for the generation of an array of behavioural characteristics that are displayed by WT(TG) and WT(KO) dams compared to the control WT(WT) cohort of dams. There initially appeared to be a stronger phenotype present in the WT(KO) dams based on the nest building and pup retrieval tasks. However, tracking plus manually scoring allowed the dissection of behaviours that were disproportionately preferred between the groups with relation to nurturing behaviour and protecting their offspring with both the WT(KO) dams



(a) Duration of time spent Self Grooming.

(b) Number of times Dam Grooms Pups.

(c) Duration of time spent Grooming Pups.

(d) The Duration of Time Spent Crouch Nursing

Figure 4.11: Manually Scored Maternal Behaviours. (a) Graph indicating the duration of time spent self grooming across the cohorts during the nest building test ($F_{2,\ 36} = 6.61$, *p-value* = 0.004). **(b)** Graph indicating the frequency of time spent grooming pups across the cohorts during the nest building test ($F_{2,\ 36} = 4.59$, *p-value* = 0.017). **(c)** Graph indicating the duration of time spent grooming pups across the cohorts during the nest building test ($F_{2,\ 36} = 4.39$, *p-value* = 0.02). **(d)** Graph indicating the duration of time spent by the three cohorts of dams performing the crouched nursing behaviour on their pups during the nest building test ($F_{2,\ 36} = 4.97$, *p-value* = 0.012). Error bars represent SEM. Statistical significance: $*p < 0.05$, $**p < 0.01$, and $***p < 0.005$.

and the WT(TG) behaving abnormally. These have been summarised in **Table** 4.3.

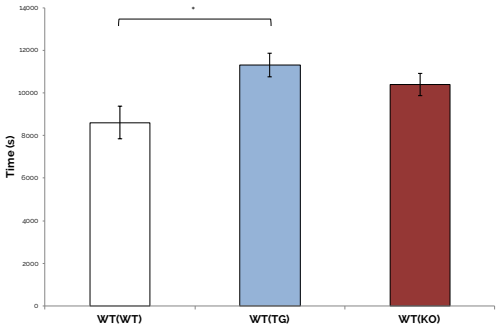
Table 4.3: Manually Scored Behaviours

Behaviour	**WT(WT) vs WT(TG)** ***p-value***	**WT(WT) vs WT(KO)** ***p-value***	**WT(TG) vs WT(KO)** ***p-value***
	↑/↓ Regulated (*p-value*)	**↑/↓ Regulated (*p-value*)**	**↑/↓ Regulated (*p-value*)**
Crouched Nursing Frequency	0.59	↑ 0.02	↑ 0.003
Crouched Nursing Duration	0.46	↑ 0.05	↑ 0.009
Arched Nursing Frequency	0.84	0.14	0.058
Arched Nursing Duration	0.74	0.22	0.070
Pup Grooming Frequency	0.28	0.21	↑ 0.012
Pup Grooming Duration	0.82	0.057	↑ 0.020
Self Grooming Frequency	0.37	0.12	↑ 0.009
Self Grooming Duration	0.64	↑ 0.02	↑ 0.003

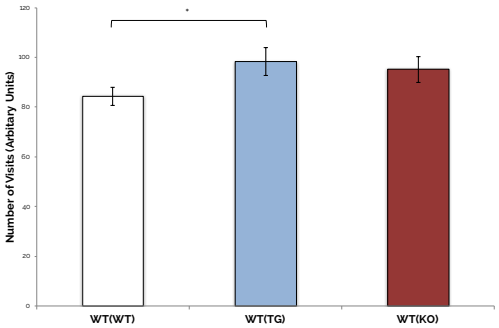
4.2.8 Ethovision - 23 Hour Monitoring - Day to Night

When analysing the tracking of the 23 hour period of the mice, the adjustment of behavioural patterns from the last two hours of "Day Time" and the first two hours of "Night Time" were analysed. This is when mice are most active as they are nocturnal animals and therefore activity around this period is heightened (Windhorst and Johansson, 2012). Analysis showed that there was an overall effect of genotype on the time spent in the nest zone during this AM-PM period ($F_{2,\,36} = 4.63$, *p-value* = 0.016), driven by the main difference between WT(WT) and WT(TG) dams (*p-value* = 0.014). WT(TG) dams spent longer in the nest zone compared to the WT(WT) dams (**Figure** 4.12). The number of visits to the nest was also seen to be significantly altered between genotypes ($F_{2,\,36} = 3.7$, *p-value* = 0.035) and was similarly driven by differences between the WT(WT) and WT(TG) cohorts (*p-value* = 0.031) where

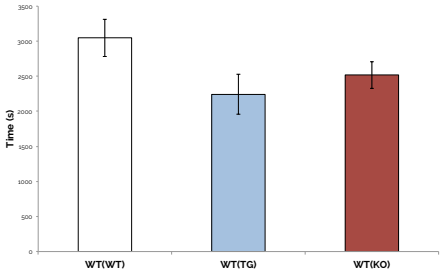
WT(TG) dams tended to visit the nest zone more often during this period compared to the WT(WT) dams (**Figure** 4.12).



(a) Duration of time spent in the nest zone during 10-14 hours of monitoring.



(b) Number of visits to the nest zone during 10-14 hours of monitoring.



(c) Duration of time spent in the food zone during 10-14 hours of monitoring.

Figure 4.12: Activity between Hour 10 to Hour 14 of Monitoring. (a) The graph shows that during the most active phase of a 24 hour day it was evident that there was a significant effect of genotype on the duration of time spent in the nest zone ($F_{2,\ 36} = 4.63$, *p-value* $= 0.016$). This was driven by the difference between WT(TG) mice and WT(WT) mice (*p-value* $= 0.014$). This difference indicated that WT(TG) dams spend longer in the nest zone compared to WT(WT) dams. **(b)** The graph indicates that the number of visits to the nest zone during this period is also significantly altered across the three cohorts ($F_{2,\ 36} = 3.7$, *p-value* $= 0.035$). WT(TG) dams drove this difference by visiting the nest zone considerably more often that WT(WT) dams with a significance of (p-value) $= 0.031$. **(c)** A barchart showing the duration of time spent in the food zone during hours 10-14 of a 24 hour monitoring period. It can be seen that WT(TG) mice show a decrease in the amount of time spent in the food zone during this period of heightened activity for mice, however this did not reach significance. Error bars represent SEM. Statistical significance: $*p < 0.05$ and $**p < 0.01$.

As well as the nest zone it was noted that the time spent feeding in the food zone during this period was reduced in the WT(TG) dams and was nearing significance (*p-value* $= 0.051$). The latency of the first visit to the food zone although not significant (*p-value* $= 0.056$), indicated that WT(TG) not only spent less time in the food zone, they also took longer to visit the food zone during this period of increased

activity (**Figure** 4.12).

4.2.9 Hour by Hour - Circadian Rhythm

Following on from the previous analysis it was apparent that reviewing the three groups of dams (n = 38) over the duration of the test monitoring on an hourly basis might uncover further information. To analyse their 23 hour pattern of behaviour, or their circadian rhythm, it was necessary to first define the 23 hour time period in the two distinct phases, "light" and "dark" as previously described by Shoji and Kato (2006). The light phase which is equivalent to day time is the period when the mice should be least active (as they are nocturnal animals). The dark phase or night time is when activity would be expected to be increased.

Using this method of analysis, there was a significant difference in the distance travelled by WT(WT) dams during the light phase compared to distance travelled in the dark phase (*p-value* < 0.001), demonstrating that WT(WT) dams displayed increased activity in the dark phase of the day. This difference in activity between the light and dark phases was not evident in the WT(TG) mice however with a *p-value* $= 0.13$. Similarly the WT(KO) dams did not show a significant difference in activity levels between day and night (*p-value* $= 0.83$). Consequently there was significant differences between the WT(WT) dams and the WT(KO) dams ($F_{1,\,24} = 6.4$, *p-value* $= 0.038$) but not between the WT(WT) and WT(TG) mice ($F_{1,\,25} = 1.93$, *p-value* $= 0.18$) dams.

The nest zone phase analysis indicated that there was an overall effect of phase on the time spent in the nest region for WT(WT) dams (*p-value* $= 0.002$), this was a result of the dramatic decrease in the duration of time in the nest between light and dark phases. WT(TG) dams spent a similar amount of time in the nest zone in the light and dark phases (*p-value* $= 0.68$). This behaviour was also evident in the WT(KO) dams (*p-value* $= 0.96$).

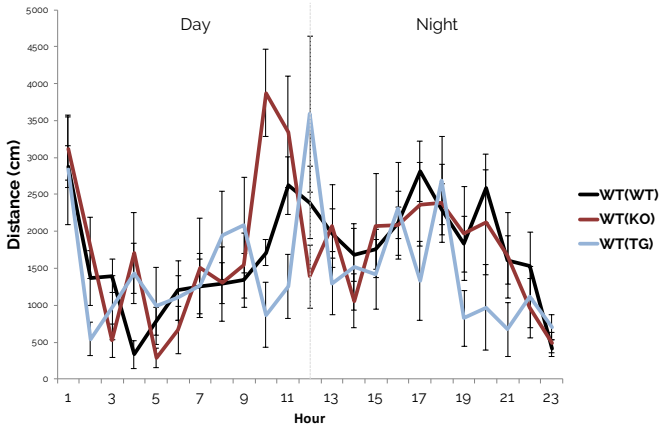


Figure 4.13: Hour by Hour distance moved across the 23hr monitoring. The graph above shows the hour by hour distances travelled by each cohort over a 23 hour period. The first 12 hours are "day" time with 12-23 hours being "night" time. There was a significant difference in distance travelled between day (light) and night (dark) phases for the WT(WT) mice ($p\text{-}value < 0.001$), but no significant difference in the distance travelled for WT(TG) and WT(KO) mice between day and night phases. There was however an effect evident between WT(WT) and WT(KO) mice, where WT(KO) dams appeared to show significantly less of a time of day phase effect compared to WT(WT) mice ($F_{1,\ 24} = 6.4$, $p\text{-}value = 0.038$). Error bars represent SEM.

When analysing the data by genotype with phase as the within-subject factor, there was a significant difference between WT(WT) and WT(TG) dams ($F_{1,\ 25} = 5.8$, $p\text{-}value = 0.024$). This was driven by the difference between time spent on the nest in the dark phase of tracking between the two cohorts ($p\text{-}value = 0.002$). WT(TG) cohort spent significantly longer on the nest during the dark phase in contrast to the WT(WT) cohort of dams (**Figure** 4.14).

The average time spent on the nest by WT(WT) and WT(KO) dams across the two distinct phases demonstrated a difference between WT(WT) dams and WT(KO) dams similar to that seen between WT(WT) and WT(TG) mice. WT(WT) dams spent less time on average in the nest during the dark phase of monitoring compared to WT(KO) dams (**Figure** 4.14).

The time spent in the food zone in both the WT(WT) and the WT(TG) mice was significantly different between the light and dark phases of the trial ($p\text{-}value < 0.001$), indicating an effect of phase on time spent in the food zone. This was not the case for the WT(KO) mice which showed no significant difference between the time spent in

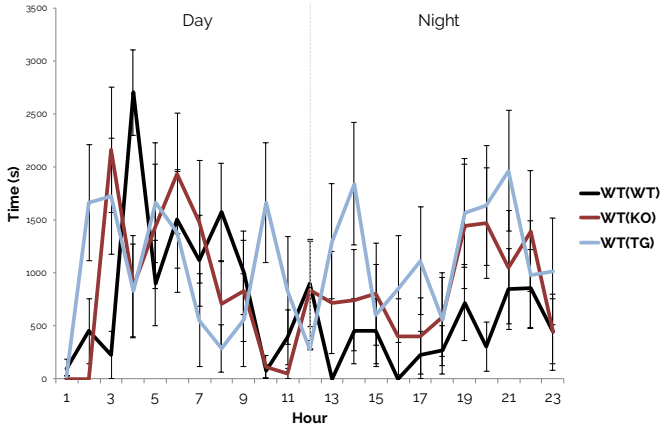


Figure 4.14: Hour by Hour time spent in the nest across the 23hr monitoring. There was an overall effect of day and night or light and dark phases upon the time spent in the nest for all the WT(WT) cohort *p-value* = 0.002), this was not seen in WT(TG) or WT(KO) dams, whom tended to spend similar amounts of time on the nest irrespective whether it was light or dark (WT(TG) *p-value* = 0.68, WT(KO) *p-value* = 0.96). This there was a significant difference between time spent on the nest for both WT(TG) and WT(KO) dams when compared to WT(WT) mice during the dark phase. The WT(TG) mice spent longer on the nest compared to WT(WT) in the dark phase ($F_{1,\ 25} = 5.8$, *p-value* = 0.024). The difference between WT(KO) and WT(WT) mice was similar with WT(KO) dams spending considerably more time in the nest zone compared to WT(WT) dams ($F_{1,\ 25} = 5.8$, *p-value* = 0.018). Error bars represent SEM.

the food zone during the light hours and the dark hours of the day (*p-value* = 0.16). The WT(WT) mice spent on average more time in the food zone during the dark phase of tracking compared to WT(TG) mice however this was only approaching significance ($F_{1,\ 25} = 3.5$, *p-value* = 0.071). In contrast when comparing WT(WT) mice with WT(KO) mice there was no effect of genotype on the duration of time in the food zone ($F_{1,\ 24} = 2.5$, *p-value* = 0.12).

4.2.10 FOOD AND WATER CONSUMPTION

Throughout the study food and water was regularly weighed for each dam. The total amounts of both food and water consumed by the three cohorts were recorded (**Figure** 4.17). When analysed there was not a significant difference in total consumption between the cohorts (*p-value* > 0.05,). There was a visible decrease in the amount of both food and water that WT(TG) dams consumed over the 48 hour period

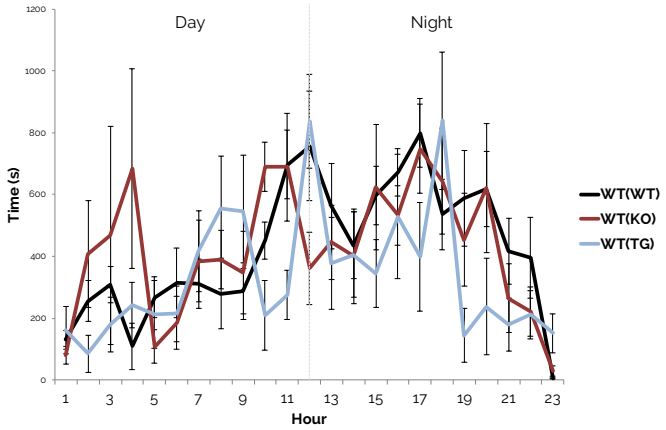


Figure 4.15: Hour by Hour time spent in the food zone across the 23hr monitoring. The time spent in the food zone per hour is displayed in the graph above. There was asignificant difference between the time spent feeding between the light and dark phases for both WT(WT) and WT(TG) mice with a *p-value* < 0.001. This was not evident in WT(KO) mice however whom showed no phase effects on feeding behaviour. There was also no significance when comparing phases between the three cohorts. WT(WT) mice tended to spend more time feeding compared to WT(TG) mice during the dark phase but this was only nearing significance ($F_{1,\ 25} = 3.5$, *p-value* $= 0.071$). Error bars represent SEM.

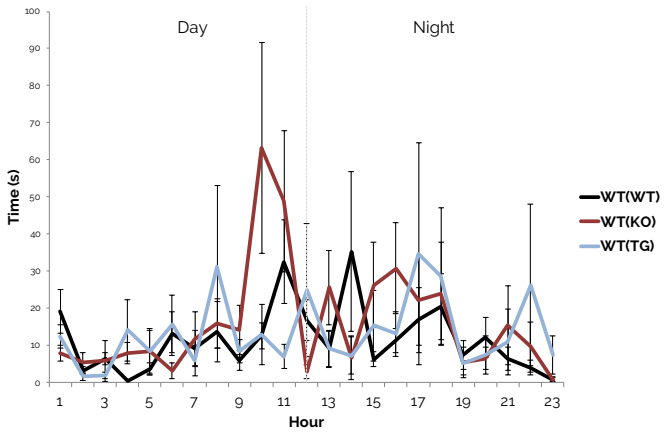
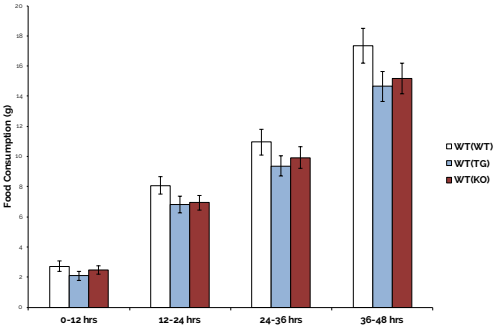
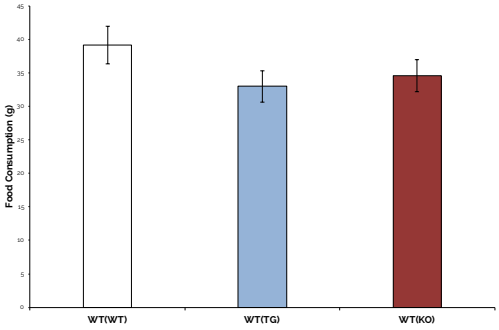


Figure 4.16: Hour by Hour time spent in the water zone across the 23hr monitoring. This graph shows the time spent in the water zone across the 23 hours of monitoring. There was no overall effect of phase on the time spent in the water zone for WT(WT), WT(TG) or WT(KO) mice (*p-value* $= 0.18$). WT(TG) mice compared to WT(WT) mice display no difference between time spent in the water zone within either the light or dark phases of the 23 hour monitoring ($F_{1,\ 25} = 0.29$, *p-value* $= 0.59$), as was the case for WT(KO) mice when compared to WT(WT) dams for both the light and dark phases of the monitoring ($F_{1,\ 25} = 0.26$, *p-value* $= 0.62$). Error bars represent SEM.

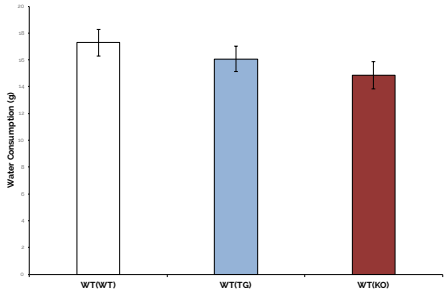
compared to WT(WT) dams, eating around 5-10 g less, but this didn't reach significance. WT(KO) dams didn't appear to consume any more than WT(WT) dams despite spending a larger proportion of time feeding, and actually showed a slight decrease in total consumption, however this was not significant.



(a) The total food consumed every 12 hours across the cohorts.



(b) The total amount of food consumed over 48 hrs.



(c) The total amount of water consumed over 48 hours.

Figure 4.17: Food and Water Consumption over 48 hrs. (a) This graph shows that whilst there is no significant difference between the groups over the course of each 12 hour period; there does seem to be a tendency for the WT(TG) dams to consume less food. **(b)** This graph summaries the previous graph and shows the total consumption of food over 48 hours for the three cohorts. There was no significant difference in total food intake between the groups of dams. **(c)** This graph shows the total water consumed by each of the cohorts over the 48 hour period. There was no significant difference between the groups, however WT(TG) mice did have a visibly lower total consumption than both WT(WT) and WT(KO) dams.

4.3 DISCUSSION

Overall, these series of assessments revealed phenotypic consequences related to the behaviour of WT dams exposed to placenta with either an enlarged or a reduced SpT.

By maintaining the WT status of the dams, these phenotypes can be attributed to the genotype of the transferred embryos. The major change driven by altered dosage of *Phlda2* is in the size of the SpT, a lineage which expresses *Prls*. Considerable data links these *Prls* to maternal care. WT(TG) dams performed better at the pup retrieval task and the nest building task compared to WT(KO) dams, and out performed the WT(WT) control group in relocating pups into the newly constructed nest in the nest building assay. This was contrary to what was predicted based upon the placental signalling hypothesis. The prediction was that, when the SpT was reduced (TG placental model), the WT dams would be exposed to less placental hormones including the Prls and would show an impaired maternal phenotype. Conversely, it was anticipated that dams carrying the *Phlda2* KO placenta with an enlarged SpT would be exposed to higher levels of placental hormones enhancing maternal behaviour. However, the WT(KO) cohort appeared to display differential care of their pups.

Interpretation of Behavioural Tests

Pregnancy in both humans and rodents is associated with changes in anxiety-related behaviours (Brunton and Russell, 2008, Grattan, 2011). Anxiety is generally increased in the postpartum period of pregnancy and is an often neglected aspect of psychopathology in new mothers (Farr *et al.*, 2014, O'Hara and Wisner, 2014). Although in recent times it is far more widely acknowledged (Matthey *et al.*, 2003, Miller *et al.*, 2006). For this reason it was critical to assess the anxiety levels of the dams. The initial behavioural testing focussed on the EPM, a well established anxiety assay in rodents (Walf and Frye, 2007). Using this assay it was not clear whether any of the cohorts exhibited an anxious phenotype. None of the models showed statistically significant differences in anxiogenic or anxiolytic behaviour. When looking at the duration of time spent in the three zones of the EPM it was clear that all three cohorts spent significantly more time in the closed arm compared

to the time spent in the open arm, which was indication that the assay fundamentally worked.

It is hypothesised that any potential phenotype could have been masked through the inclusion of the middle zone of the EPM. This is because the findings showed a possible increase in anxiety in both the WT(TG) and WT(KO) dams, but due to the presence of the middle zone the significance may have been masked by floor effects (**Figure** 4.4). The EPM is based around the paradigm that mice that are more anxious should spend more time in the closed arms of the maze and less anxious mice should spend more time in the open arms. The middle is a confounding section, however generally animals do spend time there. The dams demonstrated abnormal behaviour by spending the majority of their time in the middle zone of the EPM and very little time in the open zone. This behaviour was evident across all the cohorts. By including the middle zone, a zone that is neither open nor closed, it results in us being unable to determine the levels of anxiety an animal may be experiencing. This is due to the middle zone being either anxiolytic or anxiogenic. Thus, the floor effect of the animals spending very little time in the open zone and so much time in the middle means it is difficult to tease apart any group differences. It is possible that the reason the dams spent so much time in the middle is due to separation anxiety from their new offspring, however this test fails to confirm this.

The EPM is one of the commonest tests used to measure anxiety but may not be the most appropriate test to use in our model. As mentioned, the lack of clear results may be due to the timing of the assay, so close to parturition. Whilst the EPM has been used to study C57BL/6 BL6 mice, and at least one study on pregnant and postpartum females (George *et al.*, 2010, Golub *et al.*, 2016), it has not been regularly used previously with 129 mice and not at all with postpartum 129 females. The experimental work required the use of 129 mice due the the phenotypic differences between 129 and BL6 placenta focussing on the SpT compartment (Tunster *et al.*, 2012). Consequently, it is not possible to interpret the results of this test as a lack of an affect on anxiety. A second potential confounder is the timing of the test

procedure, on P2. Mice tested so soon after giving birth may have elevated stress that disguised any effect from placental dysfunction. Similarly, 129 mice are generally more anxious (Champagne *et al.*, 2007), which could be a factor in interpreting the results. Given the costs and complexities of generating the dams, it was not possible to repeat the assessment on a second set of animals but future work could involve a different time point, potentially both prenatally and post-natally. It may be possible to repeat this experiment using the transgenic modification bred in BL6. Alternatively, a modified version of EPM could be applied where there is no middle zone such as the Elevated Zero Maze (EZM) (Braun *et al.*, 2011, Kulkarni *et al.*, 2007). It would be worth trying altered levels of light in the test room as well to determine the optimum testing conditions to encourage greater exploration and activity. There are also other behavioural assays which measure anxiety such as the light dark box test or open field. Taking these test results together would help provide a more reliable anxiety profile of the dams.

It is usual that the pregnant state will generally have an anxiolytic effect on dams. This is to help them prioritise defending their offspring against otherwise anxiety inducing predators or danger, the aptly named "fight or flight" response. A typical arousal of the "fight" response behaviour is known to be induced by prolactin and oxytocin (Woodside, 2016). The EPM did not reveal alterations which could be used as a indicator of anxiety since all the dams essentially exhibited anxious behaviours. Despite the absence of a distinct anxiety phenotype, there were differences in the speed at which WT(KO) dams moved in the anxiogenic open zone with a mean velocity of 7 cm/s compared with both the WT(WT) and WT(TG) cohorts of dams moving slower, at 3 cm/s. This was only evident in the open arm and velocity was similar in the closed and middle zones for the WT(KO) dams. Although this difference in speed did not reach significance in a rigorous test ($p\text{-}value = 0.069$), the results warrant further investigation. While speed was potentially increased, the distance moved in the open zone during the EPM for WT(KO) dams was unchanged.

Two specific mothering behavioural tests were performed to determine levels of

maternal care in the dams. The pup retrieval task is perceived as a test for maternal responsiveness. This task highlighted a potential difference in the latency of time it took for WT(KO) mothers to locate then retrieve their pups. There was no initial difference in the time taken between the three cohorts to sniff their offspring, suggesting that there were no issues regarding any of the cohorts ability to recognise their pups. Crucially, however it doesn't distinguish how the dams initially recognise and respond to their pups. This could be due to their smell or the levels of vocalisation/calls produced by the pups being different. The observation that WT(KO) took significantly longer than both WT(WT) and WT(TG) dams to relocate the first pup into the heart of the nest indicated an impaired ability of WT(KO) mice to respond to their pups. This may be explained by the different genotypes of the pups born to WT(KO) and WT(TG) dams. *Phlda2* KO pups may be more or less recognisable by smell or call more or less to the dam. Testing this using ultra-vocalisation recordings and measuring pheromones given off by the offspring would help determine if this was the case. Either way this is an unlikely explanation since *Phlda2* is primarily expressed and imprinted only in the placenta and yolk sac (Tunster *et al.*, 2015). Another way to address this rigorously would be in a cross-fostering assay, replacing the dams pups with WT pups born naturally to WT dams, however, due to money and time contraints, this was not possible. As described in the next chapter, work was performed pre-parturition to explore this in more detail.

The second specific mothering behavioural task was nest building. This uncovered a significant deficit in nest building behaviour. WT(KO) dams only built a nest 40% of the time compared to WT(WT) dams that built a nest 60% of the time. In contrast, WT(TG) mice showed a much greater tendency to build a nest than WT(WT) and WT(KO) dams. WT(TG) dams successfully built a nest 85% of the time. All rodents build nests, but mothers are particularly likely to build a nest, to provide a warm and secure environment for the newborn (Deacon, 2006). Therefore nest building is commonly interpreted to represent an important feature of the maternal instinct. One interpretation of the contrasting nest building between

all three cohorts was to assign a hierarchy of "better" dams. Using this method it would follow that based solely on this test WT(TG) dams were inherently better nest builders and thus protected and cared for their pups better that WT(KO) mice and even WT(WT) dams. This is a dangerous stance to take however, and the term "better" even more so. When describing a behavioural phenotype, it is critical to be aware that there are, very often, numerous other behavioural differences that are affected in a knock-on response to another behavioural affect.

Nest building is a phenomenon that can be induced in virgin female mice through the presentation of young although their nest is smaller in size (Gandelman, 1973, Gandelman *et al.*, 1979). Gandelman and his colleagues also identified a distinct correlation between litter size and nest size in new dams (Gandelman, 1975). The latter may imply that any differences in nest quality could be attributed to litter size. In this study, there was no significant difference in litter sizes between the three groups so this can be excluded. It is important consider the interpretation of both nest building and pup retrieval. These assays are used to measure maternal care behaviour with slower retrieval/poor nest building used as an indicator of an inexperienced or unmotivated female. However, this interpretation in isolation should be made cautiously since potentially these behaviours could also reflect a reduced motivation to protect pups against predators manifesting in reduced agression or anxiety.

Ethovision - Monitoring of Behaviours

With the help of tracking software and manual scoring of the other key rodent behaviours described earlier (**Table** 2.10), additional alterations in behaviour were uncovered. These findings questioned the obvious conclusions that could have been drawn from the lack of nest building by WT(KO) mice and their apparent disinterest in their pups. In contrast to WT(TG) and WT(WT) dams, the WT(KO) dams spent

a significantly longer duration of their time grooming their pups and themselves during the nest building task, and on a more frequent basis. Licking and grooming of pups is a key maternal behaviour. Importantly, Zhang *et al.* (2013) showed that licking grooming in rats protects offspring from later life stress something that is further explored in chapter 6. The theory of differentially prioritising certain behaviours is further supported through the tracking of the three cohorts movement throughout the pup retrieval task. It can be clearly seen that during this test the WT(KO) dams took longer to retrieve their pups but they were quicker to visit the water and food zones compared to WT(WT) mice. This is interesting as it offers more support to the hypothesis that the placental endocrine compartment can influence maternal behaviour. Thus there are several interpretations of these marked differences in behaviour that WT(KO) dams display during Ethovision tracking. WT(KO) spent a larger proportion of their time on activities related to feeding and drinking, perhaps these dams are less receptive to the immediate needs of their pups (warmth and protection) and instead prioritise self-nurturing behaviours. Alternatively, there may be a secondary phenotype that requires dams to intake more food and water than either of the other cohorts. The third possibility is that these dams are less anxious and protective of their pups. Interpreting these findings is challenging in the absence of reliable data from the EPM test. Tracking of movement during the nest building task similarly indicated that WT(KO) mice travelled a significantly greater distance and visited the food zone more frequently.

The final behavioural assessment involved examining 23 hours of behavioural tracking analysis data from P3-4 for each dam. This was utilised to examine the behavioural phenotype over a natural daily cycle of "light" and "dark" or "day" and "night". Mice are typically nocturnal animals so it was expected that activity would naturally increase during the night time hours and decrease during the day. This was confirmed for WT(WT) mice with regards to the distances travelled between these two phases and similarly the duration of time spent in the nest. This was not the case for the WT(TG) and WT(KO) dams neither of whom displayed phase effects between

day and night. These observations moreover lent further support to the earlier observation that WT(TG) mice tended to spend more time building a nest and less time feeding themselves during the nest building task. It is important to note however, that there was no difference in overall food intake between groups, there was just a preoccupation by some groups to perform certain behaviours more regularly and for longer than other groups.

The lack of a phase effect during the 23 hour monitoring supports the idea that WT(TG) mice spend a larger proportion of their time nesting, suggesting perhaps that this behaviour is a persistent trait not brought on through stress. This is evident as during this period there was no tasks given to the dams and the behaviour was done entirely without direction from external stimuli. Similarly when looking at the time spent in the food zone across the two phases it is evident that unlike WT(WT) and WT(TG) cohorts that exhibit obvious phase effects in relation to time spent in the food zone, WT(KO) dams did not. In contrast, WT(KO) dams tended to spend more of their time feeding consistently over both light and dark phases.

Caveats to Behavioural Characterisation

This work focused on the maternal behaviours exhibited by the mice. However, it should be appreciated that other biological processes are involved that may be dictating behaviours independently of the maternal instinct. The metabolism of the dam may be altered during pregnancy with post natal consequences. Tunster *et al.* (2010) showed that *Phlda2* manipulates the placenta's demands for maternal resources by the fetus and that the SpT signals to stimulate the global accumulation of glycogen in the placenta. The demand for these resources by the fetus may put stress on the maternal metabolism resulting in the dams needing to obtain more food more frequently postnatally. Prls stimulate development of the mammary gland for lactation (Bridges, 1994, Flietstra and Voogt, 1996). Increased or decreased

milk production could influence the time the dams spend feeding themselves and their pups. Flietstra and Voogt (1996) showed that rat Prls initiate and maintain lactation but inhibit suckling-induced prolactin release. If this is the case in our WT(KO) dams, it could mean that the dams are getting less positive feedback from suckling their pups meaning a less intense release of oxytocin, a known stimulator of maternal behaviour (Ramos *et al.*, 2008). These factors may contribute to the altered behaviour displayed by these dams, including the more frequent trips to the food and water zones. There is also the genotype of the pups to consider, as discussed earlier. Some complex experiments will be required to further tease out cause and effect relationships, which will be discuss in greater detail in chapter 6.

Conclusion

The data presented in this chapter lend support to the hypothesis that a structurally altered placental endocrine compartment impacts the behavioural phenotype of the dam. The research presented allows for a role of the maternal genotype to be excluded, as all the dams were genetically WT and all cohorts were generated by the same RET protocol. Litter size can be excluded as there was no significant differences between the cohort. It is not possible to exclude a role for pup genotype in the behavioural changes. In the next chapter we explore whether the behavioural changes seen in this chapter are due to an alterations in the maternal brain or other physiological factors related to pregnancy. Importantly, the major behavioural changes seen do not necessarily confer an advantage or a disadvantage to any of the three cohorts of dams. Despite WT(TG) being more effective at retrieving their pups, building a nest and moving pups into their nest, this may not equivalent to beneficial nurturing. These dams may be neglecting their pups in different ways not explicitly tested for in the chosen behavioural assessments, although weight data at P7 showing catch up growth does indicate that they are doing a good job. Conversely the reduced retrieval and nest building behaviour of the WT(KO) dams

may not be equivalent to maternal neglect. Evidence that this might be the case is demonstrated by the increased levels of grooming and feeding behaviour displayed by WT(KO) dams during nest building. It should also be noted that WT(KO) mice tended to increase in activity when presented with a stressful or different task. At this point the mechanisms by which these behavioural changes are initiated are not well understood. Research beginning to address this deficit will be presented in the next chapter.

Summary of Findings

- The EPM did not uncover a group specific anxiety phenotype.
- WT(KO) dams took significantly longer to retrieve their offspring in the pup retrieval task.
- During pup retrieval WT(KO) mice prioritised trips to the food and water zones over retrieving their offspring.
- Throughout the Pup retrieval task WT(TG) dams performed much the same way as WT(WT) mice.
- WT(TG) dams displayed a heightened tendency to construct a well developed nest and move their pups inside the new nest compared to both WT(KO) and WT(WT) dams.
- WT(KO) mice failed to build a nest the majority of the time during the nest building assessment.
- During the hour long nest building task WT(KO) dams tended to spend a larger proportion of their time grooming their pups and themselves as well as travelling a greater distance and paying more frequent trips to the food and water zones.
- There was a lack of phase effect for WT(TG) dams in relation to time spent in the nest over a 23 hour cycle.
- There was also a lack of phase effect for WT(KO) mice with regards to the time spent visiting the food zone during the chosen 23 hour period.

Maternal Biomolecular Characterisation

5

5.1 Overview

The aim of this chapter was to perform an initial basic biomolecular characterisation and begin to provide insight into the mechanisms driving the behavioural phenotype from the previous chapter (Chapter 4). It is known that experimental infusion with prolactin and placental lactogen stimulates maternal behaviour in non-pregnant females (Bridges and Grattan, 2003, Larsen and Grattan, 2010). The evidence previously described in chapter 1, supports the idea that prolactin and the Prls are crucial in the induction of the maternal instinct but the exact mechanism is not well understood. It is known that prolactin and/or the Prlr are involved with pregnancy related adult neurogenesis in the SVZ of the hippocampus (Larsen and Grattan, 2012, Shingo, 2003, Walker *et al.*, 2012). The SpT compartment expresses a number of placental hormones and our findings in the previous chapter (Chapter 3) lend support to the hypothesis that Prls are important in the development of maternal instinct.

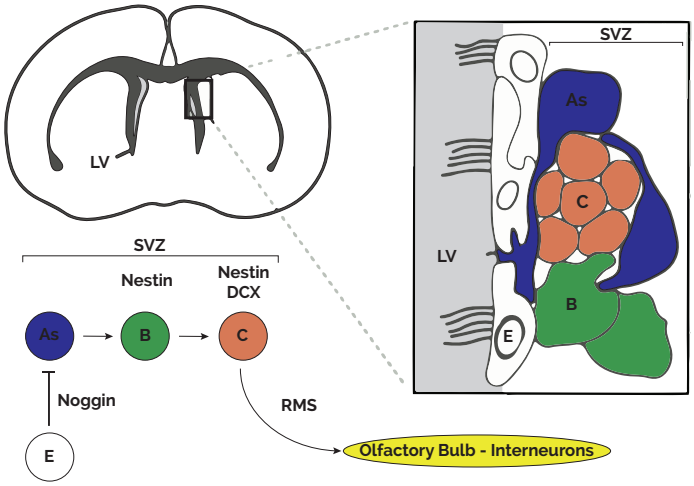
The majority of studies that look at prolactin during pregnancy have focussed on the indirect effect that prolactin infusions have upon maternal behaviour (Bridges, 1994, Bridges and Freemark, 1995, Bridges *et al.*, 1990). In this study, neurogenesis was not examined, but has since been cited by Bridges and Grattan (2003) as an exciting idea to investigate. Using the novel *Phlda2* mouse model, in which solely the SpT lineage of the placenta is altered (Tunster *et al.*, 2015), we examined both gene

expression and neurogenesis levels in the brain prenatally across the three cohorts of mice i.e. preceding the time at which behavioural changes were discovered and prior to exposure to the genetically altered pups. The identification of changes during pregnancy, at a time when placental signalling is altered, would lend support to the hypothesis that altering the size of the SpT drives the altered behaviour rather than exposure to the genetically altered pups.

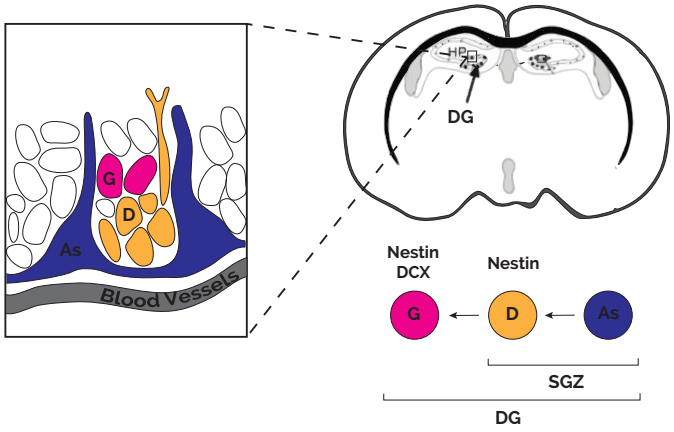
A series of biomolecular and genetic assays were performed to build up a basic characterisation of the cohorts at E16.5, the time when the placenta is known to be signalling most intensely and before any behavioural phenotypes were examined in chapter 4. It allows a direct assessment of an *in vivo* alteration of these hormones which makes it a useful way to assess the biomolecular outcome at E16.5 in the brains of the dams. Importantly the persistency of any key gene changes that are seen were tested again at P7 in the brain of the dams.

5.1.1 Maternal Neurogenesis and Pregnancy

In the adult mammalian brain two distinct regions retain neural stem cells that can continually, and do, generate new neurons. Along the walls of the Lateral Ventricles (LV) you find the largest of the two germinal zones, the SVZ (Alvarez-Buylla and Garcia-Verdugo, 2002, Doetsch and Alvarez-Buylla, 1996). The second region is the SGZ, found in the dentate gyrus part of the hippocampus. These specialised micro environments or "niches" are spatially isolated but they have common themes that have helped define what it takes to be an adult neurogenic niche (Alvarez-Buylla and Lim, 2004). The differing locations of these niches and the simplified cascades that lead astrocytes to turn from neural stem cells into progenitors or neural precursors and then into new neurons are outlined in **Figure** 5.1.



(a) Neurogenesis Schematic of SVZ.



(b) Neurogenesis Schematic of SGZ.

Figure 5.1: Neurogenesis. A schematic digram showing adult neurogenesis, "As" Cells (blue) are the astrocytes in both coronal sections in **(a)** and **(b)**. **(a)** Shows the lateral ventricle (LV, light grey) which is filled with cerebrospinal fluid. The LV and SVZ is enlarged in the box. As Cells act as the SVZ stem cell and also serve as niche cells. Ependymal cells (E, white) release noggin that helps in the initiation of the As cells becoming the precursor cells (B, green). Precursor cells are rapidly dividing, transit-amplifying cells derived from the As cells. Precursor cells give rise to neuroblasts (C, red), these neuroblasts then migrate to the olfactory bulb via the Rostral Migratory Stream (RMS), where they become local interneurons. **(b)** This coronal section through the adult mouse brain at the level of the hippocampus (HP) indicates the dentate gyrus (DG). The enlarged cross section shows the architecture of the SGZ. Astrocytes give rise to progenitors (D, orange), which mature into new granule cells (G, pink). These newly born granule cells integrate into the DG. Adapted from Alvarez-Buylla and Lim (2004).

The SVZ

As one of only two areas of the adult brain that has been shown to experience neurogenesis, the SVZ has the largest population of proliferating neuronal cells. The new neurons born in the SVZ migrate along the Rostral Migratory Stream (RMS) where they mature into local interneurons (Altman, 1969, Pencea *et al.*, 2001). SVZ neural stem cells have been shown to be able to be grown in culture medium (Kempermann and Gage, 2000). This is important as it demonstrates that the SVZ is an important source of these progenitors that not only add to the plasticity of the adult brain but also may be a reservoir of cells that may ultimately be useful in combating neurodegenerative disease.

The new neurons born in the SVZ that move into the RMS end up in the Olfactory Bulb (OB). A phenomenon that has been well studied in rodents but similar pathways have been shown in primates (Kornack and Rakic, 2001, Pencea *et al.*, 2001), and suggested may occur in infant human brains (Weickert *et al.*, 2000). The migration along the RMS occurs through the neurons moving along each other using microtubule polymerization and depolymerization (Alvarez-Buylla and Garcia-Verdugo, 2002). Doublecortin (DCX) is a microtubule protein that is found to be key in neural migration in development. It is predominately expressed by chains of cells in the RMS suggesting that it plays a role in the translocation of the new neurons to the OB (Alvarez-Buylla and Garcia-Verdugo, 2002, Gleeson *et al.*, 1999, Magavi *et al.*, 2000). The total number of neurons that migrate to the OB far outweighs the number that survive and become functional. Less is known about what these neurons are invloved in once they are fully functional, compared to the new neurons that are born in the DG of the hippocampus. The general consensus is that through the replacement of neurons in the OB, it is possible to maintain the plasticity necessary to adjust the olfactory circuitry based on new environments and experiences (Alvarez-Buylla and Garcia-Verdugo, 2002). It follows therefore that postnatally the mother has generated new neurons in order to effectively adapt to the change in environment

and novel experiences that are commonplace with newborn offspring. Once these new connections are made and the circuitry remodelled there is a period of intense cell death that adds to this theory's weight (Cecchi *et al.*, 2001).

The SGZ

The SGZ of the hippocampal DG has been shown to generate new neurons robustly in both rodents and humans throughout life (Eriksson *et al.*, 1998). The new neurons leaving the SGZ migrate into the adjacent DG granule cell layer. Neurogenesis in these regions is regulated by physiological factors including sex hormones, glucocorticoids, growth factors and stress (Cameron and McKay, 1999, Kirschenbaum *et al.*, 1999, Kuhn *et al.*, 1997, Shingo, 2003, Tanapat *et al.*, 1999). There is considerably less neurogenesis in the SGZ compared to the SVZ, something that is not well understood (Curtis *et al.*, 2012). Despite this it is considered an extremely important source of new neurons, perhaps due to the role that the hipocampus as a whole plays in the functioning brain. The hippocampus is part of the limbic system, which is involved in a host of higher order brain functions. These include emotionality, behaviours, memory and learning (Curtis *et al.*, 2012, O'Keefe and Nadel, 1978). Importantly neurogenesis in the hippocampus and DG cell proliferation have been demonstrated experimentally to impact upon learning and social behaviours (Hockly *et al.*, 2002, Malberg and Duman, 2003) also neurogenesis has been linked to environmental enrichment and depression (Lieberwirth *et al.*, 2012, Lucassen *et al.*, 2010). Interestingly studies have shown that social environment can influence rate of neurogenesis, reviewed by Lieberwirth *et al.* (2012). Positive social interaction such as mating increases neurogenesis, whilst negative interactions such as social isolation causes a reduced rate of adult neurogenesis in the limbic system, which highlights a correlation between the phenomenon and mental health outcomes.

5.1.2 Biomolecular Characterisation Assays

When considering behavioural phenotypes it is essential to assess changes in the brain regions that might be associated with these behaviours. The two areas we looked at were: the hypothalamus, which is associated with maternal behavioural adaptations and the hippocampus, which is a known location for neurogenesis and is strongly associated with psychotic illness. Therefore these are the two brain regions that were focussed upon for the majority of the characterisation.

DNA microarray is a high throughput technology that allows the expression levels of a vast number of genes to be measured simultaneously. It has been used for many years and is a helpful method in developing hypotheses and also in helping answer proposed hypotheses (Hoheisel, 2006). There can be problems with microarray analysis in the *post hoc* stages of the analysis, however the development of programs and an active online community means that statistical analysis using various statistical programs is becoming more and more precise and reliable. One such platform that uses the R statistical package is the Bioconductor platform. The LIMMA analysis method was the preferred statistical package used to analyse the microarray data in this chapter. This is because LIMMA offers an unbiased approach to microarray data generated through an Affymetrix array of the mouse genome (mogene) (Zhang *et al.*, 2009).

Microarray is useful in determining gene expression changes within a given system, whilst RT-qPCR or qPCR is the staple method for confirming and quantifying gene expression changes highlighted through other higher throughput means. This is because by measuring the amount of cellular RNA it is possible to accurately determine the extent of a given genes expression. In order to quantify this expression the end point of the qPCR is needed, this is known as the threshold cycle (C_T). The C_T is the point at which the fluorescence signal of the reporter dye (e.g. SYBR Green) passes an arbitrary value in the exponential phase of PCR amplification. The C_T value is inversely related to the amount of amplicon in the reaction (Schmittgen

and Livak, 2008). Therefore a subset of genes identified in the microarray needed to be confirmed by qPCR in order to validate the results.

It was necessary to establish the effects, if any, these different placenta had upon normal neurogenesis levels present in the maternal brain during pregnancy. There are many methods that can be used to assess neurogenesis in the adult rodent brain, most of which are outlined by Pan *et al.* (2013) in their paper. The most common method, due to its ability to "birthdate" cells as they proliferate and/or differentiate into a specific cell type, is Brdu (BrdU) labelling. BrdU labelling of cells is permanent and marks cells in the S-phase of the cell cycle. This method is flexible and convenient due to its ability to work hours or months after administration of BrdU into the animal and helps identify Adult Neural Stem Cells (aNSCs) (Ming and Song, 2005, Taupin, 2006). Therefore mice were injected with BrdU as described in chapter 2, and tissues harvested 3 hours later. IHC staining performed on the relevant brain sections to look at cells positive for BrdU. Importantly, this approach allowed assessment for Neuroectodermal Stem Cell Marker (Nestin) (a label for neural precursor cells) and DCX (a label for the cytoskeleton of new neurons) in order to further examine neurogenesis. The behavioural changes exhibited by the dams meant that HPLC analysis was undertaken on the full range of brain regions that were dissected. This generated data regarding neurotransmitter levels and there metabolites, this would enable us to determine whether behavioural changes could be attributed to changes in neurotransmitter levels in the dams brains.

5.2 RESULTS

Mice were generated following the same RET methodology previously outlined in chapter 2 and 4. They were all carefully monitored throughout pregnancy in order to check for health and determine pregnancy by regularly measuring their weight every two days from P 8.5 and comparing it to pre-pregnancy weight. Mice were sacrificed

on E16.5 of gestation, a time at which the SpT is maximally expanded and placental signalling presumed to be at its maximum. The relevant biological samples were collected. Fetuses were also collected and weighed. For the WT(TG) model, fetuses were also genotyped as these litters were generated by crossing heterozyogus parents. Only litters with $> 60\%$ TG fetuses were used for downstream analysis (**Figure** 5.2). Dams were only used if there litter was between 6 and 12 embryos (**Table** 5.1).

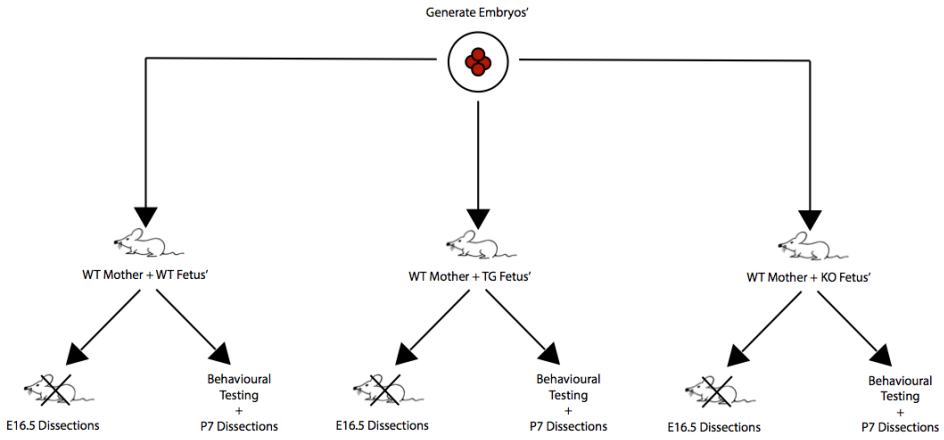


Figure 5.2: Flow Diagram Showing Experimental Design. A flow diagram illustrating the overall methodology and procedure used to generate the material and resulting data for the maternal behavioural characterisation and the biomolecular characterisation of the dams at E16.5.

Table 5.1: E16.5 Litter Sizes

Embryo Genotype	Litter Size (SEM)
WT	7.14 (± 0.63)
TG	6.91 (± 0.25)
KO	7.08 (± 0.35)

5.2.1 MICROARRAY

For each cohort of dams RNA was extracted from the hippocampus and hypothalamus. There was an n = 4 for each group. The samples were all sent to Cardiff Biotechnology Service for quality control and microarray. RNA was only used if it had a RIN value of higher than 7 (**Table** 5.3, **Table** 5.2 and **Figure** 5.3) (Schroeder *et al.*, 2006).

Table 5.2: RNA Values for E16.5 Microarray of Hippocampus

Sample ID	Tissue	RNA ng/µl	RIN Value
WT(WT)1	Hippocampus	487.0	10
WT(WT)2	Hippocampus	427.4	10
WT(WT)3	Hippocampus	325.0	10
WT(WT)4	Hippocampus	340.7	10
WT(TG)1	Hippocampus	248.2	10
WT(TG)2	Hippocampus	282.6	9.9
WT(TG)3	Hippocampus	411.2	10
WT(TG)4	Hippocampus	445.2	10
WT(KO)1	Hippocampus	336.5	10
WT(KO)2	Hippocampus	251.8	10
WT(KO)3	Hippocampus	254.1	10
WT(KO)4	Hippocampus	693.0	10

Table 5.3: RNA Values for E16.5 Microarray of Hypothalamus

Sample ID	Tissue	RNA ng/µl	RIN Value
WT(WT)1	Hypothalamus	309.9	10
WT(WT)2	Hypothalamus	217.9	10
WT(WT)3	Hypothalamus	145.9	10
WT(WT)4	Hypothalamus	188.7	10
WT(TG)1	Hypothalamus	171.1	10
WT(TG)2	Hypothalamus	151.0	9.7
WT(TG)3	Hypothalamus	218.3	10
WT(TG)4	Hypothalamus	142.5	10
WT(KO)1	Hypothalamus	192.3	10
WT(KO)2	Hypothalamus	297.1	10
WT(KO)3	Hypothalamus	280.6	10
WT(KO)4	Hypothalamus	154.7	10

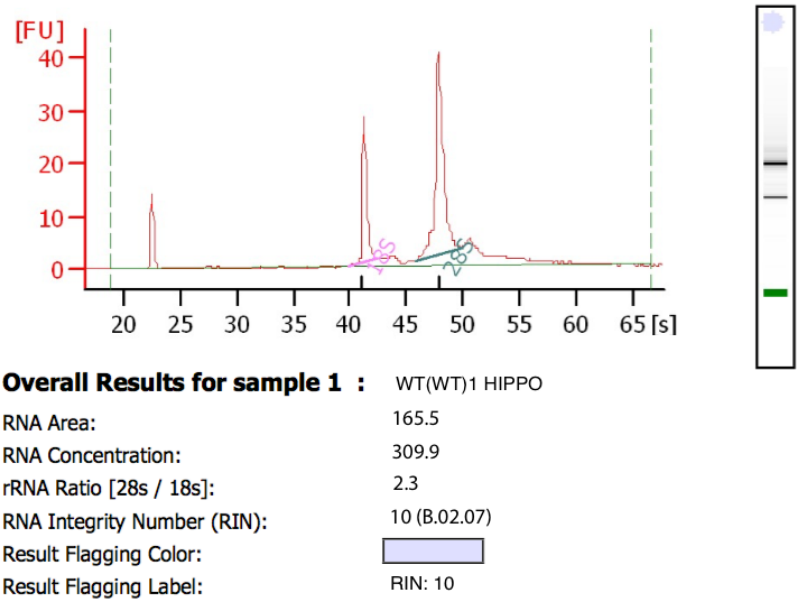


Figure 5.3: RIN Value Graph. An example of the RIN value graph used to determine RNA quality.

Before analysis took place images of the Affy chips were generated from the .CEL files, these were then assessed for any damage or visual inconsistencies that may have affected the analysis and read outs from the chips. Two examples of the images generated can be seen in **Figure** 5.4. There were no visual inconsistencies present on the chips and no obvious signs of damage that would cause issues with the further analysis of gene expression levels.

Heat Maps

Using the .CEL files it was then possible to write a custom script using both python and R Statistical software language to extract the information on the chips about gene expression levels at a genome wide level (**Figure** 5.5).

Initially, heat maps were generated using the Heatplus package on R statistical software. This was to immediately identify visually whether there were any distinct

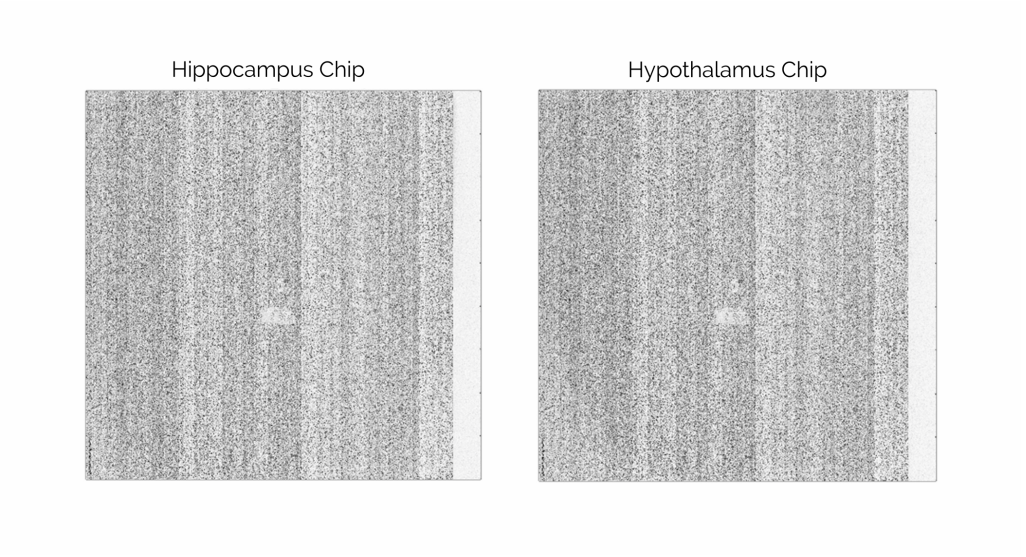


Figure 5.4: Example of Affy Chips used for Microarray. An example of the Chips used for both the Hippocampus and the Hypothalamus. Chips were assessed for any damage before being included in the microarray analysis.

gene changes between the three groups. Heat maps were plotted for the genes that showed changes in expression levels at a p-value < 0.05 in the hippocampus and the hypothalamus. Specifically, the LIMMA analysis organised the subjects independently of their genotype, based upon similar gene expression levels. This resulted in the dendrogram placing the WT(WT), WT(TG) and WT(KO) gene profiles together (**Figure** 5.6 and **Figure** 5.7). The only exception was WT(KO)-1 in the hippocampus that appeared more similar in gene expression profile to that of the WT(WT) controls.

The heat maps provided a visualisation of the general pattern of fold changes for the gene chips across the samples and between the cohorts.

Venn Diagrams and Gene Lists

Further analysis allowed the generation of a table of genes based on the heat maps that showed the altered expression across the WT(WT), WT(TG) and WT(KO) dams and between each of these cohorts (**Table** 5.4). This table was plotted into a

hippocampus.r — Microarray R Scripts

```
#=============================================================================
# R workflow for analysing Affymetrix Mogene 2 Chips.
#
#                                                                  Hugo Creeth
#=============================================================================



#=============================================================================
# Install packages
#=============================================================================
#source("http://bioconductor.org/biocLite.R")
#biocLite("oligo")
#biocLite("Heatplus")
#biocLite("mogene20sttranscriptcluster.db")
#biocLite("limma")


#=============================================================================
# Import needed libraries
#=============================================================================
library(oligo)
library(Heatplus)
library(limma)
library(mogene20sttranscriptcluster.db)
library(xtable)
library(sigPathway)
library(goTools)


#=============================================================================
# Make a variable containing every possible annotation for the chip for
# use later when outputting gene names.
# Currently adding GENENAME, SYMBOL and ENSEMBL data only.
# For further options see the mogene20sttranscriptcluster datasheet online.
#=============================================================================
allannotations <-
data.frame(GENENAME=sapply(contents(mogene20sttranscriptclusterGENENAME), paste,
collapse=", "), SYMBOL=sapply(contents(mogene20sttranscriptclusterSYMBOL), paste,
collapse=", "), ENSEMBL=sapply(contents(mogene20sttranscriptclusterENSEMBL), paste,
collapse=", "))


#=============================================================================
# Import CEL files
#=============================================================================
celFilesDirectory <- "../CELfiles"
celFiles <- list.celfiles(celFilesDirectory,full.names=TRUE)
affyRaw <- read.celfiles(celFiles[14:25])
```

Line: 4:73 | R | Tab Size: 4

Figure 5.5: Microarray R Script. A screenshot of the microarray R script used to analyse the .CEL files

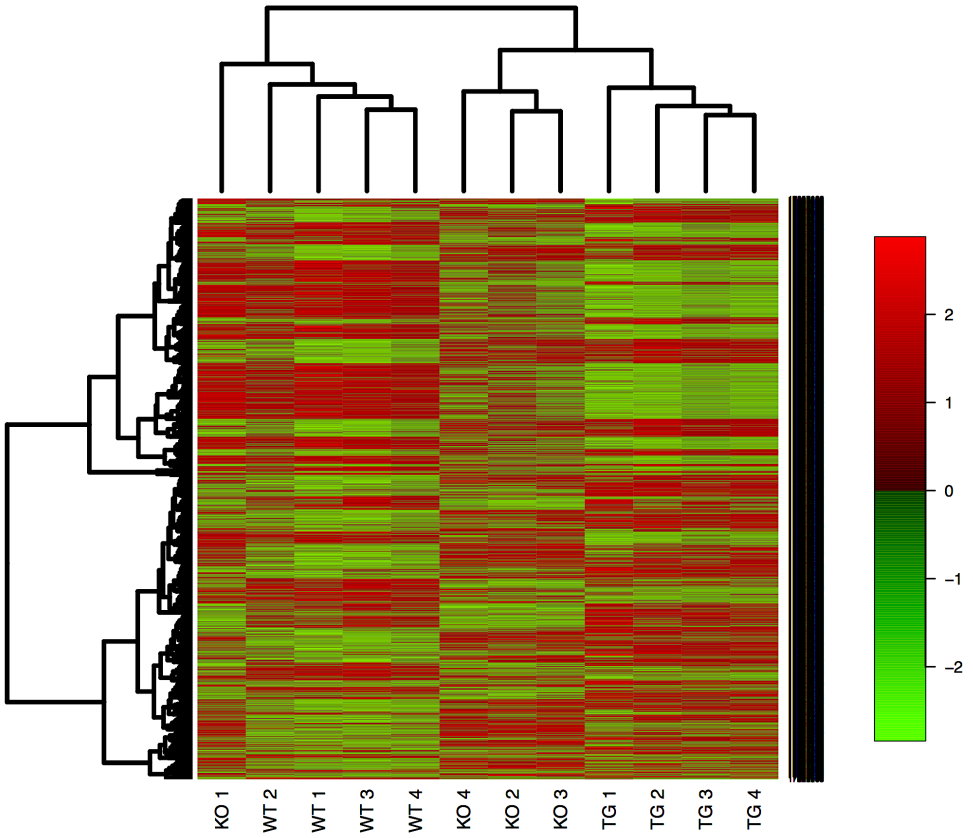


Figure 5.6: Heat map indicating expression levels of genes in the Hippocampus. A three way heat map showing the differing gene expression levels from the microarray across the three cohorts in the hippocampus. Red indicates up regulated genes and green represents down regulated genes.

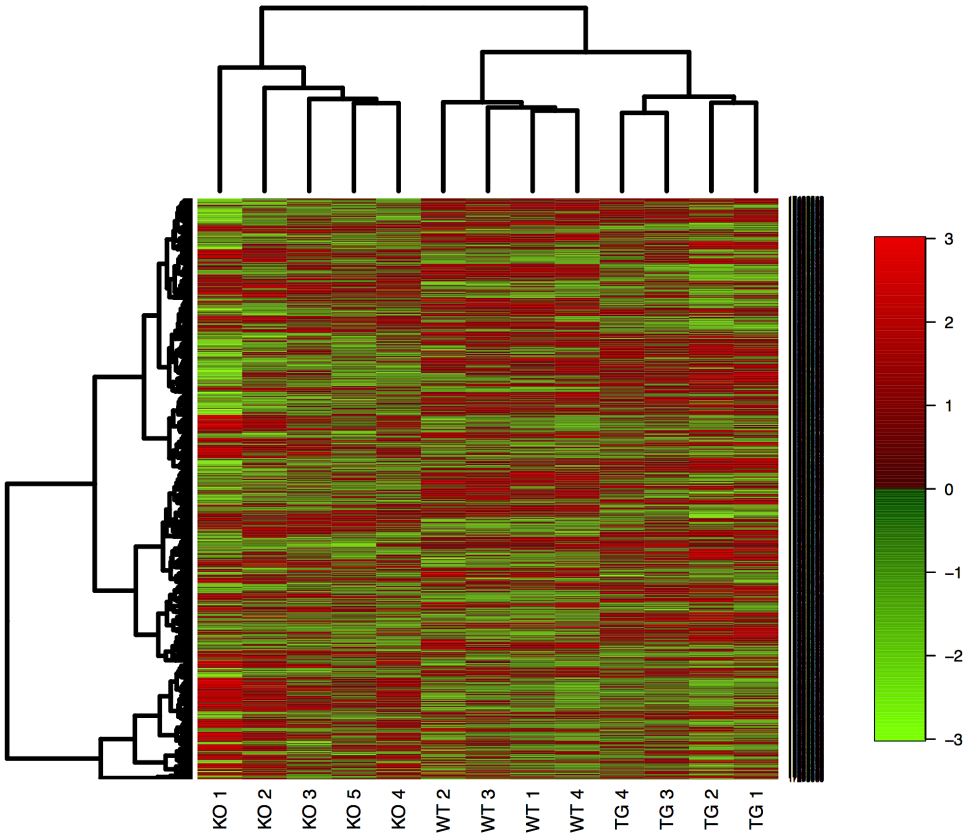


Figure 5.7: Heat map indicating expression levels of genes in the Hypothalamus. A three way heat map showing the differing gene expression levels from the microarray across the three cohorts in the hypothalamus. Red indicates up regulated genes and green represents down regulated genes.

series of Venn diagrams (**Figure** 5.8) according to the tissue type being analysed. The number of genes that are listed were selected based on the standardised p = 0.05 significance level and show the pairwise comparisons across all the samples. The number of significant genes that showed altered gene expression between all three cohorts in the hypothalamus was 8 and the hippocampus was 54. The genes that differed from WT(WT) mice in both the WT(TG) and WT(KO) cohorts totalled 288 and 1279, in the hypothalamus and hippocampus respectively. The number of genes that were uniquely different in expression levels between the WT(TG) dams hypothalamus and the WT(WT) dams hypothalamus was 640. This number increased dramatically in the hippocampus where 3297 specific genes showed altered expression between the WT(WT) and WT(TG) dams alone. In contrast the unique differences between the WT(KO) dams and the WT(WT) dams remained fairly similar in number between the hypothalamus and the hippocampus. There were 1138 genes specifically that showed differing expression between WT(WT) and WT(KO) mice in the hypothalamus and 1472 genes that were uniquely different between the two in the hippocampus.

Taking this table of uniquely altered gene expression levels meant that entire lists of these genes could be produced based on the specific comparisons that were of interest. These two tables showing a selection of the top gene changes between WT(WT) dams and both WT(KO) and WT(TG) dams in both the hippocampus and hypothalamus, that were then taken on into qPCR analysis to confirm theses gene changes.

Pathway Anaylsis

In order to identify scientifically relevant biological pathways of importance an *in silico* pathway analysis was performed online using DAVID. To use DAVID a gene list is inputted into the online portal and various parameters selected based on the type of gene list that is submitted. The software provides a comprehensive set of

Table 5.4: Gene Expression Changes

Genotype Comparisons	Brain Region	Number of Genes
WT(WT) vs WT(TG) vs WT(KO) - Top gene changes shared between all groups	Hippocampus	54
WT(WT) vs WT(TG) vs WT(KO) - Top gene changes shared between all groups	Hypothalamus	8
WT(WT) vs WT(TG):WT(KO) - Gene changes that were different between WT(TG) and WT(KO) compared to WT(WT)	Hippocampus	1279
WT(WT) vs WT(TG):WT(KO) - Gene changes that were different between WT(TG) and WT(KO) compared to WT(WT)	Hypothalamus	288
WT(WT) vs WT(TG) - Gene changes that were uniquely different between WT(WT) and WT(TG)	Hippocampus	3297
WT(WT) vs WT(TG) - Gene changes that were uniquely different between WT(WT) and WT(TG)	Hypothalamus	640
WT(WT) vs WT(KO) - Gene changes that were uniquely different between WT(WT) and WT(KO)	Hippocampus	1472
WT(WT) vs WT(KO) - Gene changes that were uniquely different between WT(WT) and WT(KO)	Hypothalamus	1138

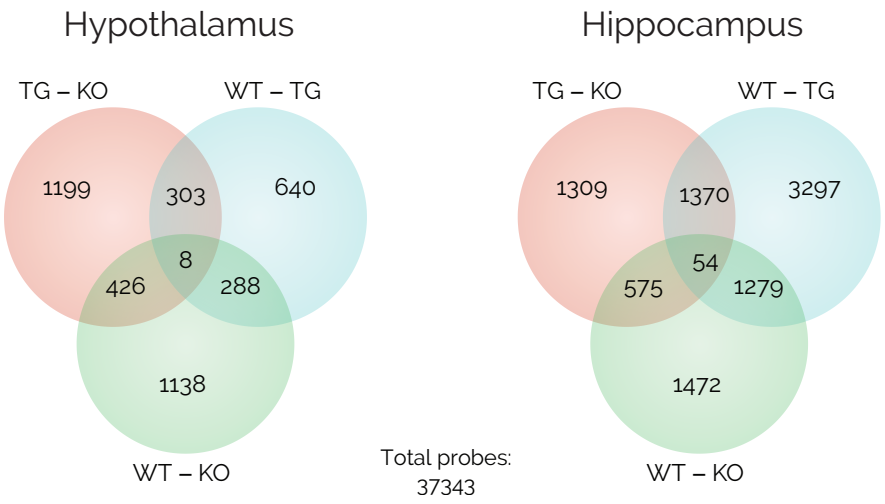


Figure 5.8: Venn Diagrams for Number of Significantly Altered Genes in the Hippocampus and Hypothalamus. The Venn diagrams from the Limma analysis showing the genes that are significantly altered across the genotypes and between genotypes ($p < 0.05$). **A** is the Hippocampus and **B** is the hypothalamus. The total number of probes for each array can be seen in the bottom of the diagram.

Table 5.5: Top Genes of Interest in the Hippocampus Microarray

Gene name	**Symbol**	↑ ↓ Regulated and **p-value**
Solute Carrier Family 6 Member 3	*Dat*	↓ 0.02
Urocortin 3	*Ucn3*	↓ 0.02
Potassium Voltage-Gated Channel Subfamily H Member 2	*Kcnh2*	↓ 0.01
Tyrosine Hydroxylase	*Th*	↓ 0.02
Tryptophan Hydroxylase 2	*Tph2*	↓ 0.02
Endonuclease Domain Containing 1	*Endod1*	↓ 0.01
Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta	*Ywhaz*	↑ 0.03
5-Hydroxytryptamine Receptor 2A	*Htr2 (5-HT2)*	↑ 0.03

Table 5.6: Top Genes of Interest in the Hypothalamus Microarray

Gene name	**Symbol**	↑ ↓ Regulated and **p-value**
Olfactory Receptor 45	*Olfr45*	↑ 0.001
Olfactory Receptor 59	*Olfr59*	↑ 0.003
Olfactory Receptor 347	*Olfr347*	↑ 0.04
Olfactory Receptor 481	*Olfr481*	↑ 0.05
Olfactory Receptor 615	*Olfr615*	↑ 0.007
Olfactory Receptor 686	*Olfr686*	↓ 0.002
Olfactory Receptor 945	*Olfr945*	↑ 0.02
Olfactory Receptor 1239	*Olfr1239*	↑ 0.02
Olfactory Receptor 1410	*Olfr1410*	↑ 0.004
Olfactory Receptor 1491	*Olfr1491*	↑ 0.002
Histocompatibility 2 M region locus 2	*H2-M2*	↓ 0.04
Histocompatibility 2 M region locus 9	*H2-M9*	↑ 0.006
Histocompatibility 2 Q region locus 10	*H2-Q10*	↓ 0.02

tools that allow for any number of the following:

- Identify enriched biological themes/pathways, particularly GO terms.
- Discover enriched functional-related gene groups.
- Cluster redundant annotation terms.
- Visualize genes on BioCarta and KEGG pathway maps.
- Display related many-genes-to-many-terms on 2-D view.
- Search for other functionally related genes not in the list.
- List interacting proteins.
- Link gene-disease associations.

Gene lists that identified gene expression level changes between WT(WT) and both WT(TG) and WT(KO) dams were submitted generating lists of potentially affected biological signalling pathways that appear to have been altered between the three cohorts. The lists for both brain regions can be seen in **Table** 5.2.1 and **Table** 5.2.1.

Table 5.7: Hippocampus Signalling Pathways

Term	Count	%	*p-value*	Genes List	Gene	Total	Pop Hits	Pop Total	Fold Enrichment	Bonferroni	Benjamini	FDR
				ENSMUSG00000067038	*Rps12-ps3*							
				ENSMUSG00000039221	*Rpl22l1*							
Ribosome	5	1.26	0.09	ENSMUSG00000029614	*Rpl6*	113	89	5738	2.852739385502635	0.999955616995393	0.999955616995393	67.12471840125333
				ENSMUSG00000061983	*Rps12*							
				ENSMUSG00000000740	*Rpl13*							
				ENSMUSG00000062997	*Rpl35*							
				ENSMUSG00000078193	*Gm2000*							

Table 5.8: Hypothalamus Signalling Pathways

Term	Count	%	*p-value*	Genes List	Gene	Total	Pop Hits	Pop Total	Fold Enrichment	Bonferroni	Benjamini	FDR
				ENSMUSG00000049334	*Olfr902*							
				ENSMUSG00000061961	*Olfr815*							
				ENSMUSG00000057351	*Olfr347*							
				ENSMUSG00000054236	*Olfr481*							
				ENSMUSG00000045150	*Olfr1161*							
				ENSMUSG00000063380	*Olfr945*							
				ENSMUSG00000075088	*Olfr1239*							
Olfactory transduction	15	15.15	≤ 0.001	ENSMUSG00000059303	*Olfr836*	32	960	5738	2.8017578125	0.007371621970343023	0.007371621970343023	0.17581019457602043
				ENSMUSG00000073947	*Olfr615*							
				ENSMUSG00000070374	*Olfr59*							
				ENSMUSG00000066122	*Olfr45*							
				ENSMUSG00000063583	*Olfr1410*							
				ENSMUSG00000051172	*Olfr672*							
				ENSMUSG00000048425	*Olfr686*							
				ENSMUSG00000051156	*Olfr1491*							
				ENSMUSG00000067201	*H2-M9*							
Allograft rejection	3	3.03	0.04	ENSMUSG00000067235	*H2-Q10*	32	58	5738	9.274784482758621	0.776961542297281	0.5277305242737798	30.011214136503405
				ENSMUSG00000016283	*H2-M2*							
				ENSMUSG00000067201	*H2-M9*							
Graft-versus-host disease	3	3.03	0.04	ENSMUSG00000067235	*H2-Q10*	32	58	5738	9.274784482758621	0.776961542297281	0.5277305242737798	30.011214136503405
				ENSMUSG00000016283	*H2-M2*							
				ENSMUSG00000067201	*H2-M9*							
Type I diabetes mellitus	3	3.03	0.05	ENSMUSG00000067235	*H2-Q10*	32	63	5738	8.538690476190476	0.8260949051414685	0.44182450198195444	34.03291003241298
				ENSMUSG00000016283	*H2-M2*							
				ENSMUSG00000067201	*H2-M9*							
Autoimmune thyroid disease	3	3.03	0.06	ENSMUSG00000067235	*H2-Q10*	32	72	5738	7.471354166666667	0.893150810348408	0.4282675948938529	41.24868587153916
				ENSMUSG00000016283	*H2-M2*							
				ENSMUSG00000067201	*H2-M9*							
Antigen processing	3	3.03	0.09	ENSMUSG00000067235	*H2-Q10*	32	91	5738	5.911401098901099	0.9672102727309312	0.4951672037836681	55.63857938994323
				ENSMUSG00000016283	*H2-M2*							
				ENSMUSG00000067201	*H2-M9*							
Viral myocarditis	3	3.03	0.09	ENSMUSG00000067235	*H2-Q10*	32	94	5738	5.722739361702128	0.9732663062596048	0.45318154760493823	57.741370591514716
				ENSMUSG00000016283	*H2-M2*							

Collating the gene expression changes highlighted the signalling pathways that were enriched in the hypothalamus. There were 13 genes that have linked been with olfactory transduction, a critical element involved with maternal behaviour that were highly significant ($p\text{-}value < 0.001$). Other pathways that showed significant enrichment included: allograft rejection, graft-versus-host disease and type-1 diabetes mellitus ($p\text{-}value's < 0.05$). All of these pathways shared the same three genes H2-M9, H2-Q10 and H2-M2. These signalling pathways are also functionally relevant as pregnancy is an un-natural state in which the host (in this case the dam) is carrying an immunologically different entity (in this case the offspring/fetus) this may explain the relevance of these pathways showing enrichment. In contrast the hippocampus showed no significant enrichment in any biological pathways, although certain genes that grouped together, related to ribosome biology, were seen to be commonly altered between the WT(KO) and WT(TG) cohorts. Ribosomal proteins have been associated with neurogenesis (Ueno *et al.*, 2002).

5.2.2 CONFIRMATION OF RESULTS USING RT-QPCR

Having performed microarray, a number of genes with significant alteration in expression were identified. The gene expression levels for these subsets of genes were then quantified using qPCR. In order to verify the robust nature of these gene changes qPCR was performed on an entirely newly generated cohort of dams. There were 12 dams in total, with n = 4 for each group. RNA was obtained as described previously and processed to cDNA as described in chapter 2. By performing qPCR on this entirely new cohort of dams enabled any changes that concurred with the microarray results from the previous cohort to be confirmed via independent verification.

E16.5 Hippocampus qPCR

The genes assayed by qPCR, chosen to confirm the microarray calls, at E16.5 in the hippocampus demonstrated that *Ucn3, Kcnh2, Endod1, Th, Tph2* and *Dat (Slc6a3)* all showed significant fold change differences ($p\text{-}value < 0.05$) in the newly generated cohorts of WT(TG) and WT(KO) dams when compared to the WT(WT) dams (**Figure** 5.9, **Table** 5.9 and **Table** 5.10). This equated to a 75% concordance rate across the cohorts between the genes highlighted in microarray compared to the qPCR results.

Th decreased in expression level in WT(KO) mice compared to WT(WT) dams, with a $p\text{-}value = 0.022$. This was also significantly changed ($p\text{-}value = 0.036$) in the WT(TG) when compared to WT(WT) dams indicating a decrease in expression levels from the control group. *Dat* showed a highly significant reduction in expression in WT(TG) mice compared to the baseline WT(WT) dams ($p\text{-}value = 0.004$). WT(KO) mice also showed a reduction in expression of *Dat* with a $p\text{-}value = 0.043$. *Endod1* was similar to *Dat* with WT(TG) dams showing a highly significant decrease in expression level, when compared to WT(WT) mice ($p\text{-}value = 0.004$). This gene was also significantly reduced in WT(KO) mice ($p\text{-}value = 0.02$). The most prominent reduction in expression levels for both groups was *Ucn3* with very low expression in the hippocampus of WT(TG) mice ($p\text{-}value = 0.007$) and WT(KO) mice ($p\text{-}value = 0.009$). It should be noted that *Tph2* showed significant reduction in expression through qPCR for the WT(TG) dams, but not in the WT(KO) dams something that was also observed in the microarray for this cohort, despite there being a dramatic decrease in expression of *Tph2* in the WT(KO) condition.

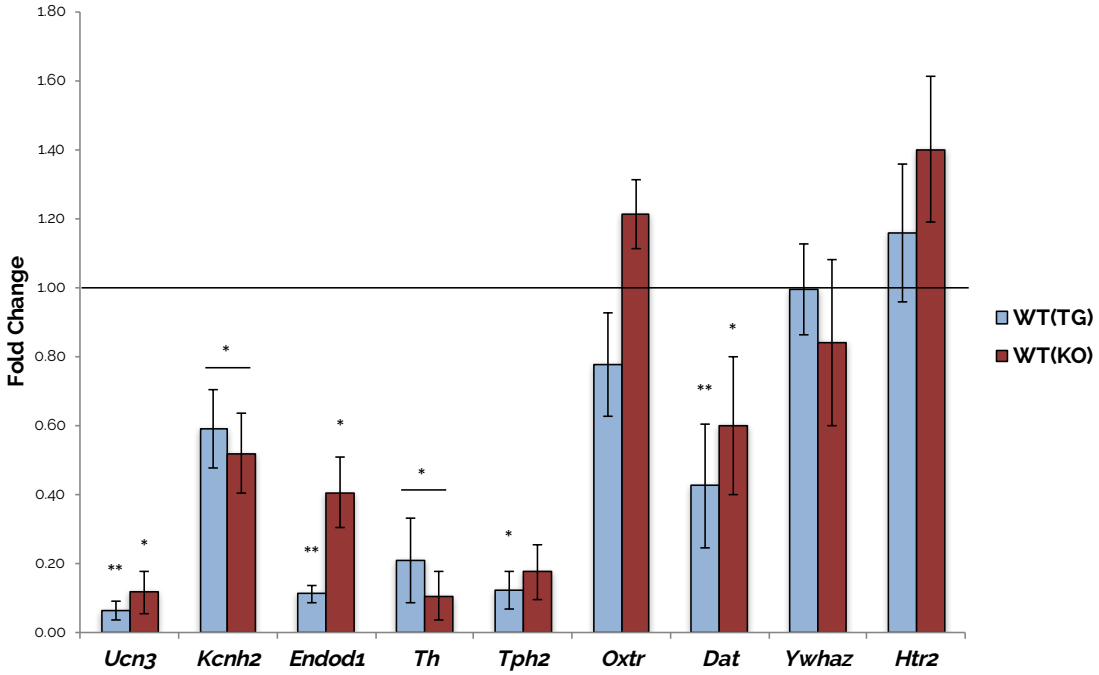


Figure 5.9: Average fold changes for specific genes shown to have differential expression in the hippocampus of dams at E16.5. A bar graph showing the average fold change for specifically chosen genes of interest in the hippocampus using qPCR that were initially shown to be significantly altered in the microarray for either WT(TG) and WT(KO) dams. Error bars represent SEM. Statistical significance: $*p < 0.05$ and $**p < 0.01$.

Table 5.9: Statistical Significance of the Fold Change in WT(TG) Hippocampus qPCR

Gene	Fold Change (± SEM)	WT(TG) p-value
Ucn3	↓ 0.06 (±0.03) ☑	0.007
Kcnh2	↓ 0.59 (±0.11) ☑	0.04
Endod1	↓ 0.11 (±0.05) ☑	0.004
Th	↓ 0.21 (±0.12) ☑	0.02
Tph2	↓0.12 (±0.06) ☑	0.04
Dat	↓ 0.43 (±0.15) ☑	0.004
Ywhaz	0.99 (±0.14)	0.96
Htr2	1.40 (±0.41)	0.70
Oxtr	1.21 (±0.27)	0.47

Note: ☑= Concordant to microarray fold change.

Table 5.10: Statistical Significance of the Fold Change in WT(KO) Hippocampus qPCR

Gene	Fold Change (± SEM)	WT(KO) p-value
Ucn3	↓ 0.12 (±0.06) ☑	0.009
Kcnh2	↓ 0.52 (±0.12) ☑	0.026
Endod1	↓ 0.40 (±0.10) ☑	0.02
Th	↓ 0.10 (±0.07) ☑	0.02
Tph2	0.17 (±0.08) ☑	0.10
Dat	↓ 0.60 (±0.20) ☑	0.04
Ywhaz	0.84 (±0.15)	0.34
Htr2	1.16 (±0.46)	0.35
Oxtr	0.78 (±0.38)	0.58

Note: ☑= Concordant to microarray fold change.

E16.5 HYPOTHALAMUS QPCR

Using the microarray of the hypothalamus from the dams sacrificed at E16.5 a list of genes that showed differing levels of expression between the cohorts was generated. A selection of these genes were also seen to be enriched in the pathway analysis. The genes that were common to the top gene expression changes and also appeared in pathway analysis were then further quantified using qPCR. The genes that appeared to be most enriched were the olfactory receptor genes. These are a type of *G-Protein Coupled Receptor* (*GPCR*) genes that have broad functional roles (Zhang and Firestein, 2002). The rate of concordance between the qPCR and microarray was 35% across all the cohorts in the hypothalamus.

Using the same newly generated cohort of mice, qPCR was used to examine gene expression in the hypothalamus. qPCR confirmed altered expression levels of all the genes tested at varying degrees of significance between the WT(TG) and WT(KO) cohorts of dams and WT(WT) control mice. *Olfr59*, *Olfr347*, *Olfr481*, *Olfr1239*, *Olfr1410* and *Olfr1491* all showed a significant increase ($p\text{-value} < 0.05$) in expression levels in the hypothalamus of WT(TG) dams compared to the WT(WT) cohort (**Figure** 5.10). Data from WT(KO) dams was not significantly different to that of the WT(WT) dams but there was a high level of variation across individuals within each cohort, which may have obscured these results. The statistical *p-values*

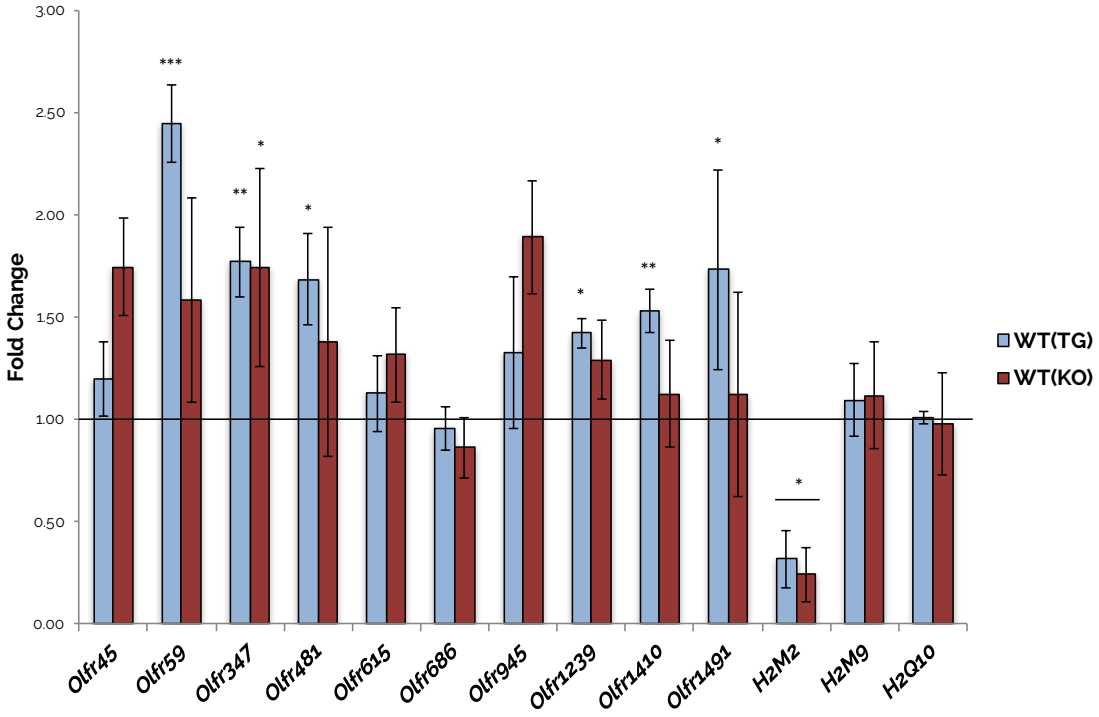


Figure 5.10: Average fold changes for specific genes shown to have differential expression in the hypothalamus of dams at E16.5. A bar graph showing the average fold change for specifically chosen genes of interest in the hypothalamus using qPCR that were initially shown to be significantly altered in the microarray for either WT(TG) and WT(KO) dams. Error bars represent SEM. Statistical significance: $*p < 0.05$, $**p < 0.01$, and $***p < 0.005$.

and relative fold changes for both WT(TG) and WT(KO) dams are summarised in **Table** 5.11 and **Table** 5.12. The use of newly generated dams for the qPCR analysis provided convincing evidence that the changed identified by microarray were genuine, by adding independent verification.

Table 5.11: Statistical Significance of the Fold Change for WT(TG) Hypothalamus qPCR

Gene	Fold Change (± SEM)	WT(TG) p-value
Olfr45	1.20 (±0.18)	0.43
Olfr59	↑ 2.44 (±0.19) ☑	0.001
Olfr347	↑ 1.77 (±0.17) ☑	0.01
Olfr481	↑ 1.69 (±0.23) ☑	0.03
Olfr615	1.13 (±0.19)	0.48
Olfr686	0.96 (±0.11)	0.68
Olfr945	1.33 (±0.37)	0.33
Olfr1239	↑ 1.42 (±0.07) ☑	0.02
Olfr1410	↑ 1.53 (±0.11) ☑	0.01
Olfr1491	↑ 1.73 (±0.49) ☑	0.04
H2-M2	↓ 0.32 (±0.14) ☑	0.02
H2-M9	1.10 (±0.17)	0.37
H2-Q10	1.01 (±0.03)	0.72

Note: ☑= Concordant to microarray fold change.

Table 5.12: Statistical Significance of the Fold Change in WT(KO) Hypothalamus qPCR

Gene	Fold Change (± SEM)	WT(KO) p-value
Olfr45	1.74 (±0.24)	0.10
Olfr59	1.58 (±0.50)	0.11
Olfr347	↑ 1.74 (±0.48) ☑	0.05
Olfr481	1.38 (±0.56)	0.21
Olfr615	1.32 (±0.23)	0.30
Olfr686	0.86 (±0.15)	0.55
Olfr945	1.89 (±0.28)	0.09
Olfr1239	1.29 (±0.19)	0.11
Olfr1410	1.12 (±0.26)	0.24
Olfr1491	1.12 (±0.50)	0.27
H2-M2	↓ 0.24 (±0.13) ☑	0.02
H2-M9	1.12 (±0.26)	0.32
H2-Q10	0.98 (±0.25)	0.87

Note: ☑= Concordant to microarray fold change.

Hypothalamus and Hippocampus P7 qPCR

After behavioural assessments were performed on the original cohorts of mice, they were culled at P7 to examine brain gene expression. The hippocampus and hypothalamus for all the groups (n = 12) was assessed by qPCR to ask whether changes identified at E16.5 persisted postpartum.

Table 5.13: Fold Changes and Statistical Significance in Hypothalamus Genes at P7

Gene	WT(TG) Fold Change	WT(KO) Fold Change	p-value
Olfr45	1.10 (±0.28)	0.61 (±0.34)	0.28
Olfr59	1.13 (±0.32)	0.68 (±0.26)	0.32
Olfr347	1.08 (±0.41)	0.74 (±0.50)	0.51
Olfr481	1.10 (±0.40)	0.74 (±0.36)	0.50
Olfr615	1.09 (±0.48)	0.72 (±0.43)	0.56
Olfr945	1.04 (±0.46)	0.70 (±0.51)	0.56
Olfr1239	1.07 (±0.39)	0.70 (±0.38)	0.49
Olfr1410	1.12 (±0.24)	0.68 (±0.25)	0.23
Olfr1491	1.09 (±0.35)	0.68 (±0.22)	0.41
H2-M2	1.05 (±0.21)	0.57 (±0.31)	0.27
H2-M9	0.79 (±0.27)	0.65 (±0.21)	1.34
H2-Q10	1.24 (±0.31)	0.73 (±0.20)	1.28

Table 5.14: Fold Changes and Statistical Significance in Hippocampal Genes at P7

Gene	WT(TG) Fold Change (± SEM)	WT(KO) Fold Change (± SEM)	p-value
Ucn3	2.31 (±0.78)	5.27 (±2.29)	>0.05
Kcnh2	0.83 (±0.27)	0.90 (±0.32)	>0.05
Endod1	0.21 (±0.11)	0.25 (±0.13)	>0.05
Th	0.96 (±0.39)	1.97 (±0.90)	>0.05
Tph2	0.34 (±0.12)	0.36 (±0.14)	>0.05
Oxtr	1.32 (±0.33)	1.69 (±0.77)	>0.05

As can be seen from the qPCR results in **Table** 5.13 and **Table** 5.14 there were no persistent gene expression changes of the same genes that were altered at the E16.5 time point. There was a high level of SEM across these samples however, which is one factor preventing some of these genes being significantly changed.

5.2.3 Immunohistochemistry

To examine proliferation and neuronal markers, brains from WT(WT) (n = 6), WT(TG) (n = 4) and WT(KO) (n = 4) dams, post BrdU injection (see Chapter 2), were paraffin embedded and sectioned coronally, generating slices 10 µm thick. A total of 18 sections per brain for each antibody were then stained using the previously described protocol in chapter 2 for BrdU, Nestin and DCX. The resulting slides were subsequently scanned using Carl Zeiss Axio Scan.Z1 slide scanner before positive cells quantified using manual counting in Fiji (Schindelin *et al.*, 2012) and Zen Blue Software (Carl Zeiss).

Bromodeoxyuridine - BrdU

BrdU labels cell proliferation and new neurons, a one-way ANOVA on the total cell counts indicated that there was a significant effect of genotype upon the number of BrdU positive cells in the SVZ of the hippocampus at E16.5 across the three cohorts ($F_{2,\,10} = 4.6$, *p-value* = 0.046). **Figure** 5.11 shows that this effect was driven by a significant decrease in the number of BrdU positively labelled neurons in WT(TG) mice compared to WT(WT) controls (*p-value* = 0.01). This reduction was also seen in the WT(KO) cohort (*p-value* = 0.05).

Examples of BrdU, Nestin and DCX positive cells are shown in **Figure** 5.16. The average numbers of positive cells for BrdU, Nestin and DCX collated in **Table** 5.15.

BrdU is a marker for cell proliferation (Kee *et al.*, 2002). A reduction in the number of cells taking up this marker at E16.5 suggests a reduction in cell proliferation in the SVZ in both the WT(TG) and WT(KO) cohorts E16.5 brain as a consequence of the changes in the placenta. There appeared to be a simultaneous increase in the number of BrdU labelled neurons as the SGZ showed in the WT(KO) dams had a

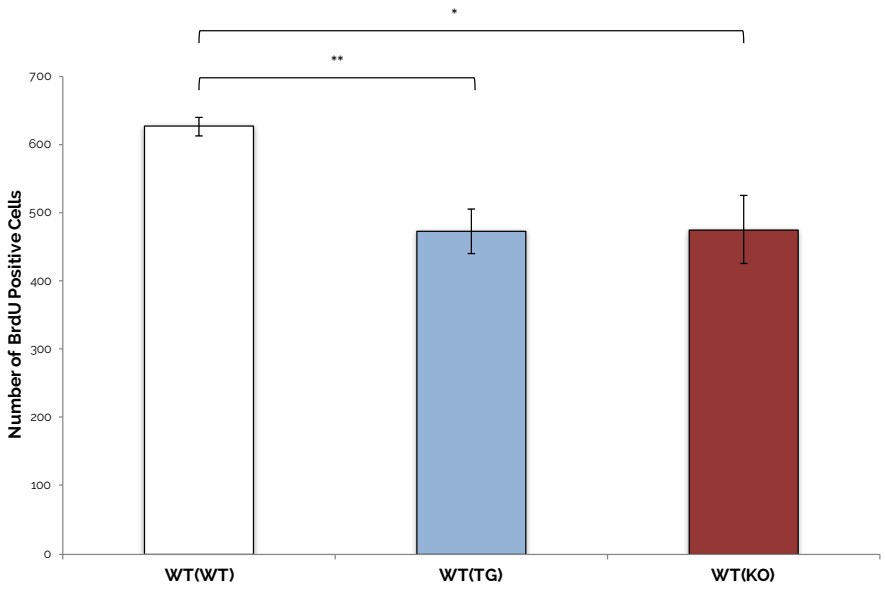


Figure 5.11: Average number of BrdU Cells across the SVZ of the 3 cohorts. A graph showing the average number of positive BrdU cells counted in the SVZ in each of the three cohorts. There was a significant difference in BrdU positive cells across the cohorts (p-*value* = 0.046). With both WT(TG) dams (p-*value* = 0.01) and WT(KO) dams (p-*value* = 0.05) exhibiting fewer BrdU positive cells than WT(WT) dams. Error bars represent SEM. Statistical significance: $*p < 0.05$, $**p < 0.01$, and $***p < 0.005$.

larger number of positively labelled cells compared to WT(WT) dams (**Figure** 5.12 and **Figure** 5.13).

NESTIN

Using Nestin, which is a label for neural stem cells (Johansson *et al.*, 1999) it was possible to quantify the number of stem cells that are in the SVZ. The results of staining sections for this marker revealed that there was a significant change in the number of positively labelled Nestin cells between the three groups of dams ($F_{2,\,10}$ = 6.3, p-*value* = 0.023). This effect was driven by the decrease in Nestin positive cells in WT(KO) dams compared to WT(WT) dams (p-*value* = 0.035) (**Figure** 5.14 and in **Table** 5.15). There was no difference in the number of Nestin labelled neural stem cells between WT(WT) and WT(TG) mice (p-*value* = 1.0).

Examples of sections stained for Nestin positive cells are given for WT(WT), WT(TG)

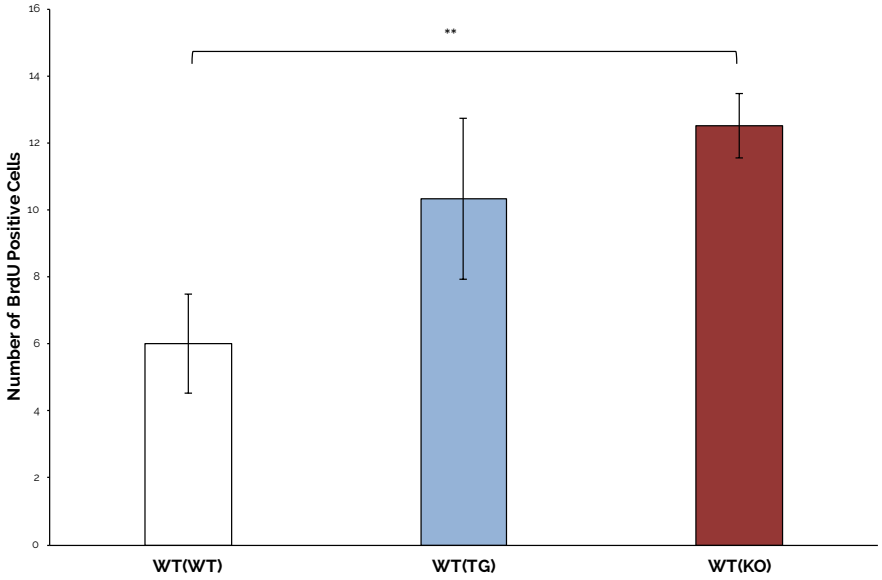


Figure 5.12: Average number of BrdU Cells across the SGZ of the 3 cohorts. A graph showing the average number of positive BrdU cells counted in the SGZ of the DG in each of the three cohorts. There was a significant difference in BrdU positive cells across the cohorts (p-*value* $= 0.046$). With WT(KO) dams (p-*value* $= 0.01$) exhibiting larger number of BrdU positive cells than WT(WT) dams in the SGZ. Error bars represent SEM. Statistical significance: $^{*}p < 0.05$ and $^{**}p < 0.01$.

and WT(KO) brains in **Figure** 5.16. The decrease in neural stem cells that is indicated through the specific staining of Nestin suggests that there are few neural stem cells present to the WT(KO) dams indicating that the reduced proliferation could be due to a deficit in total number of cells. In contrast the WT(TG) dams had no difference in neural stem cells in comparison to WT(WT) dams, thus the reduction in total cell proliferation seen in the BrdU staining may be a consequence of a delayed proliferation to generate new neurons.

DOUBLECORTIN - DCX

DCX is expressed in the cytoskeleton of new neurons, and is a microtubule associated protein (Brown *et al.*, 2003, Rao and Shetty, 2004). DCX has been linked to neural plasticity, morphological changes and migration of new neurons (Klempin *et al.*, 2011). DCX marks the period of adult neurogenesis that comes between the progenitor cell

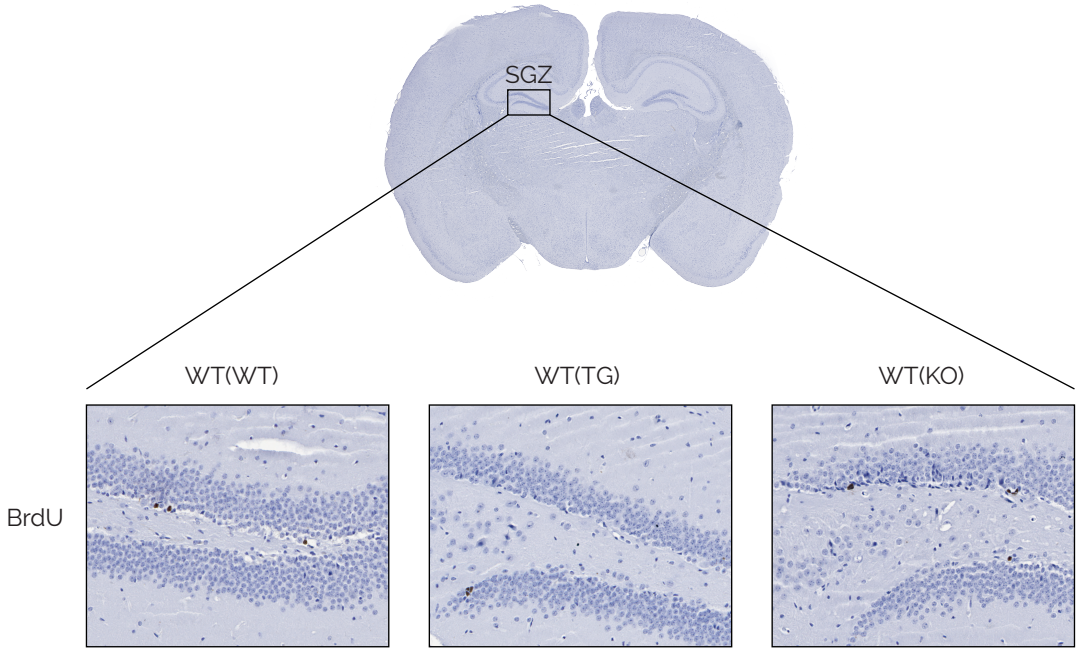


Figure 5.13: Representative images of the BrdU labelled cells in the SGZ across the 3 cohorts. The positive cells labelled for BrdU can be seen as the brown staining for all three cohorts in the SGZ.

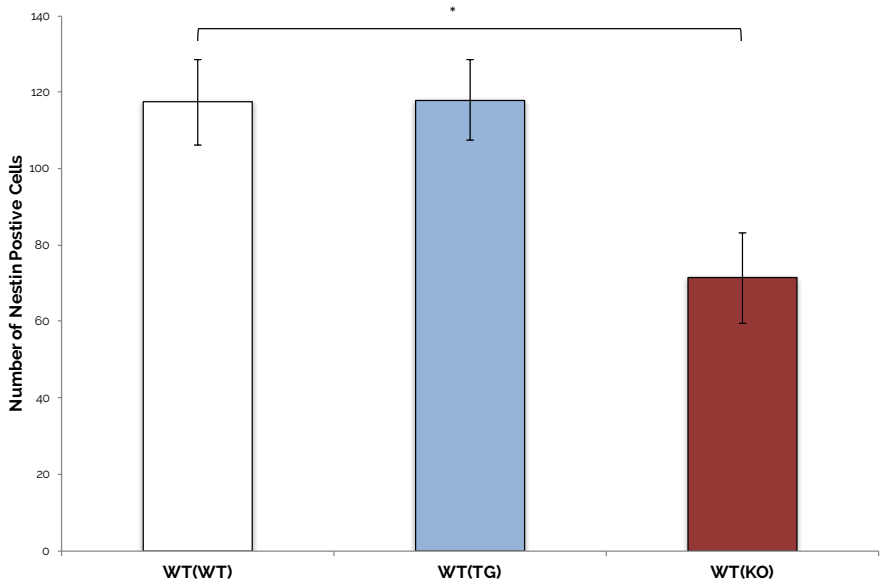


Figure 5.14: Average number of Nestin Cells across the SVZ of the 3 cohorts. A graph showing the average number of positive Nestin cells counted in the SVZ in each of the three cohorts. Error bars represent SEM. Statistical significance: $^*p < 0.05$.

stages and the early post-mitotic maturation stage. Specifically in the SVZ DCX labeled migratory cells (Brown *et al.*, 2003).

The DCX positive cells can be seen in **Figure** 5.16. The number of DCX labelled cells appeared increased in the WT(TG) cohort of dams (**Figure** 5.15). This was confirmed through a one-way ANOVA that suggested a significant effect of genotype on DCX positive cell number ($F_{2,\ 10} = 10.1$, *p-value* $= 0.009$). This was confirmed through *post hoc* tests indicating that this effect was driven by the larger number of DCX labelled cells in the WT(TG) mice's SVZ in comparison to WT(WT) dams (**Figure** 5.15, *p-value* $= 0.016$). The reduction in DCX in the WT(KO) dams is further indication of their reduced neural plasticity.

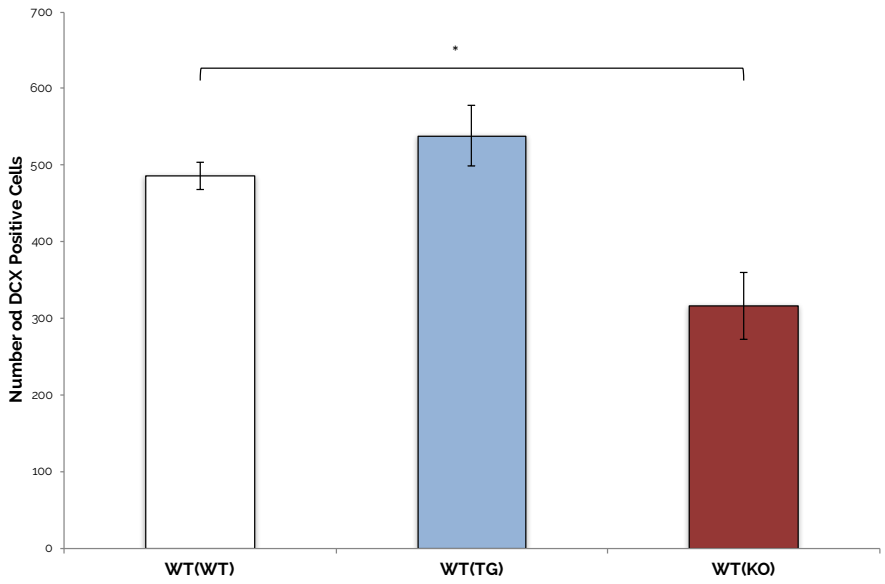


Figure 5.15: Average number of DCX Cells across the SVZ of the 3 cohorts. A graph showing the average number of positive DCX cells counted in the SVZ in each of the three cohorts. Error bars represent SEM. Statistical significance: $^*p < 0.05$.

Table 5.15: Cell Counts

Label	**Positive Cells**		
	WT(WT)	**WT(TG)**	**WT(KO)**
BrdU (SVZ)	626 (± 13.5)	472 (± 32.2)	474 (± 50.3)
BrdU (SGZ)	6 (± 1.5)	10.3 (± 2.4)	12.5 (± 1.0)
Nestin	117 (± 11.2)	117 (± 10.5)	71 (± 11.8)
DCX	485 (± 17.8)	538 (± 39.8)	316 (± 42.9)

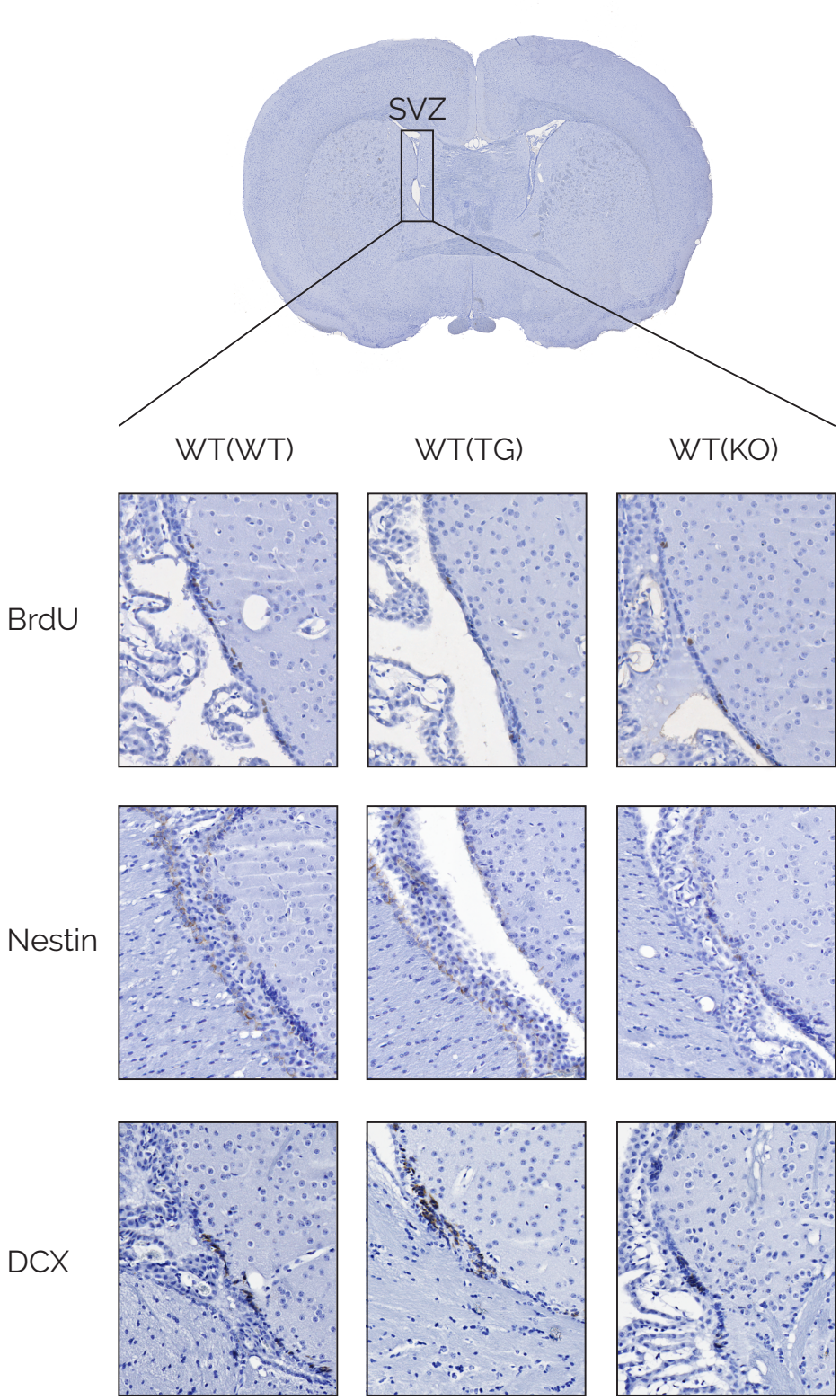


Figure 5.16: Representative images of the BrdU, Nestin and DCX labelled cells in the SVZ across the 3 cohorts. The positive cells labelled for BrdU/Nestin/DCX can be seen in the SVZ and are labelled brown in colour and non-BrdU labelled cells are purple in colour.

5.2.4 High Performance Liquid Chromatography - HPLC

The brains of each of the dams across the three cohorts were dissected into several brain regions: frontal cortex, hypothalamus, hippocampus and olfactory bulbs. This tissue was used for HPLC analysis and levels of nor-adrenaline (NA), dopamine (DA), serotonin (5-HT), glutamate (GA) and aspartate and their constituent secondary metabolites were assessed. The number of samples for each tissue are summarised in **Table** 5.16. The HPLC was performed by the Psychology Analytical Laboratory (Cambridge University, UK).

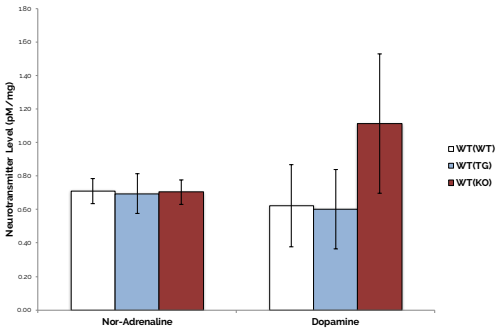
Table 5.16: Number of HPLC Samples

Region	Number of Samples (n)		
	WT(WT)	**WT(TG)**	**WT(KO)**
Frontal Cortex	5	5	5
Hippocampus	5	5	4
Hypothalamus	3	1	3
Olfactory Bulb	5	5	4

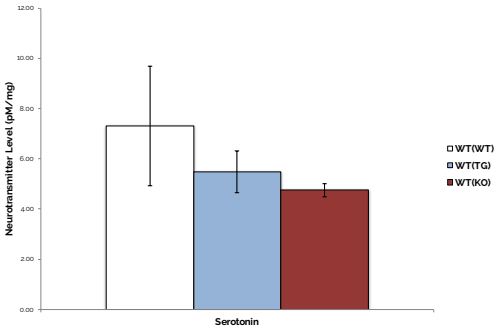
Note: Due to sample loss for WT(TG) dams the analyses for the hypothalamus from all cohorts were discarded and not shown in this thesis.

Frontal Cortex Neurotransmitter and Metabolite Levels

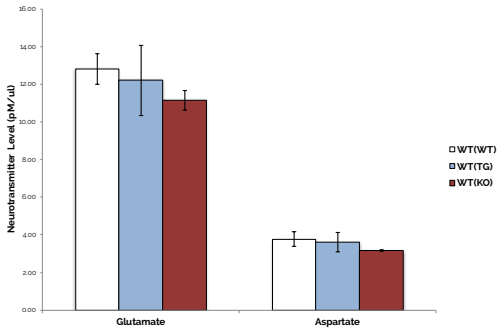
Whole tissue neurochemistry analysis of the frontal cortex was performed on an n = 5 for each cohort. The frontal cortex was tested for specific neurotransmitter levels and the subsequent metabolites. The data is displayed in **Figure** 5.17. The analysis revealed no significant change in levels of NA, DA or 5-HT between the three cohorts of dams.



(a) Levels of Nor-Adrenaline and Dopamine in the Frontal Cortex.



(b) Levels of Serotonin in the Frontal Cortex.

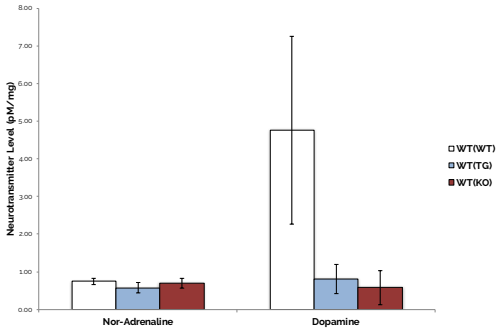


(c) Levels of Glutamate and Aspartate in the Frontal Cortex.

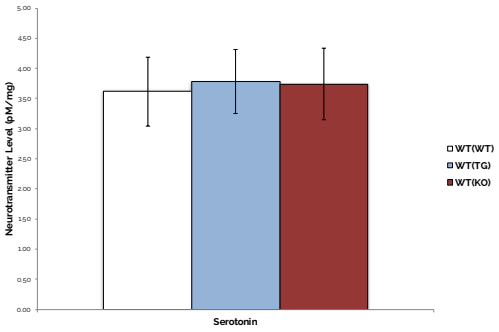
Figure 5.17: Neurotransmitter Levels in the Frontal Cortex. (a) Average levels of nor-adrenaline (NA) and dopamine (DA) in the frontal cortexes of WT(WT), WT(TG) and WT(KO) dams. **(b)** Average levels of serotonin (5-HT) in the frontal cortexes of WT(WT), WT(TG) and WT(KO) dams. **(c)** Average levels of glutamate (GA) and aspartate (ASP) in the frontal cortexes of WT(WT), WT(TG) and WT(KO) dams. Error bars represent SEM.

Hippocampus Neurotransmitter and Metabolite Levels

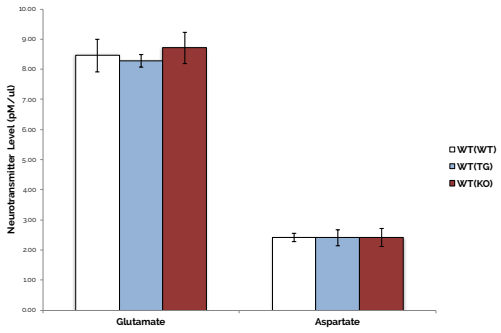
HPLC of the hippocampus for the same three neurotransmitters and their metabolites did show a decrease in dopamine levels for both WT(TG) and WT(KO) dams compared to the WT(WT) cohort, but this was not significantly different (**Figure** 5.18). The relatively high deviation in the WT(WT) samples suggested a natural variation amongst the WT(WT) dams. Consequently greater numbers will be required to determine the significance of these findings. No change in serotonin levels within the hippocampus was apparent between the three groups (**Figure** 5.18).



(a) Levels of Nor-Adrenaline and Dopamine in the Hippocampus.



(b) Levels of Serotonin in the Hippocampus.

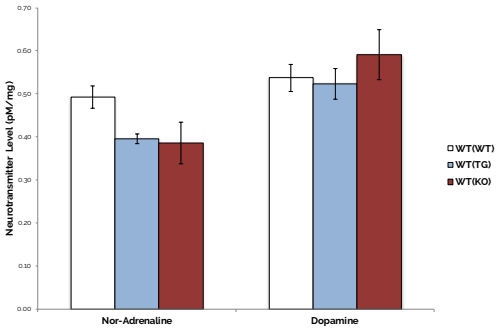


(c) Levels of Glutamate and Aspartate in the Hippocampus.

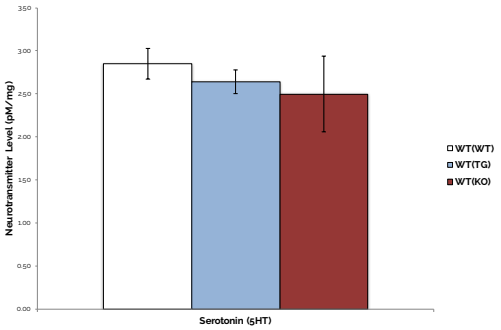
Figure 5.18: Neurotransmitter Levels in the Hippocampus. (a) Average levels of noradrenaline (NA) and dopamine (DA) in the hippocampi of WT(WT), WT(TG) and WT(KO) dams. **(b)** Average levels of serotonin (5-HT) in the hippocampi of WT(WT), WT(TG) and WT(KO) dams. **(c)** Average levels of glutamate (GA) and aspartate (ASP) in the hippocampi of WT(WT), WT(TG) and WT(KO) dams. Error bars represent SEM.

Olfactory Bulb Neurotransmitter Levels

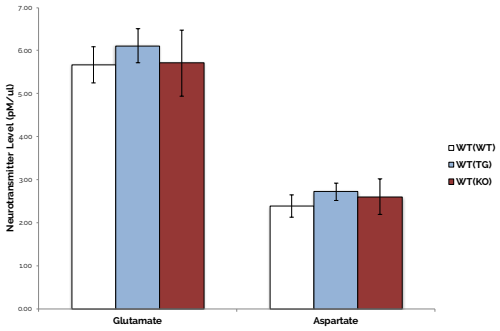
There was a consistant but not significant decrease of nor-adrenaline for both WT(TG) and WT(KO) mice in the Olfactory Bulb (OB). There was no difference between the groups in levels of dopamine 5.19. The serotonin levels were likewise not changed relative to WT(WT) 5.19.



(a) Levels of Nor-Adrenaline and Dopamine in the Olfactory Bulbs.



(b) Levels of Serotonin (5-HT) in the Olfactory Bulbs.



(c) Levels of Glutamate and Aspartate in the Olfactory Bulbs.

Figure 5.19: Neurotransmitter Levels in the Olfactory Bulbs. (a) Average levels of nor-adrenaline (NA) and dopamine (DA) in the olfactory bulbs of WT(WT), WT(TG) and WT(KO) dams. **(b)** Average levels of serotonin (5-HT) in the olfactory bulbs of WT(WT), WT(TG) and WT(KO) dams. **(c)** Average levels of glutamate (GA) and aspartate (ASP) in the olfactory bulbs of WT(WT), WT(TG) and WT(KO) dams. Error bars represent SEM.

Neurotransmitter to Metabolite Turnover

It is important to be aware of the ratio between the levels of neurotransmitter and their subsequent metabolites. This gives a representative turnover rate between the two. We only had the metabolite data for dopamine and serotonin (**Table** 5.17). There was no obvious abnormalities in the rate of turnover across the cohorts for any of the neurotransmitters to metabolites.

Table 5.17: Ratio of Neurotransmitter to Metabolites

Region	**DOPAC/DA**			**5HIAA/5HT**		
	WT(WT)	**WT(TG)**	**WT(KO)**	**WT(WT)**	**WT(TG)**	**WT(KO)**
FC	0.71	0.54	1.16	0.67	0.7	0.62
HIPPO	0.26	0.34	0.3	0.34	0.3	0.34
OB	0.31	0.34	0.31	0.34	0.33	0.34

5.3 Discussion

The results presented in this chapter highlight distinct gene expression changes in two key regions in the brain involved with maternal behaviour and also demonstrate dramatic alterations in the level of neurogenesis occurring in the E16.5 maternal brain between the three cohorts. The gene changes identified through microarray highlighted clusters of genes associated with olfaction in the hypothalamus and ribosomes/RNA binding in the hippocampus. More specifically, genes involved with the dopaminergic and serotonergic systems were significantly altered in both the microarray and then verified using qPCR. The results of the HPLC did not show any significant differences in specific neurotransmitters in several brain regions.

Gene Expression Levels in the Hippocampus and Hypothalamus

The key gene changes that were identified and then confirmed via qPCR are some that have been previously linked to mood and mental illness. In particular, there were several genes involved in mediating the monoaminergic system that were significantly down regulated in both the WT(TG) and WT(KO) dams hippocampus as well as a gene involved in mediating the stress response and also calcium signalling gene that has previously been linked to schizophrenia. These key genes and their known functions in relation to this study are summarised in **Table** 5.18.

Specifically, the dopamine transporter (Dat) is known to be critical in controlling both spatial and temporal dynamics of the neurotransmitter dopamine. It works by driving the re-uptake of the extracellular dopamine back into the presynaptic neurons (Vaughan and Foster, 2013). It is extensively reported that Dat is likely to play a crucial role in many human conditions, including depression, bipolar disorder, attention deficit hyperactivity disorder (ADHD) and Parkinson's disease (Madras *et al.*, 2005, Nutt *et al.*, 2004). This is because these diseases are all associated with abnormal levels of dopamine and therefore Dat's ability to modulate the levels of dopamine are a vital component of these diseases aetiologies. Similarly, Tyrosine hydroxylase (Th) is an enzyme that is responsible for catalysing tyrosine into L-DOPA. L-DOPA is the precursor of the catecholamines which include dopamine, noradrenaline and adrenaline. Th is the rate limiting enzyme in the production of these key neurotrophic factors. Deficiencies in this enzyme therefore are linked to the abnormal function of the dopaminergic system. The down regulation of *Dat* by 50% in the WT(TG) dams hippocampus and a similar reduction in the WT(KO) dams hippocampus, combined with the *Th* being significantly reduced by 90% in the WT(KO) dams point to a clear disruption of two key genes involved with the dopaminergic system. Thus implying a potential role of these genes in the regulation

and stability of the dams maternal behaviour.

Table 5.18: Key Functions of Genes Investigated in the Hippocampus and Hypothalamus.

Gene	Up ↑ or Down ↓ Regulated	Function/s	Reference/s
Ucn3	WT(TG)/WT(KO) ↓	Regulates food uptake during stress and feeding behaviour. Itself and other CRF family members play a role in anxiety related behaviours.	Reyes *et al.* (2001), Ushikai *et al.* (2011), Venihaki *et al.* (2004), Zheng *et al.* (2016)
Kcnh2	WT(TG)/WT(KO) ↓	Identified as a risk gene for schizophrenia. Can cause differences in microcircuit function in the hippocampus and structure and number of dendrites.	Carr *et al.* (2016), Hashimoto *et al.* (2013), Heide *et al.* (2016), Vereczkei and Mirnics (2011)
Endod1	WT(TG)/WT(KO) ↓	Implicated in psychiatric disorder due to function in mylein formation/neurogenesis.	Jahn *et al.* (2009), Morag *et al.* (2011)
Th	WT(TG)/WT(KO) ↓	Rate limiting enzyme in the production of dopamine/noradrenaline/adrenaline.	Lee and Voogt (1999), Voogt *et al.* (2001)
Tph2	WT(TG)/WT(KO) ↓	Linked to increased expression levels in the pancreas due to elevated PL.	Kim *et al.* (2010), Mueller and Bale (2008)
Dat	WT(TG)/WT(KO) ↓	Linked to a role in ADHD, Parkinson's, depression and bipolar disorder. Involved with spatial and temporal dynamics of dopamine.	Carlin *et al.* (2013), Peciña *et al.* (2003), Vaughan and Foster (2013)
Olfactory Receptors *Olfr*	↑ and ↓	G-protein Coupled Receptors - Implicated in a variety of signalling pathways and are likely to bind other functional ligands.	Zhang and Firestein (2002)

What is known about *Dat* and the role of the gene plus the subsequent protein may suggest that the WT(TG) dams greater down regulation is a natural response to lower levels of dopamine in the system. Perhaps, in an attempt to stabilise mood, cognitive ability and other key behaviours known to be partly controlled by dopamine. This is perpetuated by the reduction in dopamine seen in the HPLC of the hippocampus for both WT(TG) and WT(KO) dams. The less marked difference in *Dat* down regulation in the WT(KO) dams could be attributed to the differences in behaviour seen between the three cohorts. In contrast the large down regulation of *Th* could suggest that in WT(KO) dams there is less dopamine being synthesised and therefore there could be an active up-regulation of *Dat*. The fact that *Dat* is still reduced in WT(KO) dams however indicates that there may well be something else going on. Perhaps there could be a threshold response resulting from a bell shaped curve in relation to the levels of placental signalling. Too much or too little placental signalling may cause similar effects upon the maternal brain. This idea is supported by the similar decrease in *Th* in the WT(TG) dams (80%). Too much or too little of the key hormones from the SpT creates an imbalance driving maternal brain gene expression patterns away from the optimum conditions for pregnancy.

It is known that Prls influence the expression of *tryptophan hydroxylase* (*Tph2*) in the pancreas, the rate limiting enzyme in serotonin synthesis. Increased levels of Prls induce the expression of *Tph1* and *Tph2* in the pancreas, specifically in islet cells (Kim *et al.*, 2010). Biomolecular qPCR analysis of the various brain regions taken at E16.5 and P7 in this study was useful in helping identify whether changes were present in the maternal brain. The results showed that there was a decrease in both WT(TG) and WT(KO) hippocampi. Although only the down regulation of *Tph2* in WT(TG) dams was significant, there was a large decrease in expression of this gene in WT(KO) dams. This may similarly point to a bell shaped action of an aberrant placenta on maternal behaviour. There has not yet been a link between *Tph2* and postnatal depression. Although, serotonergic activity has been shown to play a role more widely in depression as a whole, and implicated in postnatal depression through

the effective treatment of the condition using selective serotonin re-uptake inhibitors. The decrease in *Tph2* in both these cohorts may indicate that SSRI's could be an effective treatment in this model and should be investigated.

Despite not being present on the microarray it was important to look at oxytocin receptors in the hippocampus. This is because there is extensive research that has demonstrated the link between oxytocin in the initiation of maternal behaviour, but not in the maintenance of this maternal behaviour (Bick and Dozier, 2010, Schaller *et al.*, 2010). Consequently any changes in the level of the oxytocin receptor in the brains of these dams may cause alterations in oxytocin levels and in neurodevelopment. There was no significant change in the oxytocin receptor expression levels across the groups. Despite this the *Oxtr* did show reciprocal expression between the WT(TG) and WT(KO) dams indicating that although not significantly different to WT(WT) dams there may be some biological relevance to the down regulation of the gene in the WT(TG) and the up regulation in WT(KO) dams and the postpartum maternal care provision. The surge in oxytocin at parturition is not all that occurs at the end of pregnancy. There is also an abrupt decrease in the activity of serotonin neurons in the brain of the dam. This is compounded by a substantially reduced availability of tryptophan. It has been suggested that this may be a contributing factor to the development of increased maternal aggression in rodents and linked to depressed mood in humans after birth (Dennis *et al.*, 2013). There was not the time to assess maternal aggression in this study, however it would be an interesting behaviour to address using the intruder task in mice and then follow it up with a directed approach to potential pathways involved with aggression.

Urocortin 3 (*Ucn3*) is a member of the *corticotropin releasing factor family* (*CRF*). The *CRF* family of genes play a role in regulating food uptake in times of stress. In the brain it may be responsible for the effects of stress on appetite. Ushikai *et al.* (2011) and colleagues showed that centrally administered Ucn3 decreased food intake in high-fat diet-fed obese mice and in lean mice, although not as strongly as Ucn1. The results indicate that Ucn3 influences feeding behaviour and gut motility.

The dams carrying different placentae and fetuses in this study showed differing feeding habits compared to WT(WT) dams, further implicating this gene in feeding behaviour. Interestingly the CRF family of peptides are recognised for there effects on anxiety related behaviour, Ucn1 has an anxiogenic profile as it has a high affinity for the CRF receptor 1, which mediates adrenocorticotropic hormone release. In contrast Ucn2 and Ucn3 have preferential affinity for the CRF receptor 2 and this mediates stress coping responses resulting in anxiolytic behaviour (Lewis *et al.*, 2001, Reyes *et al.*, 2001). Important to note however is that these studies focussed on the hypothalamus as the site of these genes, they haven't been looked at in the hippocampus which is where they have been identified as being significantly reduced in both our WT(KO) and WT(TG) dams by around 90%. The role Ucn3 has in coping with stress could help explain the heightened stress responses seen in these mice during the EPM in chapter 4.

Potassium Voltage-Gated Channel Subfamily H Member 2 (*Kcnh2*) was identified as being around 50% decreased in WT(KO) dams hippocampus and slightly less decreased (40%) in WT(TG) dams. A *KCNH2* variant was fairly recently highlighted as a risk gene for schizophrenia in a study that looked at mRNA from the hippocampus (Hashimoto *et al.*, 2013). The study used a meta analysis of 5 ethnic groups and a total n = 4329 individuals, the idea that having the *KCNH2* variant can increase the risk of developing neuropsychological deficits and schizophrenia later in life links with the fact that mothers whom develop postpartum depression or puerperal psychosis are more likely to go on and develop other mental health disorders later in life (Chu *et al.*, 2015, Jones, 2007). A mouse model investigating this variant has shown that mice with the *KCNH2* variant are normal and display usual sensory behaviours but show differences in neuronal structure and microcircuit function in the hippocampus and pre-frontal cortex, resulting in fewer mature dendrites (Carr *et al.*, 2016). Could this therefore be one aspect that is effecting the different levels of neurogenesis seen in WT(KO) dams with significantly less expressed levels of *Kcnh2*? Accumulating evidence has implicated *KCNQ2* and *KCNQ3*, both potassium voltage gated channels

similar to *KCNH2* in bipolar disorder (Kaminsky *et al.*, 2015). More evidence showing the roles that these genes play in the effectiveness of drug responses to treating such diseases add weight to their role in these disease phenotypes (Heide *et al.*, 2016). Despite these large genome wide searches for risk genes it is still debated whether such research is useful for such diverse disorders such as schizophrenia (Vereczkei and Mirnics, 2011). It is therefore critical that despite this research highlighting genes that may play a role in the development of behavioural changes and eventually lead to mood disorders in dams with aberrant placenta, that further research into their exact effects be explored. Overall it is clear that key pathways have been disrupted within the hippocampi of these dams that are known to be involved in mood and mental illness.

The hypothalamus is a brain region that is implicated in eating, locomotor activity sexual behaviour and exploratory behaviour. It is a key neuroendocrine signalling organ that regulates a vast array of autonomic bodily functions in mammals, often through its control of the anterior pituitary. There is a known cross talking link between olfaction and the hypothalamus (Gascuel *et al.*, 2012). The hypothalamus shows sensitivity to olfactory stimuli from the environment, seen most clearly in newly pregnant mice that are exposed to urine of a "strange" male mouse resulting in complete pregnancy block, in a phenomenon known as the Bruce Effect (Bruce, 1959, Bruce and M, 1960). This effect is thought to occur as a result of hypothalamic dopamine release causing termination of the pregnancy in mice (Rosser *et al.*, 1989). More subtly however the hypothalamus which is part of the limbic system is believed to be involved with emotional responses to external and internal stimuli (Hess and Akert, 1955). It was a surprise therefore to find that the most extensively up regulated genes in the microarray of the hypothalamus was a number of olfactory receptor genes. These were subsequently shown to be part of an enriched signalling pathway for olfactory transduction.

Olfactory receptors make up about 60% of all GPCRs in the mouse and rat genome and some 50% in the human genome (Hazell *et al.*, 2012). Olfactory receptors are not

known to be present in the hypothalamus. However, although they are called olfactory receptors, the fact that they are expressed in such robust levels in the hypothalamus suggests that they may simply be GPCRs that act in the hypothalamus and perhaps bind various Prls (Zhang and Firestein, 2002). Despite the fact that a qualitative analysis suggested that these olfactory receptors do not fall into particular olfactory receptor types. This observation helps generate numerous questions surrounding the functional significance of these receptors in pregnancy. We know that specific Prls (but not all) bind to the Prlr, it is therefore possible that they also have specific receptors themselves that are different from the Prlr. The current study was not able to determine whether this is actually the case but further work on this would be interesting. Another key question concerns the reasons behind the up-regulation of the olfactory receptors. Are they up-regulated in WT(TG) dams to compensate for the lower signalling from the placenta in these mice? And if so is it a self regulating system? As a self regulating system it would make sense that when the system is flooded with high levels of signalling hormones, in order to mediate the responses that are initiated by these chemical signals then the appropriate receptors would be down regulated. If this were the case it would be expected that WT(KO) dams might show less enriched receptors and that this would potentially result in a less severe maternal behavioural response. This is not the case however and supports the idea that there is an optimum state for pregnancy.

Neurogenesis

There are two brain areas from which new neurons are made in adult brain, these are both extremely plastic and are susceptible to be programmed through hormones and experience (Kinsley *et al.*, 2012, 2006). It is possible that differing levels of hormonal release may impact the levels of neurogenesis in the maternal brain and subsequently be a causal factor in specific maternal behaviours. The evidence presented in this chapter demonstrated the difference in the levels of neurogenesis across the three

cohorts of dams at E16.5. Both WT(TG) and WT(KO) dams have reduced BrdU positive cell counts in the SVZ. Whilst WT(KO) dams have an increased number of positively labelled cells in the SGZ. This is exciting as previously these findings have not been reported in the maternal brain at this later time point during gestation, with studies focussing on E6.5 (Bridges and Grattan, 2003, Larsen and Grattan, 2010).

The SVZ is known to give rise to new inter neurons that migrate along the RMS to the olfactory bulbs. Where they are purported to function in an olfactory manner and help in olfactory recognition of the new offspring. The reduction in new neurons in the SVZ for both cohorts may imply that this aspect of maternal neurogenesis has an optimum hormonal trigger. However the WT(TG) mice did seem to have similar numbers of Nestin and DCX positive cells compared to the WT(WT) dams. This contrasted to the WT(KO) dams, which maintained around a 20-40% decrease across these cell markers.

The increase in new neurons in the SGZ of the WT(KO) dams could explain the contrast in behaviour seen in the WT(KO) dams compared to the other two groups. Whilst the reduced BrdU labelled cell number may be linked to the slower pup retrieval seen in the WT(KO) dams. It would follow that the increased number of BrdU labelled cells in the SGZ may link to previously reported findings that have implicated an increase in grey matter volumes in brain regions that are involved with maternal motivation. These findings first presented by Kim *et al.* (2010) suggested that dams with increased neurogenesis in the SGZ show greater positive behaviour towards their offspring. The SGZ is associated with learning and memory (Abrous and Wojtowicz, 2008). Therefore testing more specific learning and memory behaviours of these dams would be interesting. It must also be considered that any differences in behaviour between these cohorts may not be due to the amount of neurogenesis taking place, but be a result of neurogenesis taking place at a different time point. It is plausible that WT(KO) dams may experience higher levels of neurogenesis at an earlier stage of pregnancy, perhaps as a result of higher signalling from the placenta,

meaning that the neurons are incorporated into the functional circuitry of the brain sooner. In order to test this theory a longitudinal study should be undertaken and assessment of neurogenesis at different time points during pregnancy between the cohorts.

Although this research focussed upon neurogenesis in the well documented regions of the adult maternal brain, it would be fascinating to assess levels of neuronal development in less well documented areas, that are also involved with maternal behaviour. In particular the hypothalamus. Several researchers have identified neurogenesis in the hypothalamus and linked a potential role of hormones, specifically oestrogen, on cell proliferation in hypothalamus and amygdala (Fowler *et al.*, 2002, 2008, Okuda *et al.*, 2009). If the Prls could be shown to impact upon the levels of neurogenesis of this region it may help explain the increase in olfactory receptors in the region. The amygdala has been implicated in a variety of social and reproductive-associated behaviours, including olfactory and pheromonal processing (Dudley *et al.*, 1996, Meredith, 1991) as well as social learning and memory (Cahill *et al.*, 1996). It is important to note that despite several researchers documenting neurogenesis in the cerebral neocortex there have been others that have not observed such changes, which makes this an area of debate (Bhardwaj *et al.*, 2006, Koketsu *et al.*, 2003).

In conclusion, there are many factors that may affect the timing and rate of neurogenesis in the adult brain. These range from hormonal cycles (Rasika *et al.*, 1994), physical activity (van Praag *et al.*, 2014) and living conditions (Kempermann and Gage, 1999, Sandeman and Sandeman, 2000). Recently Goergen *et al.* (2002) have demonstrated that in the crustacean brain there is an element of circadian control of neurogenesis. Their data shows that there is a diurnal rhythm of neurogenesis along olfactory projection neurons. Although this hasn't been shown in mammals it raises the question whether a similar mechanism of control could be apart of what we have seen in our dams. Thus there is still large amounts of work that needs to be done to clarify the significance in the variations in maternal neurogenesis that we have demonstrated, and the underlying mechanisms behind the differences. However

what we have shown is that there is a role of the placenta in our findings, which is an exciting step forward in understanding the programming of the maternal brain.

Caveats to Biomolecular Characterisation

An overall limitation that may affect the validity of the region specific expression changes seen through both microarray and qPCR of these genes, is that the dissection method may be too crude and unspecific to confidently say the exact regions were solely removed and analysed. This factor was controlled for through the use of a second cohort of mice to perform qPCR validation upon and the same researcher carrying out all the dissections. However perhaps the use of a more precise dissection method such as laser capture micro dissection would aid in the specificity of the dissections and allow for greater confidence (Espina *et al.*, 2006).

It is important to note that differences seen in the microarray and qPCR may not have been exactly alike (Chuaqui *et al.*, 2002). This could be down to a number of factors. RNA quality is extremely important for both qPCR and microarray. However the stringent quality testing processes applied to RNA sent for microarray and the preparation of the samples before the array itself can introduce certain discrepancies between results of the two procedures (Freeman *et al.*, 1999, Morey *et al.*, 2006, Wurmbach *et al.*, 2003). Microarray analysis can incur non-specific and/or cross hybridisations of labeled targets to array probes; an issue that isn't the case in qPCR. qPCR however has the issue of primer dimers, amplification biases and mis-priming (Freeman *et al.*, 1999, Wurmbach *et al.*, 2003). These are controlled for through normalisation of the data, but the global normalisation of microarray data can cause differences (Yang *et al.*, 2002). While there are undoubtably potential pitfalls in microarray and qPCR procedures, most sources of error can be controlled through robust experimental designs, good laboratory practices, and rigorous normalisation of the data, as was the case for these data (Morey *et al.*, 2006). If this experiment

was performed again, RNAseq would be a great addition to the protocol, as would the investigation of protein levels as the current study primarily focussed on only mRNA levels. To look at protein levels Western blots would be useful and/or immunohistochemistry assays. This would help create a greater focus on potential mechanisms behind the behaviours.

Conclusion

The overarching finding of this chapter was that there are changes in the maternal brain that preceded the changes in maternal behaviour observed in the dams in chapter 4. The genes that have been significantly down or up-regulated in both the WT(KO) and WT(TG) dams are linked to pathways associated with mood and mental illness in humans. While these changes do not prove that the maternal behavioural differences were due to placental factors, it does demonstrate alteration in the maternal brain during pregnancy some of which have been linked to maternal behaviour in previous studies (Bridges, 2016). These results do give support to the hypothesis that the altered placenta is playing a key part programming the maternal brain and the subsequent behavioural changes.

Alterations in neurogenesis taking place during gestation that may or may not confer an advantageous effect on the maternal care postnatally. One key question is the functional relevance of newborn neurons during pregnancy since those born at E16.5 would not be integrated into the nervous system in time for postnatal care. It will be of interest to perform a longitudinal study to ask whether the dams have more persistent alterations in behaviour. For example, are they better or worse mothers in the second pregnancy.

Summary of Findings

- Microarray analysis highlight distinct global differences in gene expression levels between the three cohorts of dams in both the hippocampus and the hypothalamus at E16.5.
- There were distinct pathways that were enriched in both the hippocampus and hypothalamus between the three cohorts at E16.5.
- The hippocampus had enrichment of the ribosome and RNA binding pathways although this didn't reach significance at E16.5.
- The hypothalamus showed significant global enrichment of the olfactory transduction pathway at E16.5.
- Genes that were significantly up or down regulated in both the WT(TG) and WT(KO) dams hippocampi included key genes involved in the dopaminergic and serotonergic systems. As well as *Ucn3*, *Endod1* and *Kcnh2*. All of which have roles in behaviour and neuronal development.
- The genes of interest were confirmed through qPCR. Validating the microarray and enabling a more in depth look at specific gene changes.
- Olfactory receptor genes that showed distinct changes in expression levels between the three cohorts and were enriched as a pathway were confirmed through qPCR analysis at E16.5.
- The gene changes seen at E16.5 did not persist until P7 in the brains of the dams.
- There was a decrease in BrdU labelled cells in the SVZ of both WT(TG) and WT(KO) dams at E16.5.
- There was subsequently a significant decrease in Nestin and DCX positive cells in WT(KO) dams SVZ at E16.5.

- The SGZ showed increased BrdU positive cell numbers in WT(KO) dams only at E16.5.
- HPLC analysis showed no significant differences in neurotransmitter levels or in there metabolites across the three groups at E16.5.

Offspring Outcome 6

6.1 Overview

It has been consistently reported over the last century that the early life environment is both a source of adult psychopathology and physiological disease (Bale *et al.*, 2010b, Champagne and Curley, 2009, Visentin *et al.*, 2014). The early concepts of "psychic conflict" and "toxic parenting" have been replaced with a view to genes and the epigenome, due to a greater understanding of the complex relationship between genes and the environment. This paradigm shift in knowledge has made room for an exciting and rapidly growing field of research focussed on the early life environment. The early life of an individual is a uniquely sensitive period of development, during which adverse experiences can lead to enduring effects that impact upon adult health. These range from physiological conditions such as obesity, diabetes and cardiovascular disease (Elford *et al.*, 1991, Reilly *et al.*, 2005, Stein and Charles, 1971) to psychological and neurodevelopmental conditions like schizophrenia, bipolar disorder and ADHD (Bale *et al.*, 2010a, McEwen, 2008, Mill and Petronis, 2008).

The previous two chapters (Chapter 4 and Chapter 5) identified a maternal care and LBW phenotype based upon an altered placenta, potentially as a consequence of changes that occur in the maternal brain during gestation. The knowledge that maternal care is intrinsically linked to offspring outcome opened up the question about what effect, if any, the maternal phenotype identified in chapter 4 may have

upon the offsprings developmental trajectory.

This chapter aimed to dissect the specific effects that the *in utero* environment and maternal care phenotype may have upon the offsprings behavioural and biomolecular profiles. To do this an initial behavioural characterisation was performed and genes associated with distinct behavioural outcomes assessed using qPCR for one of the maternal phenotypes described previously.

6.1.1 Maternal Care and Development

The process of development post partum is dynamic. In the majority of mammals the postnatal phase of development is characterised by a prolonged period of dependency on mothers for warmth, nutrition, protection and stimulation (Franks *et al.*, 2015, Numan, 1994, Rheingold *et al.*, 1963). These behaviours materialise in rodents in the form of nest building, elevated eating levels to help balance the demands placed on the body system through lactation (Chapter 1). The offspring of mammals however show a periodic reduction in dependence (Curley *et al.*, 2009, Rheingold and Eckerman, 1970). In laboratory offspring this includes increased locomotor activity, eye opening, play and other social interactions all of which can continue for up to 21 days post parturition, with some suggesting up to 28 days (Curley *et al.*, 2009).

The bi-directional nature of the developmental process means that variations in the interactions between mothers and their offspring can have significant effects on the growth, neural development and behavioural outcomes in offspring (Champagne *et al.*, 2007, Duque *et al.*, 2012). This has previously been shown to be the case in situations of maternal separation or deprivation (George *et al.*, 2010, Millstein and Holmes, 2007). One study highlighted the increased stress reactivity in adulthood of one group of young that suffered from maternal separation during development (Champagne, 2011). These changes in behaviour are also evident in natural variations of maternal care between mother and offspring in rats (Meaney, 2001, Meaney and

Szyf, 2005).

The maternal phenotype and *in utero* environment that was studied in this chapter was that of the WT(TG) dams. The reasoning behind this choice of phenotype is based on on several reasons (a) the offspring from this *in utero* environment are shown to be growth restricted and have LBW but then display rapid catch catch up growth after birth (Tunster *et al.*, 2014) (b) the mothers carrying a the TG placenta have reduced maternal care and (c) utilising this model allowed assessment of NON-TG litter mates to control for the direct effects of the transgene. There is increasing evidence that IUGR plays a role in adult development and is a risk factor for a spectrum of future health complications (Barker, 2006).

6.1.2 LBW and IUGR

One of the most problematic and prevalent conditions affecting up to 19% of all births in developing countries worldwide and between 5-7% of births in developed countries is, Low Birth Weight (LBW) (Valero De Bernabé *et al.*, 2004). LBW is defined as a birth weight that is less than 2500 grams in humans and is commonly used as an overall measure of both fetal and maternal health (Alexander *et al.*, 2014). This is because offspring born too small at the time of birth are usually a result of either poor growth *in utero*, a genetic abnormality or a consequence of a preterm birth (Valero De Bernabé *et al.*, 2004). Crucially several areas of research have highlighted the idea that these newborns are more susceptible to birth complications leading to an elevated mortality risk, and it is becoming more and more evident that LBW is a risk factor for later life complications such as depression and ADHD but also diabetes, obesity and other metabolic disorders (Grissom and Reyes, 2013, Loret de Mola *et al.*, 2014, Witt *et al.*, 2012a,b). Research has focussed on understanding the causes of LBW in human populations with many risk factors being discovered including: maternal diet, smoking and ethnicity (Fulda *et al.*, 2014), but also the placenta has emerged as having a role in both maternal mood and programming

childhood behavioural outcomes (Janssen *et al.*, 2016).

6.1.3 The Placenta and Fetal Programming

The placenta's role throughout pregnancy is well documented and has already been described in detail in chapter 1. An optimally functioning placenta to protect the fetus from many types of prenatal adversity. Recently however there is increasing evidence that some forms of prenatal stress can lead to changes in placental function (Blakeley *et al.*, 2013, Jensen Peña *et al.*, 2012, Mairesse *et al.*, 2007, O'Donnell *et al.*, 2012), ultimately contributing to an altered *in utero* environment and poorer outcomes for offspring (Janssen *et al.*, 2016, O'Donnell *et al.*, 2009).

The term "fetal programming" arose after David Barker and his team proposed the "thrifty phenotype hypothesis" (Hales and Barker, 1992). This hypothesis primarily focussed on the effect of poor fetal nutrition has upon metabolic organs such as the pancreas. It has since been realised that the effects that occur early in pregnancy and the post partum play a far wider reaching role upon offspring development, as reviewed by Janssen *et al.* (2016).

Imprinted genes have been implicated in fetal growth, placental development, adult behaviour and metabolism (Peters, 2014). It is this array of roles and the epigenetic plasticity of these genes that led to Keverne (2010) suggesting their importance in fetal programming. Moore *et al.* (2015) summarises the role of imprinted genes on IUGR and LBW, whilst Green *et al.* (2015) and others (Marsit *et al.*, 2012) have suggested a link between imprinted gene expression in the placenta with infant neuro-behavioural outcomes. This evidence provides a strong foundation to the hypothesis that the offspring from mothers with a compromised placenta due to a genetic alteration of the placenta will display the effects of fetal programming on their behavioural outcome.

The aim of this chapter was to characterise the behavioural outcome of both TG, NON-TG and WT offspring that have been exposed to different placenta, *in utero* environments and different maternal care. It sought to determine whether there was any transgenerational effect that altered maternal care has upon the later life outcomes of their offspring. The tests used will range from basic motoric and anxiolytic assays to determine the natural states of the animals basic behaviour and also use the LCA test to measure mood and hedonic response.

6.2 Results

6.2.1 Offspring Development

In order to determine whether there was a developmental deficit between the three genotypes a comprehensive screening of the development was carried out on a day to day basis using the JAX developmental chart that pinpoints milestones in a mouses development from birth (**Figure** 6.1).

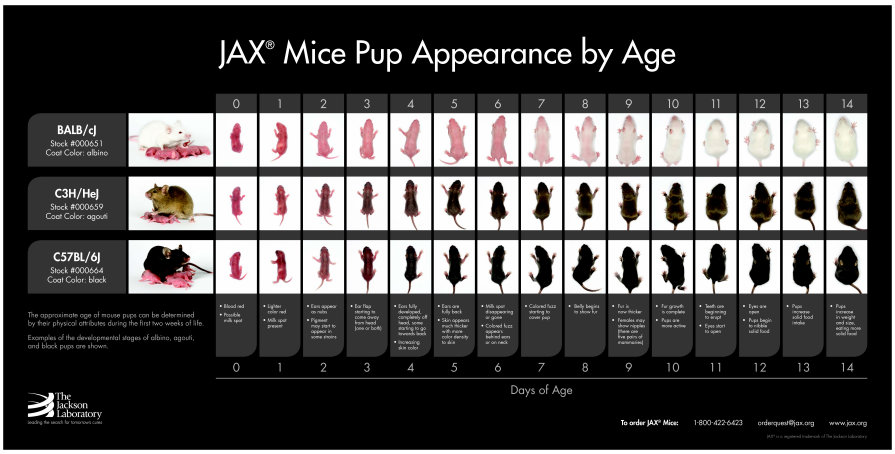


Figure 6.1: JAX Development Table. A schematic diagram illustrating the stages of development for newborn mice over the first two week period.

Mice were scored blindly without knowledge of their genotype on whether they had

reached the milestone or not by 1pm each day. The findings showed that there was no developmental phenotype between the WT and NON-TG mice (which are genetically wildtype) but also importantly that there was no difference in developmental trajectory for the TG mice that were identified after genotyping and before weaning.

6.2.2 Behavioural Characterisation

Genotyping of the TG, NON-TG and WT mice was performed before weaning so that at time of weaning male mice could be separated according to their genotype and sex. Female mice were used for a different study. A maximum n = 4 for each genotype was housed in a single cage and initially no less than n = 2. The exact numbers of each genotype used throughout the behavioural characterisation can be seen in **Table** 6.1.

Table 6.1: Numbers of Mice in each Cohort of Offspring

Genotype	Total Number (n)
WT	19
TG	12
NON-TG	17

Each cage was assigned a letter from the alphabet at random. A total of 16 cages were initially used however this number did increase due to in fighting within cages resulting in certain animals having to be separated and moved into another cage. The letters were used to ensure that the genotypes of each cage were kept blind for testing so that there was no investigator bias during manual scoring of behaviours.

Elevated Plus Maze - EPM

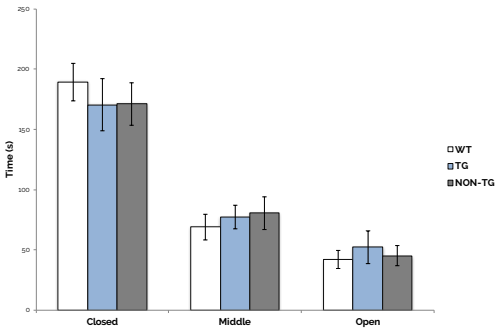
The elevated plus maze is an anxiety assay that can be used to decipher differences between anxiolytic and anxiogenic behaviour in rodents. Mice from each cohort were tested after a period of habituation to the testing room. There were n = 48

mice in total. These were all run through the EPM blind by genotype. The findings from the EPM showed that all the three genotypes had a preference for the least anxiety inducing closed arm of the EPM (*p-value* < 0.05). This was indicated by the amount of time spent on the closed arm compared to the middle zone and open arm (**Figure** 6.2). There was no difference between the duration of time spent in each zone between the three genotypes, closed arm ($F_{2,\ 46} = 0.433$, *p-value* $= 0.43$), middle zone ($F_{2,\ 46} = 0.295$, *p-value* $= 0.74$) and the open arm ($F_{2,\ 46} = 0.399$, *p-value* $= 0.67$) of the EPM. Indicating that there was no impact of genotype on this aspect of behaviour.

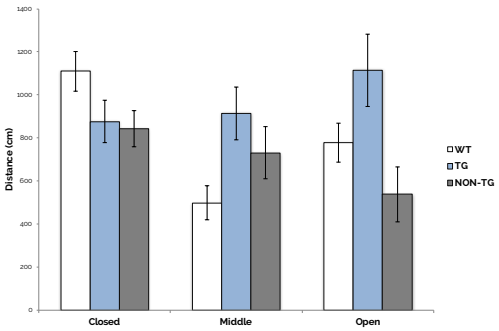
The distances that each of the three genotypes travelled in the open ($F_{2,\ 46} = 0.82$, *p-value* $= 0.45$), closed ($F_{2,\ 46} = 2.18$, *p-value* $= 0.13$) and middle ($F_{2,\ 46} = 2.08$, *p-value* $= 0.14$) zones was not seen to be significantly different by genotype (**Figure** 6.2). There was also no significant difference between the zones for WT mice ($t_{1,\ 18} = 0.24$, *p-value* $= 0.21$) TG mice ($t_{1,\ 11} = 0.54$, *p-value* $= 0.61$) and NON-TG mice ($t_{1,\ 16} = 0.12$, *p-value* $= 0.19$).

There was no difference in the velocity/speed that the mice of each group moved at within each of the three zones (**Figure** 6.2). The speed of movement was not significantly reduced in the open arm between the genotypes ($F_{2,\ 46} = 1.52$, *p-value* $= 0.23$), this was despite what appeared to be an obvious reduction in speed for the NON-TG animals. This was also the case for the closed arm ($F_{2,\ 46} = 6.64$, *p-value* $= 0.63$) and the middle zone ($F_{2,\ 46} = 4.60$, *p-value* $= 0.63$).

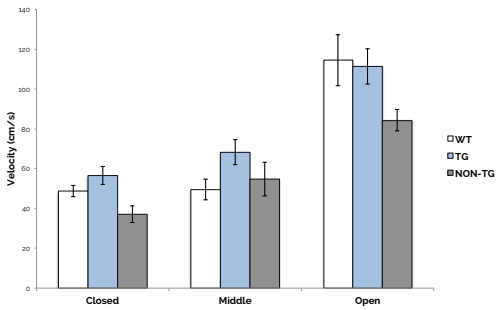
Manually scored behaviours were also recorded in each zone for the duration of the test. These included rearing, stretching and head dipping (**Figure** 6.3 and **Figure** 6.3), there was no significant effect of genotype on any of these behaviours. There was a significant effect of zone on the number of rears performed ($F_{2,\ 46} = 3.84$, *p-value* $= 0.025$), this corresponded to the reduction in the number of rears in the open zone compared to the number of rears performed in the less anxiogenic closed zone across all the genotypes. This decrease was also evident in the number of stretches that were



(a) Duration of time spent in each zone across cohorts.



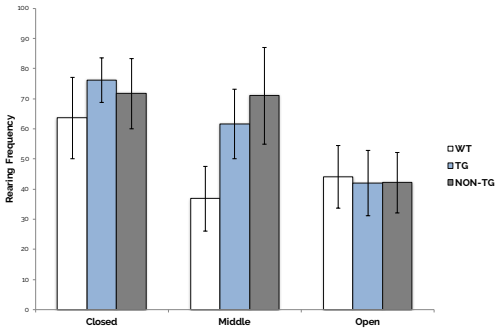
(b) Total distance travelled in each zone across cohorts.



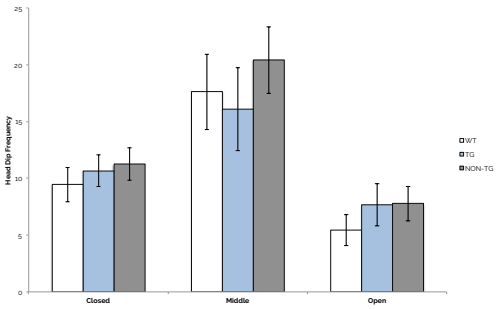
(c) Speed of movement within each zone across cohorts.

Figure 6.2: Offspring EPM. (a) A Bar-Chart illustrating the duration of time each cohort spent in the three zones of the EPM. There was no significant difference between the groups in relation to time spent in each zone $p\text{-}value > 0.05$. **(b)** A Bar-Chart showing the average distance travelled by each cohort over the course of the EPM assay. There was no significant effect of genotype on the distance travelled across all three cohorts. **(c)** A graph demonstrating the varying velocity by each group within the three zones of the EPM. There was no significant difference within zones but there was the expected difference between zones. Error bars represent SEM.

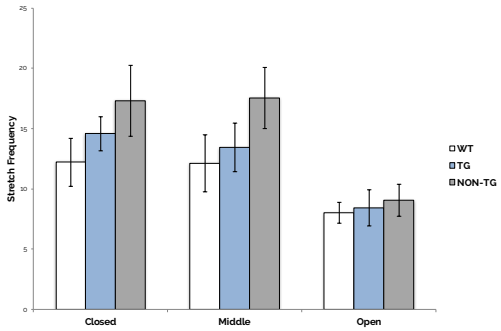
performed by each cohort in the open zone (**Figure** 6.3) in contrast to the closed and middle zones ($F_{2,\,46} = 13.77$, *p-value* < 0.001). Similarly there was an effect of zone on the number of head dips performed, with all three cohorts performing more head dips in the middle zone compared to both the closed and open zones ($F_{2,\,46} = 23.76$, p-value < 0.001). The number of fetal boli was recorded during the test but was not statistically different across the groups.



(a) Total number of Rears performed in each zone across cohorts.



(b) Total number of Head Dips performed in each zone across cohorts.



(c) Total number of Stretches performed in each zone across cohorts.

Figure 6.3: EPM - Manually Scored Behaviours. (a) A simple bar chart to display the number of rears performed within each zone of the EPM for each cohort of mice. **(b)** A simple bar chart to display the number of head dips performed within each zone of the EPM for each cohort of mice. **(c)** A simple bar chart to display the number of stretches performed within each zone of the EPM for each cohort of mice. Error bars represent SEM.

OPEN FIELD - OF

The OF test, like the EPM, is an anxiety assay that exploits the natural aversion of rodents to spending time in an open space when they can otherwise stay closely

around the edge of the arena (Chapter 2). The same numbers for each cohort used for the EPM were used for the OF (**Table** 6.1).

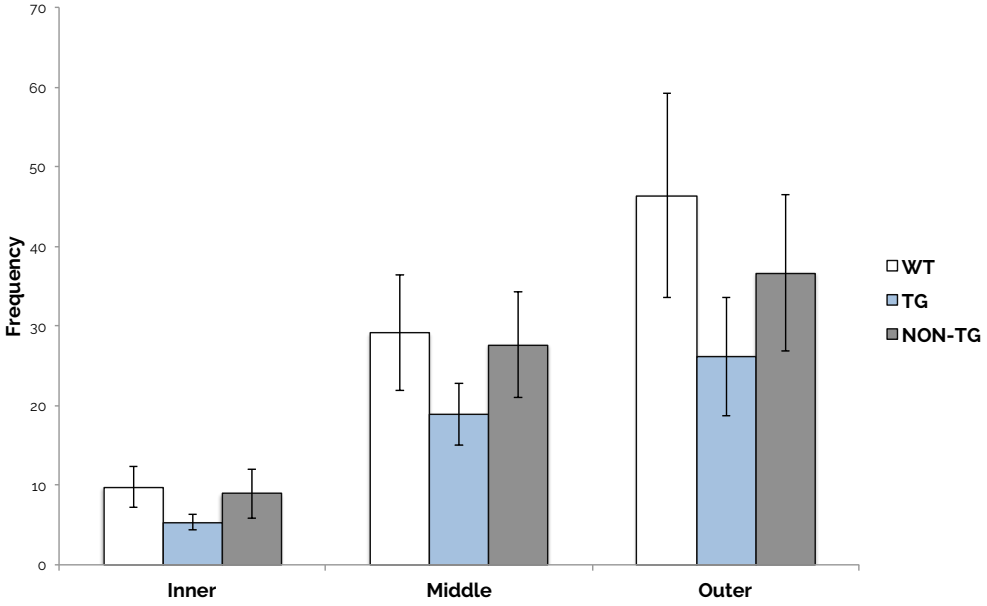


Figure 6.4: OF - Number of visits to each zone across cohorts. A barchart illustrating the number of visits into each predefined zone of the OF across the groups. There was no significant difference between the three cohorts. Error bars represent SEM.

All of the mice displayed standard behaviour, that remained consistent across the three groups of mice. However TG mice showed a trend, where they visited each zone less frequently on average compared to both WT and NON-TG mice. Despite the trend it was not statistically significant (**Figure** 6.4).

The three cohorts conformed to the expected paradigm regarding the duration of time they would spend in the various zones. All three displayed the expected thigmotaxis behaviour (tracking around the edge of the OF box in the outer zone), by spending 6 times as long in the outer zone compared to each of the other zones (inner and middle). Similarly all three cohorts travelled 3 times as far in the outer zone compared to each of the other zones (**Figure** 6.6).

The OF further confirmed the findings of the EPM. There were no ascertainable differences in anxiety related behaviour between the genotypes. There were the expected natural variations across the groups and the standard responses to the

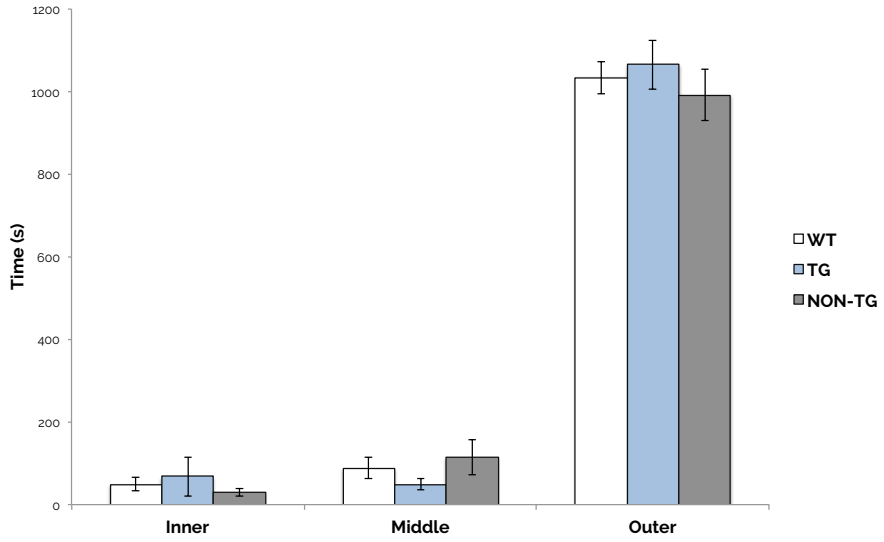


Figure 6.5: OF - Duration of time spent in each zone across cohorts. A barchart showing the duration of time each cohort spent within each zone of the OF. There was no significant difference between any of the groups. Error bars represent SEM.

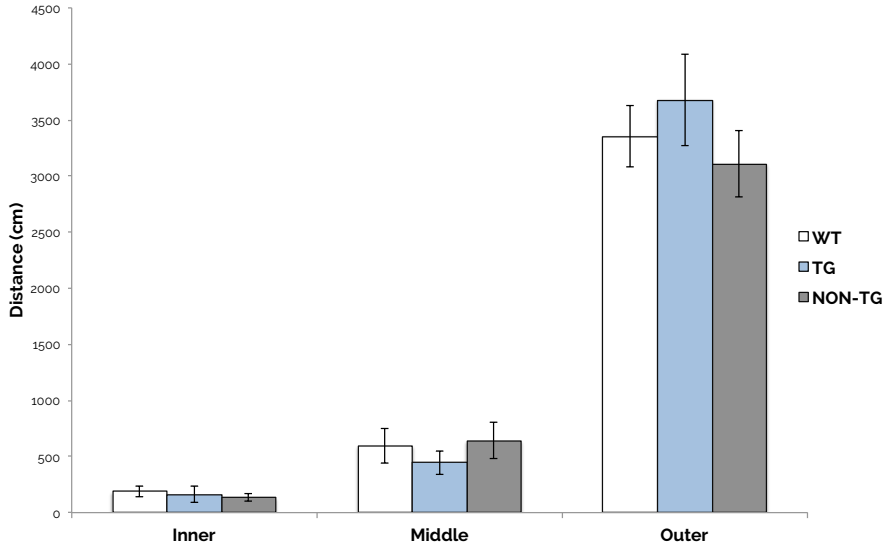


Figure 6.6: OF - Total distance travelled in each zone across cohorts. A barchart showing the total distance travelled by each cohort within each zone of the OF. There was no significant difference between any of the groups. Error bars represent SEM.

tests indicating that the tests themselves were appropriate. This suggests that any behavioural differences that are seen are unlikely to be linked to an underlying anxiety phenotype.

Locomotor Activity - LMA

The LMA task revealed that across the three cohorts all the animals exhibited the expected habituation phenotype over the course of the three days of testing. This was obvious due to the significant reduction in activity over time, represented by the number of breaks (**Figure** 6.7, $F_{1,\ 47} = 47.40$, *p-value* < 0.001) and the number of runs (**Figure** 6.8, $F_{1,\ 47} = 24.92$, *p-value* < 0.001).

When comparing between the genotypes, it was seen there was no difference between the number of runs for WT (n = 19), TG (n = 12) and NON-TG (n = 17) mice on day one ($F_{2,\ 48} = 0.77$, *p-value* $= 0.47$) and similarly on day three ($F_{2,\ 48} = 1.00$, *p-value* $= 0.37$). This pattern continued for the number of breaks performed by all three genotypes on day one ($F_{2,\ 48} = 0.85$, *p-value* $= 0.43$) and day three ($F_{1,\ 47} = 0.26$, *p-value* $= 0.78$). Summarised in **Figure** 6.7 and **Figure** 6.8.

Further analysis of the LMA sessions that looked at the entire 120 minute session by breaking it down into 5 minute bins for the number of breaks (**Figure** 6.9) and runs (**Figure** 6.10) over the duration of time that the test was running on each day. This confirmed that both breaks and runs declined across time bin for day 1 and day 3. There was no significant difference (*p-value* > 0.05) between the three genotypes in the number of breaks on day 1 and day 3. Furthermore there was likewise no significant difference (*p-value* > 0.05) between WT, TG and NON-TG regarding the number of runs on day 1 and on day 3. This indicates that neither TG or NON-TG mice display altered locomotor reactivity to a novel environment, and that their behaviour is analogous to WT subjects in the same situation.

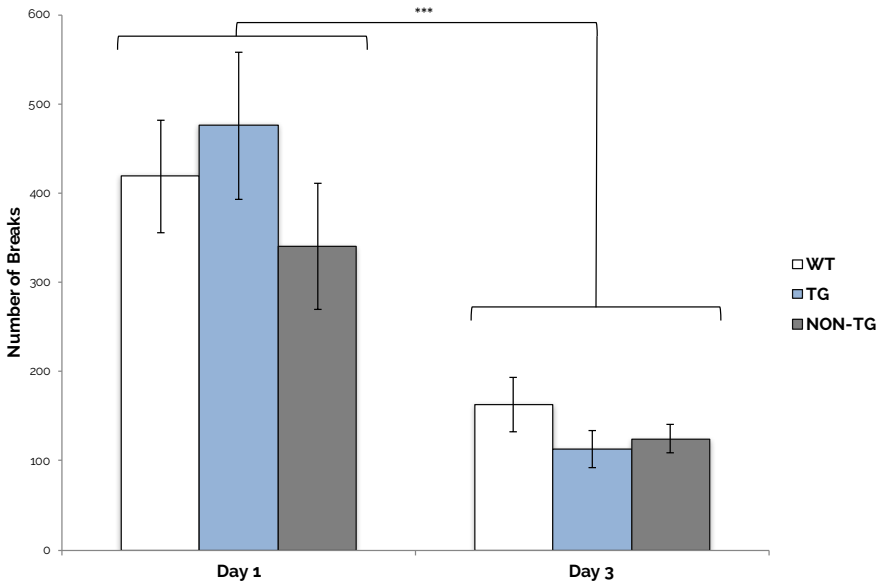


Figure 6.7: Number of Breaks Performed during LMA Task. Basic barchart illustrating that there is no significant difference in the number of breaks performed between the three cohorts. There was a significant difference across the days with the number of breaks reducing by over half for all three genotypes between day 1 and day 3. Error bars represent SEM. Statistical significance: $^{***}p < 0.005$.

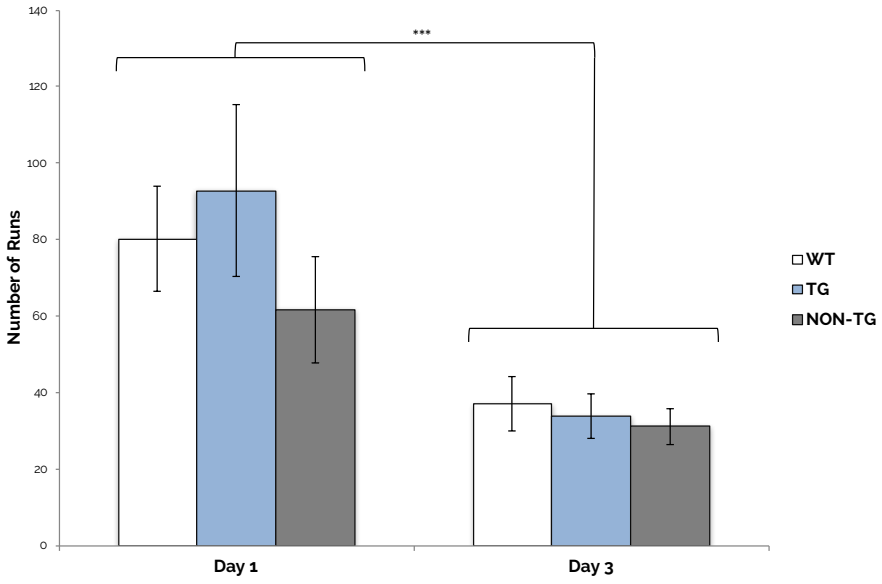
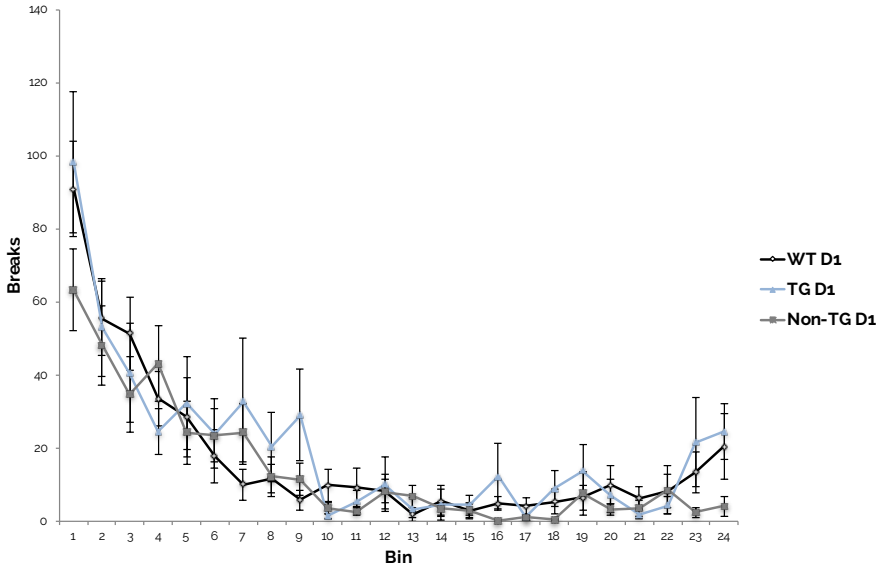
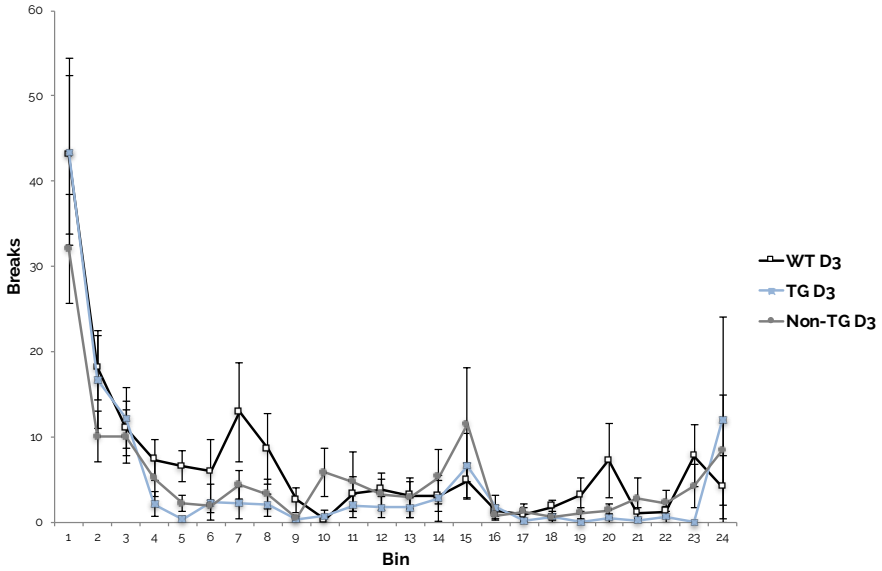


Figure 6.8: Number of Runs Performed during the LMA Task. Basic barchart illustrating that there is no significant difference across the three cohorts. There was a significant difference between days with the number of runs halving for all three genotypes between day 1 and day 3. Error bars represent SEM. Statistical significance: $^{***}p < 0.005$.



(a) Locomotor Activity Breaks Day 1 - In 5 minute Bins.



(b) Locomotor Activity Breaks Day 3 - In 5 minute Bins.

Figure 6.9: LMA Breaks between Day 1 and Day 3 in 5 minute Bins. **(a)** Graph depicting the number of breaks over day 1 broken down into 5 minute bins. **(b)** Graph depicting the number of breaks over day 3 broken down into 5 minute bins. There was no significant difference between the cohorts. Error bars represent SEM.

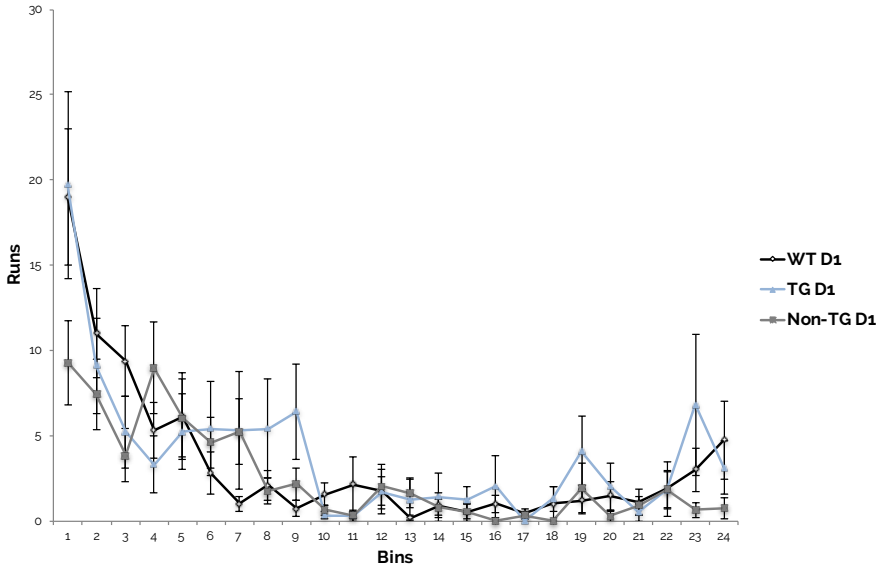
Acoustic Startle Response and Pre-pulse Inhibition - ASR and PPI

The ASR and PPI assays assess a subject's ability to filter out the unnecessary information. Deficits in this ability have been linked to abnormalities in sensorimotor gating and have been noted to occur in human patients suffering from schizophrenia and Alzheimer's disease (Braff *et al.*, 2001, Ueki *et al.*, 2006) and subsequently modelled in rodents (Powell *et al.*, 2009, van den Buuse, 2010).

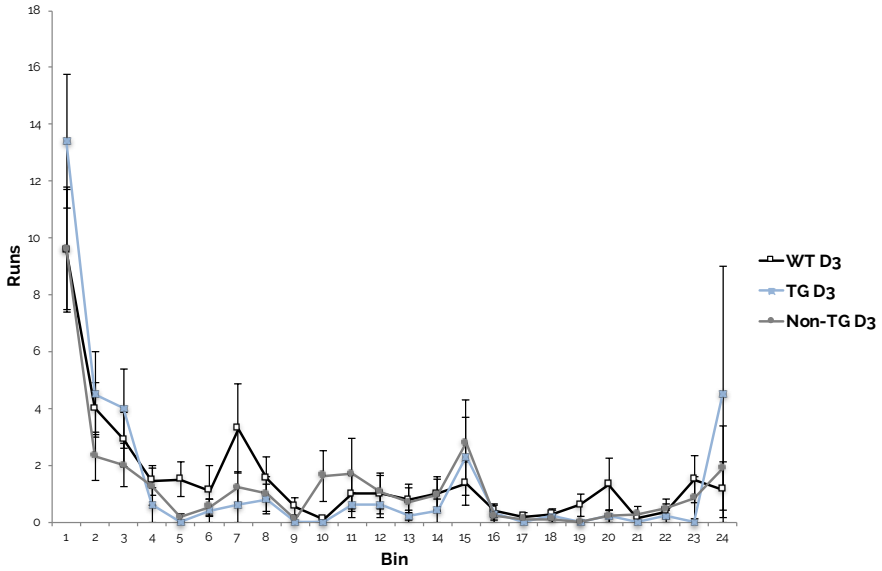
To test these responses each mouse was securely placed in the tube so there was no room for residual movement during the test. The findings showed there was an overall effect of trial number on the Vmax startle response for all three genotypes at 105 dB ($F_{1,\,11} = 3.18$, *p-value* $= 0.024$). There was no interaction between trial number and genotype, suggesting that genotype had no effect upon Vmax startle at 105 dB (**Figure** 6.11).

The same was the case for the trials at 120 dB (**Figure** 6.12), there was an effect of trial number with ASR decreasing across the trials as habituation occurred ($F_{1,\,11} = 8.65$, *p-value* < 0.001), there was also a significant interaction between genotype and trial ($F_{1,\,11} = 1.85$, *p-value* $= 0.05$). This was driven by the heightened response to the 120 dB startle of the TG mice at the beginning of the test (*p-value* $= 0.05$).

When assessing PPI at both 105 dB and 120 dB there were a series of pre-pulses at either 4 dB, 8 dB and 16 dB. The test indicated that there was no significant blunting of the responses of either of the three cohorts based on any of the pre-pulses (**Figure** 6.13 and **Figure** 6.14).



(a) Locomotor Activity Runs Day 1 - In 5 minute Bins.



(b) Locomotor Activity Runs Day 3 - In 5 minute Bins

Figure 6.10: LMA Runs between Day 1 and Day 3 in 5 minute Bins. (a) Graph depicting the number of runs over day 1 broken down into 5 minute bins. **(b)** Graph depicting the number of runs over day 3 broken down into 5 minute bins. There was no significant difference between the cohorts. Error bars represent SEM.

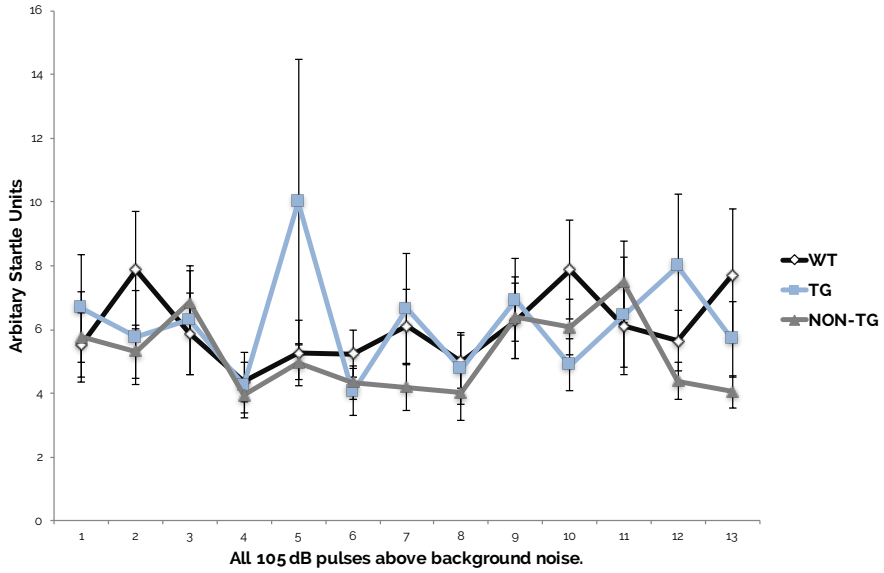


Figure 6.11: Acoustic Startle Response - 105 dB. Barchart illustrating that there is no significant difference across the three cohorts. Error bars represent SEM.

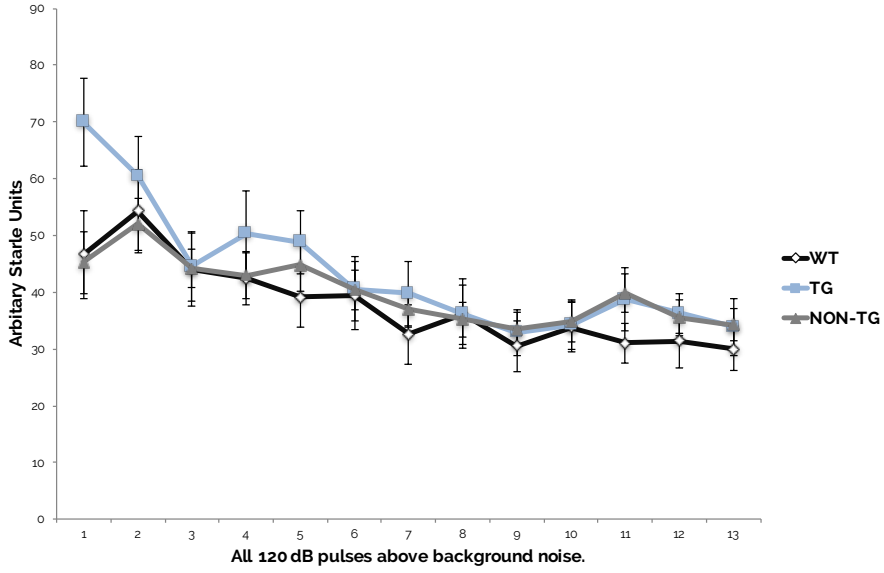


Figure 6.12: Acoustic Startle Response - 120 dB. Barchart illustrating the heightened response to the 120 dB startle of the TG mice compared to WT mice ($p\text{-}value = 0.05$). Error bars represent SEM.

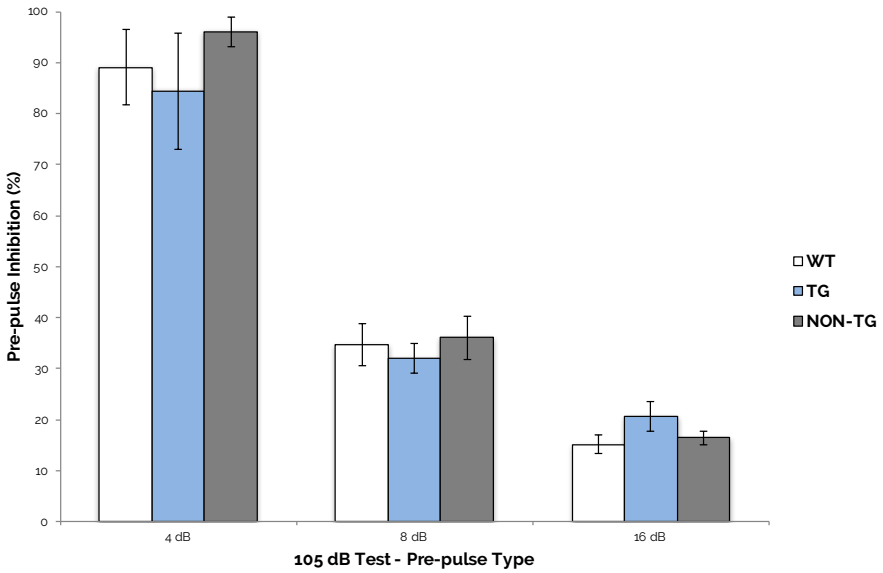


Figure 6.13: Prepulse Inhibition - 105 dB. Barchart illustrating that there is no significant difference across the three cohorts in the PPI of the mice at 105 dB. Error bars represent SEM.

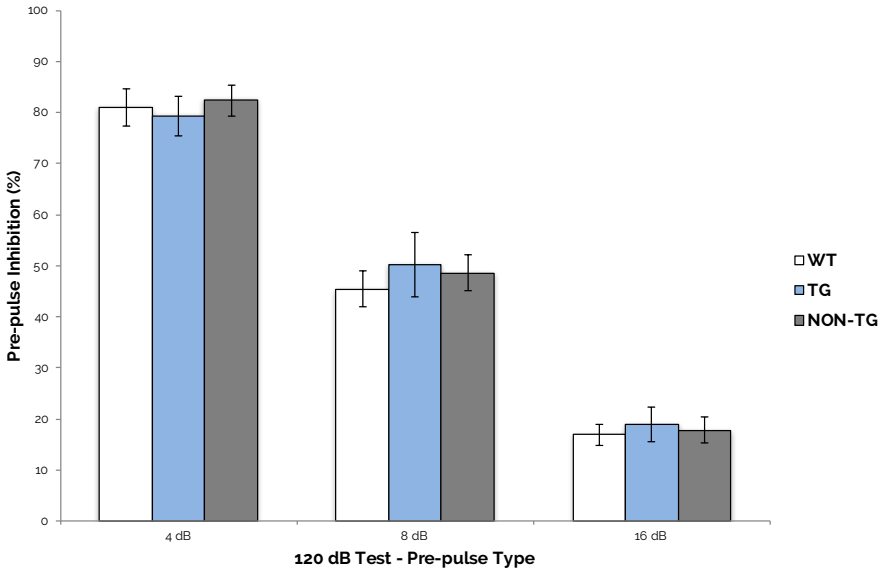


Figure 6.14: Prepulse Inhibition - 120 dB. Barchart illustrating that there is no significant difference across the three cohorts of the PPI of the mice at 120 dB. Error bars represent SEM.

Lick Cluster Analysis - LCA

The LCA is used to assess the hedonic response of the mice. It was performed after animals were trained to consume freely available 8% sucrose for 30 minutes after 12 hours of food deprivation. Only when individuals had shown stable consumption for at least two days were they used for the testing phase. There was no dropout from the mice during training. The exact numbers of mice used for the LCA for each cohort can be seen in **Table** 6.1.

The lick cluster analysis had several measures that were able to be taken throughout the test these are summarised in (**Table** 6.2) below:

Table 6.2: Lick Cluster Analysis Measures

LCA Measure	Abbreviation
Consumption	C
Total Licks	TL
Total Bouts	TB
Lick Cluster Size	LCS
Total Bout Time	TBT
1 Lick Bouts	1LB
Average Inter-Lick Interval	ALI
Total Volume Consumed	TVC

There was a significant effect of sucrose concentration on the consumption ($F_{2,\,37} = 4.49$, *p-value* $= 0.041$) that was independent of genotype. There was no difference in the TL between the two test solutions ($F_{2,\,37} = 1.89$, *p-value* $= 0.178$) and similarly no effect of genotype on TL ($F_{2,\,37} = 0.91$, *p-value* $= 0.41$).

The measure that is most important when distinguishing between hedonic response is the Lick Cluster Size (LCS). The overall pattern of consumption for both test concentrations indicated increased palpability of the 16% solution compared to the 4% solution as can be seen in **Figure** 6.15.

This led to a significant increase in the LCS for all three cohorts (WT, TG and

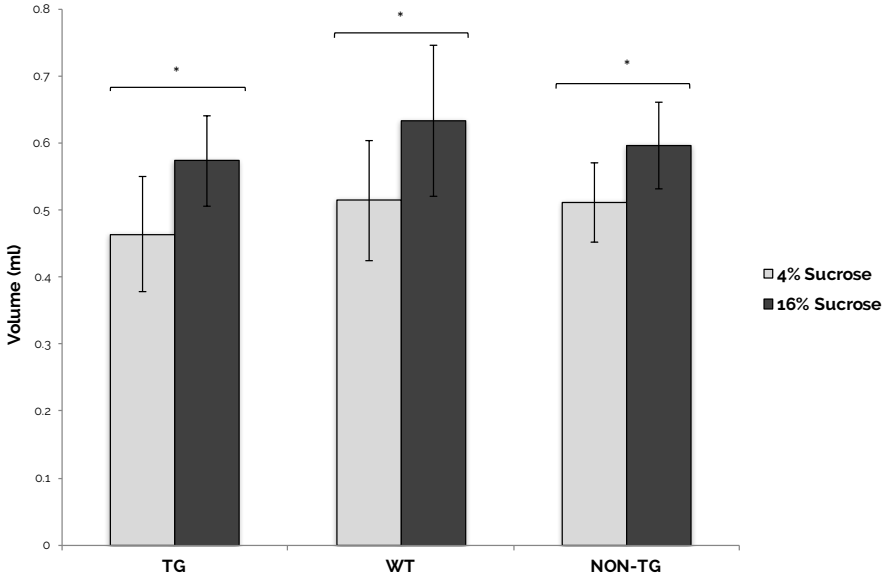


Figure 6.15: Consumption at 4% and 16% Sucrose. Line graph demonstrating distinct differences between the volume consumed by the groups for the two different concentrations of sucrose. Error bars represent SEM. Statistical significance: $*p < 0.05$.

NON-TG) with the main effect being solution ($F_{2,\,37} = 7.41$, *p-value* < 0.05). There was also a significant effect of genotype on the LCS ($F_{2,\,37} = 8.30$, *p-value* $= 0.007$). This was driven by the decreased LCS of the TG mice at the 4% sucrose solution compared to WT mice (*p-value* $= 0.001$) as well as at the 16% solution (*p-value* $= 0.033$). This difference was diminished in the wildtype NON-TG litter mates when compared to the TG mice at 4% (*p-value* $= 0.146$) and 16% (*p-value* $= 0.073$) solutions. There was a difference between WT and NON-TG mice at 4% sucrose (*p-value* $= 0.025$) that wasn't evident at 16% sucrose (**Figure** 6.16).

A second test was performed using 0.1% saccharin solution in order to determine whether there was any form of calorie seeking behaviour evident amongst the three cohorts of mice. This showed that there was also a difference between the cohorts LCS for saccharin ($F_{2,\,37} = 10.35$, *p-value* < 0.001). This was due to the dramatic reduction in LCS for TG mice relative to WT mice (*p-value* < 0.001) and also the difference between WT and NON-TG mice (*p-value* $= 0.01$), **Figure** 6.17. There was a notable difference between the Average Inter-Lick Interval (ALI) of the TG mice compared to WT and NON-TG mice (*p-value* $= 0.045$ and 0.036 respectively).

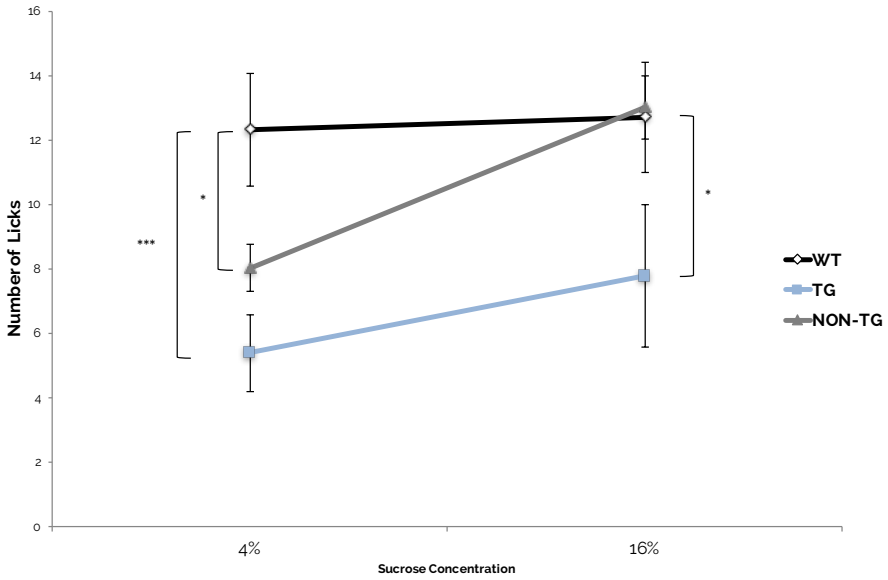


Figure 6.16: LCS at 4% and 16% Sucrose. Line graph demonstrating distinct differences between the LCS for the two different concentrations of sucrose. Error bars represent SEM. Statistical significance: $*p < 0.05$, $**p < 0.01$, and $***p < 0.005$.

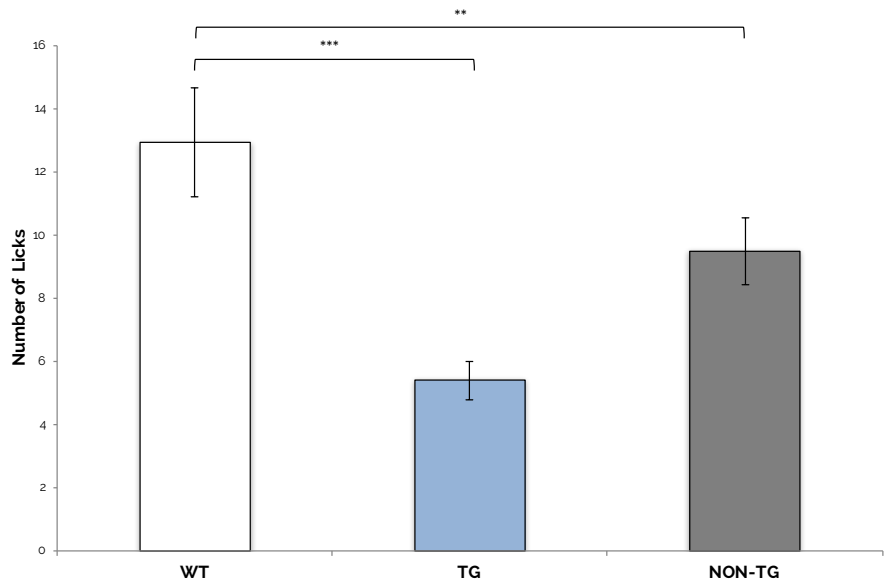


Figure 6.17: LCS for 0.1% Saccharin. Line graph demonstrating distinct differences between the hedonistic response between the groups regarding number of licks per bout for 0.1% saccharin solution. Error bars represent SEM. Statistical significance: $*p < 0.05$ and $**p < 0.01$.

Suggesting that TG mice take significantly longer between each lick than both WT and NON-TG mice.

6.2.3 Gene Expression Analysis of Ventral Striatum and Hypothalamus

For each cohort of offspring the RNA was extracted and the ng/ml values for the total RNA per µl checked. There was an n = 6 in each for each group. qPCR of the Ventral Striatum (VS) and hypothalamus was performed; analysing the expression levels of some opioid (*Oprd1*, *Oprk1* and *Oprm1*) receptors associated with hedonistic response in mice. Levels of some similarly associated dopamine receptors (*Drd1*, *Drd2* and *Drd5*) and the dopamine transporter (*Dat* or *Slc6a3*) were assessed in the VS.

The VS showed that there was no significant fold changes in any of these genes for either TG or their NON-TG littermates. *Drd1* appeared to show a 50% decrease in the TG mice and also a 25% decrease in the NON-TG mice, however this was not significant (**Figure** 6.18).

Analysis of the hypothalamus for opioid receptor differences revealed no changes in their levels of expression for either TG or NON-TG mice (**Figure** 6.19).

6.3 Discussion

The basic characterisation of behaviours for the three offspring cohorts in this chapter indicated that there were no differences between the groups for the EPM, OF, LMA assessment, PPI and ASR assays. The major discovery was that the LCA test assessing hedonic response in the offspring highlighted a distinct phenotype in both

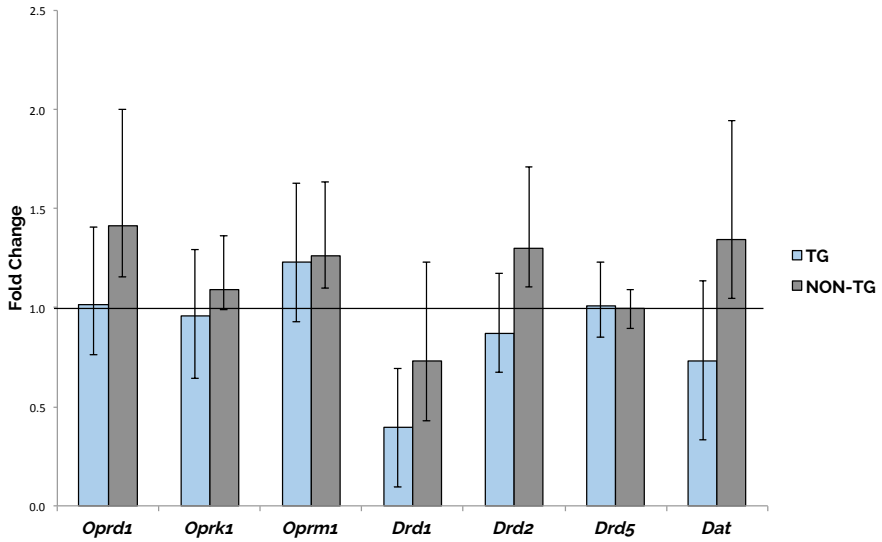


Figure 6.18: qPCR of Ventral Striatum. Bar chart showing the fold changes of opioid and dopamine receptor genes for the TG and NON-TG mice compared to WT in the ventral striatum. Error bars represent SEM.

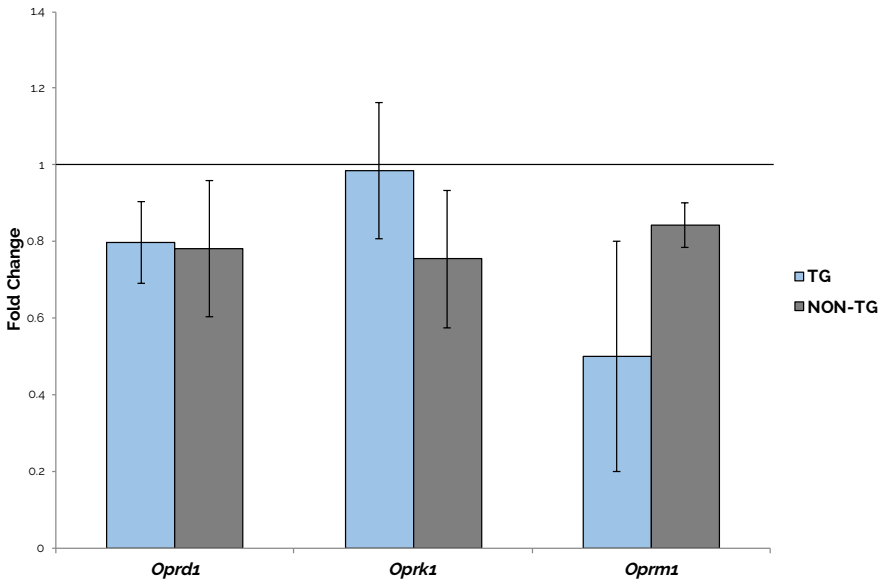


Figure 6.19: qPCR of Offspring Hypothalamus. Bar chart showing the fold changes of opioid receptor genes for the TG and NON-TG mice compared to WT in the hypothalamus. Error bars represent SEM.

the TG and NON-TG mice. Both the TG and NON-TG mice had a smaller LCS in comparison to WT mice, suggesting that both these groups had a reduced hedonic response when directly compared to WT offspring. The qPCR analysis that looked at the expression of key genes known to be associated with deficits in the behaviours assessed, showed no significant changes in of the dopamine or opioid receptors tested.

Behavioural and Biomolecular Findings

The EPM and OF both confirmed that the groups of mice responded normally throughout the tests. The lack of any significant findings regarding any abnormal anxiolytic or anxiogenic behaviour within the cohorts meant that any differences that were observed, for example how TG mice tended to move farther in the open arm of the EPM, were down to the natural variation seen between the mice. The LMA indicated that TG and NON-TG mice were not compromised in relation to their basic motor function. This is interesting as in previous studies IUGR has been known to lead to locomotor deficits (Bellinger *et al.*, 2006, Curley *et al.*, 2009). This data implies that an aberrant placenta, which leads to IUGR and altered maternal care postnatally doesn't impact upon the locomotor development of the offspring. Similarly the PPI assay, which assessed the mice's ability to filter out irrelevant information showed no deficits in any of the groups.

The PPI and ASR are sensitive to neuronal levels of dopamine. Rodents and humans possess an involuntary motoric response to an unexpected acoustic noise or startle. The response is blunted when the pulse is preceded by a pre-pulse that does not evoke a response by itself (Groves *et al.*, 1974). The reduced ability to show inhibition to a startling acoustic noise that is preceded by a quieter pre-pulse, is one characteristic of a hyper-dopaminergic state in humans (Hutchison and Swift, 1999) and rodents (Ralph *et al.*, 2001). As a result it has been shown that D1 and D2 receptor antagonists decrease the percentage inhibition of rodents (Swerdlow *et al.*,

2005).

Anhedonia is the diminished ability to experience pleasure and is a common symptom in both schizophrenia and depression (American Psychiatric Association *et al.*, 2013). It is also linked to the dopaminergic system which is intricately associated with motivation and reward (Wise, 2008, Wise and Rompre, 1989). The development of the LCA test has meant that it is possible to generate quantitative data that relates to the animals hedonic response to a palatable solution that is independent of consumption volume. Two-bottle choice tests have previously been used extensively in the literature in order to help categorise and understand "pleasure" in rodent models. The test predominately focusses on the animals preference for the two bottles of different solutions. Typically however the assay works on the basis of consumption, where total consumption of a solution is taken to relate to the palatability (Drew *et al.*, 2007, Hajnal *et al.*, 2004, Vucetic *et al.*, 2010). These approaches have been criticised however due to the inverted-U shaped function of palatability confounding the results (Dwyer, 2012). Another method that has been used, to avoid this confusion, is the study of orofacial movements during consumption (Faure *et al.*, 2010, Peciña *et al.*, 2003, Shin *et al.*, 2011). The benefits of this method is that it can reliably inform on the animals hedonic response, however it is more technically challenging in mice than in rats where the test was first established and is open to subjective analysis.

The LCA test works by exploiting the known phenomenon that rodents will freely consume palatable solutions, such as sucrose, in a predictable way over a course of time (Dotson and Spector, 2005, Lin *et al.*, 2013). When the animal is presented with a solution, its pattern of licks is made in a group, burst or "cluster". The size of these clusters are indicative of the palatability of the solution. As concentration increases so to does the number of licks per cluster, resulting in a linear increase so avoiding the inverted-U problem described earlier and providing an accurate, quantifiable and reliable way to determine the hedonic state of rodents Dwyer (2012), Dwyer *et al.* (2009), Wright *et al.* (2013), Yoneda *et al.* (2009). In our cohorts there was an obvious difference in palatability of the 16% sucrose solution compared to the less

concentrated 4% sucrose solution with all three cohorts consuming more of the more concentrated (16%) sucrose solution. This corresponded to an increase in LCS for all three cohorts indicating a heightened hedonic response to the sweeter solution and ultimately showing that all three cohorts prefer the 16% solution. Crucially there was a significant effect of genotype on the LCS. TG mice had a smaller number of licks per cluster for both 4% sucrose and 16% sucrose compared to WT mice, an effect that was also evident at 4% sucrose for the NON-TG cohort of mice. The latter observation indicates that the overall hedonic responses of both cohorts are likely to be programmed through a combination of genetic and environmental factors. The lack of motor dysfunction exhibited by the three cohorts in the LMA suggests there is unlikely to be a difference in motor speed contributing to the reduced LCS and consequently licking speed between the groups. This would need to be confirmed however, in a test more explicitly looking at the motor ability of the orofacial muscle ands movements.

The lack of increased preference between the two concentrations of sucrose in the WT mice was unexpected and was problematic for the analysis. In developing this test Dwyer (2012) highlighted that that the learned preference between the test concentrations of the solutions should be a clear indicator that the test itself worked. This work is focussed predominately on rats (Dwyer *et al.*, 2009, Lydall *et al.*, 2010). Therefore it is likely that this issue was due to standardised ceiling effects in the mouses ability to lick faster in the given time. The saccharin assessment was ultimately used in this case to show the linear difference in preferences between the three groups to a palatable solution. If this was to be performed again it would be important to include more sucrose concentrations to confirm these suspicions.

The intimate link between the dopaminergic system and both hedonic response and pre-pulse inhibition meant that genes involved with this system were investigated. There are two main families of dopaminergic receptors. These are known as D1 and D2, and are based on their members structural similarities and intracellular messengers. *DRD1* and *DRD5* make up the D1-like group and couple to Gα s intra-

cellularly, increasing cAMP levels (Dearry *et al.*, 1990, Zhou *et al.*, 1990). Whilst *DRD2*, *DRD3* and *DRD4* make up the D2-like group. D2 receptors couple to Gα intra-cellularly, decreasing cAMP levels (Sokoloff *et al.*, 1990). *DRD1* and *DRD2* are the most abundantly expressed dopaminergic receptors in the human brain (Hurley and Jenner, 2006). *DRD1* and *DRD2* receptors differ in their binding capacity for dopamine. *DRD2* has a greater affinity for dopamine compared to *DRD1* (Baik, 2013). This introduces flexibility in the dopaminergic system. As different receptors are activated depending on whether firing is phasic or tonic.

qPCR analysis didn't highlight any significant changes in D1 or D2 receptors (specifically *Drd1*, *Drd5* and *Drd2*) and the *Dat* in the ventral striatum of the brains of the TG or NON-TG offspring compared to the WT mice. It did raise some interesting questions regarding the mechanisms controlling the altered behaviour displayed by both TG and NON-TG mice in the LCA and lack of behavioural changes in the other behavioural tests.

Dopamine has often been associated with reward processing, predominantly through evidence from drug and genetic studies in humans and model organisms. Berridge (2009) theorised that dopamine has a role in the incentive salience of a reinforcer, but not necessarily the hedonistic response to it. In adult rats viral over expression of *Drd1* in prefrontal dopaminergic neurons results in an increase in sucrose and saccharin preference plus an improved motivation to work for cocaine in a progressive ratio task (Sonntag *et al.*, 2014). Whilst D2 receptors have been linked to food addiction (Baik, 2013). It followed that these receptors could be critical in distinguishing the behavioural responses of TG and NON-TG mice have to either sucrose and saccharin. The lack in differences between these dopamine receptors supported previous findings that *Drd1* is not involved in the hedonistic response and explains the lack of differences between the cohorts in the PPI and ASR tests. Therefore it is possible to say that the behavioural phenotype observed in TG and NON-TG mice is likely independent from the dopaminergic system and supports the findings of the saccharin test that the phenotype is not based upon calorie seeking behaviour.

Additionally we also looked at the potential role of the opioid system in the observed behaviours. The opioid system is synonymous with addiction, pain and reward (Dickenson, 1991, Wise, 1996). It has also been linked to emotionality and mood disorders (Filliol *et al.*, 2000), which in turn effect the success of bonding and social attachment between the mother and infant (Curley, 2011, Higham *et al.*, 2011). The opioid system is mediated by the opioid receptors, specifically μ, δ and κ receptors. KO models of these receptors have identified specific roles (Filliol *et al.*, 2000, Matthes *et al.*, 1996, Simonin *et al.*, 1998). Moles *et al.* (2004) identified that μ-opioid receptor knockout mice pups had a possible attachment deficit as they emit fewer distress vocalisations than WT mice when separated from their mothers. It follows that the behavioural phenotype identified in this chapter, which is one measure of mood and emotionality in mice may be a result of a deficit in these receptors. The results of the qPCR analysis of these 3 opioid receptors in the hypothalamus showed no significant changes in levels of expression of any of these receptors. This suggests that the mechanism that underlies the behavioural phenotype needs further assessment. Although this initial analysis didn't indicate any changes in the opioid receptors they can't be ruled out as a potential mechanism for the behavioural phenotype, thus it would be appropriate to look at them in other brain regions and at protein level.

CAVEATS

Animal models are, or at least have been, key tools in the studying the biological mechanisms that underpin many psychiatric disorders and have aided in the development of clinically effective treatments (Lewis and Lieberman, 2000). Despite this there has also been much debate about whether they are truly useful (McArthur and Borsini, 2006). Perhaps the major concern with regards to psychiatric research using mice is whether emotionality can be behaviourally assessed affectively. Several experimental models of depression, for example those focussing on chronic stress, result in reduced sucrose consumption (Kompagne *et al.*, 2008, Rygula *et al.*, 2005).

This supports the idea that sucrose consumption has surface validity when looking at anhedonia. The mice were re-housed in a previous rat room, in a situation that was out of our control for the duration of the behavioural testing. This may have induced stress that confounded our results. The fact that we still observed a significant change in the LCA in the NON-TG and TG mice and not in the other tests makes this test result valid. In order to fully validity it would be important to perform these behavioural assessments again in a less stressful environment.

Conclusion

The findings of this chapter have offered evidence that the non-genetic maternal care phenotype described in chapter 4 and the biomolecular changes which likely contribute to the maternal behavioural phenotype described in chapter 5 have lasting consequences on the behavioural trajectory of the offspring. The research presented points to two major causal factors behind the offsprings behavioural phenotype, it indicates that either the shared *in utero* environment or post natal care influences this area of development. The research however does not allow for these two factors to be deciphered between as the main contributor and due to the phenotype of the NON-TG mice it is possible to be a combination of the two. In order to be able to distinguish between the postnatal care phenotype or the *in utero* environment phenotype it would be important to carry out some cross fostering studies on the pups.

Summary of Findings

- Neither the EPM and OF assays uncovered a group specific anxiety phenotype.
- PPI and ASR were no different between the groups.
- LCA identified a specific group effect in hedonic response to 4% and 16%

sucrose in the TG mice compared to WT mice.

- NON-TG litter mates also displayed a reduced hedonic response with 4% sucrose in the LCA test compared to WT mice.
- The LCA of 0.1% saccharin confirmed the findings by identifying differing responses to this solution that mirrored the responses of all the groups at 4% sucrose. TG mice had the greatest reduction in LCS compared to WT mice and then NON-TG mice.
- qPCR analysis of *dopamine receptors* and *opioid receptors* in the ventral striatum showed that these genes were not significantly altered in expression.
- Similarly qPCR of the hypothalamus showed no genotype specific changes in gene expression of the *opioid receptors*.

General Discussion

7

The placenta is fundamental in maintaining the pregnant state in mammals and serves numerous roles in ensuring that the appropriate physiological and behavioural adaptations occur in the mother throughout pregnancy and in preparation for birth (John and Hemberger, 2012). It expresses many imprinted genes whose altered expression are associated with various developmental phenotypes in the offspring including LBW and IUGR (Bressan *et al.*, 2009, John, 2013, Tunster *et al.*, 2013). The elevated expression of the imprinted gene *Phlda2* has been shown to result in the asymmetric late fetal growth restriction of mice and regulate extra-embryonic energy stores (Tunster *et al.*, 2010, 2014). Understanding the role *Phlda2* has in modulating the SpT endocrine lineage of the mouse placenta (Tunster *et al.*, 2015, 2016) was instrumental in the development of the hypothesis that: "The placenta plays a vital in the development of the maternal instinct via this key endocrine compartment and this is modulated by an imprinted gene". The key discoveries from each chapter during this study are summarised in the tables at the beginning of each section. These findings have answered questions concerning this hypothesis and also raised several questions that are discussed in the following pages.

7.1 Placental Endocrine Lineage Analysis

Key Findings (Chapter 3)	• *Phlda2* acts exclusively to constrain the expansion of the SpT compartment of the mature mouse placenta. • *Phlda2* negatively regulates the expression of a number of key placental hormones, including the *Prls*. • ELISA analysis of levels of the *Prls* and prolactin were inconclusive, but both did show an increase in the blood of WT(KO) dams.

This chapter describes the role *Phlda2* plays in regulating the SpT lineage and the SpT's role in modulating the *Prls*. The evidence that the imprinted gene *Phlda2* has this specific function and that this directly impacts the levels of a key set of hormones that are known to play roles in the maternal adaptations to pregnancy. Some of which bind directly to the Prlr, this begs the question whether other imprinted genes may play a similar role? Recent research by Tunster *et al.* (2016) has identified another maternally expressed gene, *Ascl2*, within the same mechanistically distinct imprinted domain that acts on the SpT. This takes the number of genes identified by the John lab acting on the SpT to three, *Phlda2*, *Ascl2* and *Cdkn1c*. *Phlda2* and *Ascl2* repress the expansion of this lineage (**Figure** 7.1) whilst *Cdkn1c* is required for this lineage to develop normally (Salas *et al.*, 2004, Tunster *et al.*, 2015, 2010, 2011, 2014). These findings, combined with numerous experiments highlighting a role for lactogenic hormones and the Prlr in programming maternal care in rodents (Bridges and Freemark, 1995, Bridges *et al.*, 1990, Lucas *et al.*, 1998, Walker *et al.*, 2012), suggested that imprinted genes expressed in the placenta might regulate the extent to which dams care for their young.

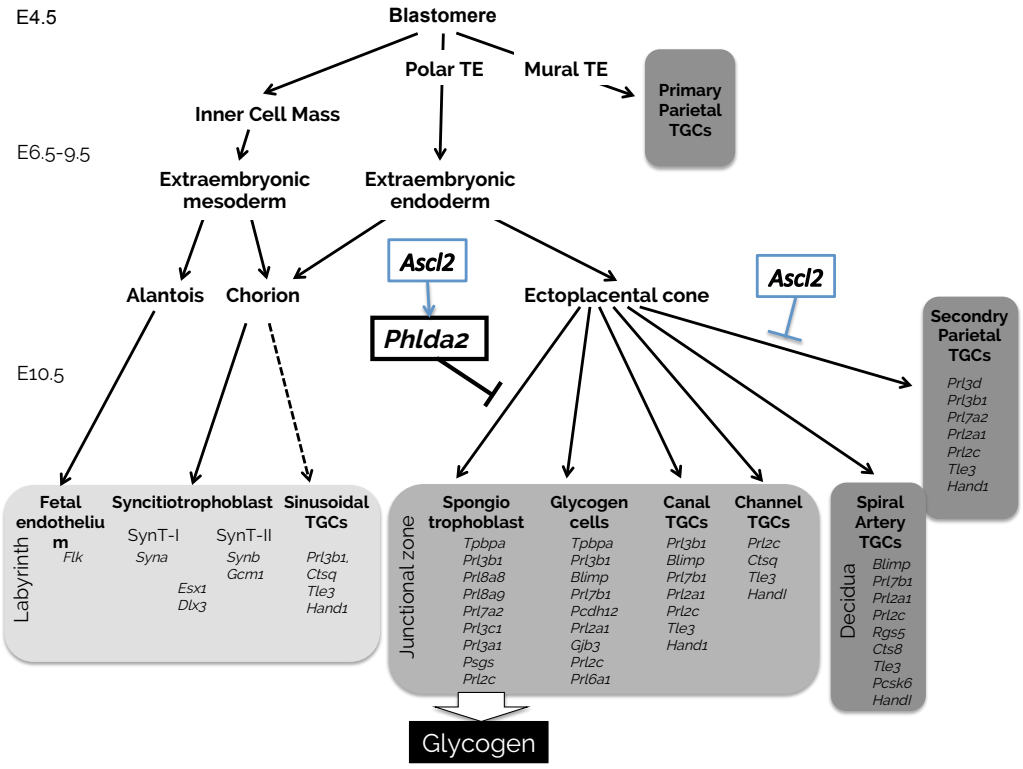


Figure 7.1: *Ascl2* and *Phlda2* Expression. Schematic summarising the cell autonomous and non-cell autonomous functions of *Phlda2* and *Ascl2* on the SpT. Taken from Tunster *et al.* (2016).

7.2 Maternal Behavioural Characterisation

Key Findings (Chapter 4)	• Altered *Phlda2* expression in the fetal placenta leads to abnormal pup retrieval and nest building in WT dams. • Altered *Phlda2* expression in the fetal placenta disrupts normal circadian nesting and feeding behaviour WT dams.

The results of the maternal behavioural characterisation defined a distinct behavioural phenotype across the three cohorts. The behaviours that were altered depended on the tests that were used and the interpretation of the results. In some respects the initial tests used were basic, however it was necessary to first establish whether there were alterations in mothering behaviours in mice and rodents in general (Deacon, 2006, Franks *et al.*, 2011). The traditional measures of maternal behaviour postnatally, such as pup retrieval and nest building are considered informative in relation to the mechanisms of maternal behaviour (Gammie, 2005). Therefore these tests were considered the appropriate starting point for this study. They have previously been used in genetic and non-genetic models looking at maternal behaviours in mice and rats. Stressed dams have been shown to retrieve their pups more rapidly and be very aggressive towards an intruder compared to non-stressed dams (Meek *et al.*, 2001). Whilst studies on a mouse model of the *Oxtr* reported that these dams took longer to retrieve pups (Takayanagi *et al.*, 2005). It is important to discuss the strain differences that are known between mice laboratory mice. Champagne *et al.* (2007) investigated differences in the maternal behaviours of 129, BL6 and Swiss mice using very similar protocols that were implemented during this study (**Figure** 7.2). There significant differences in baseline behaviour between the three strains that were tested. It was noted that 129 mice didn't show any decline in normal levels of postpartum

care for the first 6 days after parturition (Champagne *et al.*, 2007). This was key to the decision to perform the testing within the first week after birth on the 129 mice. BL6 and Swiss mice both show a decline in normal care in the first 6 days after birth. Understanding strain differences is therefore important when interpreting data on maternal care and choosing when to perform specific tests. Pseudopregnant mice can display maternal nest-building behaviour, however their nests tended to be smaller but qualitatively identical to those constructed by pregnant animals (Gandelman *et al.*, 1979). This raises issues concerning how certain we can be regarding the extent of the influence of the placenta in initiating this behaviour. It would therefore be beneficial if this research was repeated to perform the assessments on WT dams and also pseudopregnant females. Similarly, although care was taken to minimise confounding factors in the pup retrieval task and nest building, by accounting for litter size and modifying the original nest building protocol described by Deacon (2006), it would be useful to assess the general quality of nests longitudinally over the entire pregnancy period. Some studies have similarly combined the two maternal behavioural tests and used more pups for the pup retrieval task. Champagne *et al.* (2007) scattered three pups and original nest building material within the home cage on the day of birth and scored the success of both retrieval and nest building.

Voci and Carlson (1973) showed that prolactin and progesterone infusions improved nest building behaviour. The prediction that was originally formulated based on research into prolactin, specifically work by Voci and Carlson (1973) and Bridges and Freemark (1995), was that dams possessing a placenta that showed a higher expression of *Prls* and therefore posessessing theoretically greater placental signalling (WT(KO) dams) would be "better", and display a more developed maternal instinct than those dams with a WT or TG placenta. The findings of the pup retrieval and nest building demonstrated that on the surface WT(KO) dams displayed behaviour that was contrary to what was anticipated, by building nests less and retrieving pups slower than both other cohorts.

Upon taking a closer look at the Ethovision data and video recordings it became

clear it was necessary to further explore the maternal phenotypes and look at the grooming, feeding and general nurturing behaviour in each of these cohorts both within a specific test period and over the course of the assessment. Meek *et al.* (2001) reported that stressed dams raising stressed pups exhibited high levels of nursing and grooming. Therefore the discovery that the WT(KO) dams showed a similar phenotype during the nest building task, may imply that these mice have a predisposition to stress or may possess an anxiety phenotype. Unfortunately the EPM was inconclusive and therefore it would be necessary to perform a more thorough characterisation of the anxiety profile of these mothers, pre- and post-natally. Despite this it became clear that WT(KO) dams presented a more pup focussed nurturing phenotype compared to WT(TG) dams that carried out the more basic maternal behavioural tasks similarly well, or better than WT(WT) dams.

As previously mentioned infusion of prolactin decreases the latency to initiation of maternal behaviour in steroid-primed rats (Bridges *et al.*, 1990). The increased signalling of the Prls from the WT(KO) dams placenta may be an example of the decreased latency for mothers to initiate postpartum care. This evidence demonstrates the importance of utilising several behavioural assays and observations to fully understand the behaviours of a mouse model. It demonstrated that dams can prioritise behaviours dependent on their environment (Aubert *et al.*, 1997), or that hormones help differentially prime specific behaviours. The decision to perform these maternal behavioural tests and observations between PND 2-4 was based around previous studies that used this time period. Other studies that have observed differences in maternal care behaviour have looked at later timepoints or more prolonged stages and daily manual scoring (Champagne *et al.*, 2007, Chourbaji *et al.*, 2011). This would be something that could be worth investigating, through the use of Ethovision tracking and videoing longitudinally. This would prove useful in stopping any bias or disruption caused by the observer entering the room to score manually, and potentially skewing the results.

Perhaps two of the main questions that come to light from this study are: (1) is

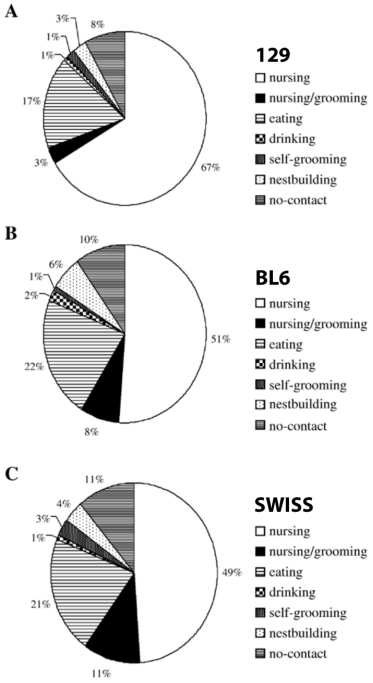


Figure 7.2: Mouse Strain Differences in Maternal Behaviour. Pie charts demonstrating the strain differences between 129, BL6 and Swiss mice. Adapted from Champagne *et al.* (2007).

maternal behaviour a direct result of the pregnancy and aberrant placenta? (2) Or does the pups genotype have a consequence upon the behavioural outcomes of the dams? In the study by Takayanagi *et al.* (2005), infant *Oxtr* KO males emitted fewer ultrasonic vocalisations than wild-type litter mates in response to social isolation. This presented the idea that the transgenic offspring in this study may potentially have vocal deficits that could result in differential maternal nurturing behaviour. The developmental findings of the offspring outcome study (Chapter 6) offer some evidence that there are no motor or anxiety deficits in TG pups. They are initially growth restricted, which may illicit a different response from the dams. The KO pups have not been behaviourally characterised within this research and this would be important to assess. An effective way to test whether the pups genotype plays a role in the observed maternal behaviour would be to perform a cross fostering study. This would allow dams to foster pups of different genotypes postnatally after being exposed to a different placenta during gestation and the results collected. Meek *et al.* (2001) performed a cross fostering study during their research and this helped

distinguish the effects of the maternal phenotype as well as the effects upon the offsprings development.

It is possible that the varying levels of Prls may impact upon the lactation of the dams. Prls are known to be important in mammary development and milk production helping to maintain lactation after it has been initiated by prolactin (Flietstra and Voogt, 1996). The suppression of prolactin by bromocriptine rapidly causes a reduction in milk secretion (Forsyth and Wallis, 2002). *Src* KO mice show a failure in milk secretion but not mammary development due to a failure in expression of the *Prlr* and subsequent downstream signalling (Watkin *et al.*, 2008). The role that the Prls play in maintaining lactation make it plausible that milk secretion and/or milk quality may be affected. This could potentially explain the reduced nursing behaviours exhibited by the WT(TG) dams, which are thought to have reduced levels of *Prls*. The fact that the offspring don't differ in weight after catch up from the IUGR, may suggest this not to be the case. Behaviourally, a cross fostering study would help decipher any lactation differences; physiologically, the mammary glands should be fully appraised and milk quality tested for each of the cohorts of dams using whole mounting and oxytocin induced milk ejection followed by the appropriate biomolecular assays (Plante *et al.*, 2011). If less prolactin and Prls are transferred to the offspring could this impact on the offsprings development? Prolactin has been shown to be involved in the development of neuroendocrine, immunological and reproductive systems of the young (Melo *et al.*, 2009). Melo *et al.* (2009) showed that deficiency in prolactin intake during development of female rats negatively affected the maternal care provision they provided when they themselves became mothers. This would be interesting to assess in the female offspring from this study. Future experiments to address the issues highlighted in this section could involve the use of the newly available *Prlr* conditional KO (Brown *et al.*, 2016). Using the *Prlr* conditional KO to attempt to restore maternal care through the genetic normalisation of signalling via the Prlr would allow us to determine the extent to which placental *Phlda2* regulates maternal care via the maternal Prlr.

7.3 Maternal Biomolecular Characterisation

Key Findings (Chapter 5)	• Microarray analysis highlight distinct global differences in gene expression levels between the three cohorts of dams in both the hippocampus and the hypothalamus at E16.5. • Altered placental *Phlda2* expression leads to changes in gene expression in maternal brain. • Altered placental *Phlda2* expression leads to changes in maternal neurogenesis. • HPLC analysis showed no significant differences in neurotransmitter levels or in there metabolites across the three groups at E16.5.

The maternal biomolecular chapter identified changes in the maternal brain that preceded the behavioural changes identified (Chapter 4). The microarray analysis highlighted distinct differences in many of genes between the three cohorts with genes that were up regulated in the WT(TG) brains and down regulated in the brains of WT(KO) and vice versa. These differences led to a more detailed look at the pathways linked to the gene changes seen in the hypothalamus and hippocampus across the groups. The hypothalamus showed an enrichment in *Olfr* genes, that were involved in olfactory transduction. Previous work by Del Punta *et al.* (2002) has linked a cluster of the vomeronasal receptor genes to a deficit in pheromone responses by mice. Pheromones themselves have been linked to increased neurogenesis and improved maternal care in mice (Larsen *et al.*, 2008). The fact that there are hundreds of Olfr's that have no known ligands, begs the question whether any of these receptors have a role in a similar pathway or whether they are not relevant at all (Zhang and Firestein, 2002). Are these Olfr's ultimately responding to the

pheromones of the pups causing the dams to respond in different ways with their care towards the offspring? It was an initial surprise that these receptors were present in the hypothalamus, however there is an established link between the olfactory system and the hypothalamus with projections from the olfactory bulb branching into the hypothalamus (Yoon *et al.*, 2005).

The hippocampal gene expression highlighted genes that were associated with behavioural disorders, including schizophrenia and feeding behaviour, as well as some linked to the dopaminergic and serotonergic system. These key genes appeared to be down regulated in both the WT(TG) and WT(KO) conditions in comparison to the WT(WT) dams. This was confirmed through qPCR indicating that the gene expression patterns are seemingly not associated with gene dosage relationships. We know that placental hormones are produced by offspring, but act on receptors of mothers. As such, placental hormones and maternal receptors are prime candidates for the expression of parent-offspring conflict (Haig, 1993, 1996). Prolactin-dependent signalling occurs as the result of ligand-induced dimerisation of the Prlr. Several alternatively spliced transcript variants encoding different membrane-bound and soluble isoforms have been described for the *Prlr* gene, which may function to modulate the endocrine and autocrine effects of prolactin in tissues, including the brain. Prolactin (like GH) has two separate binding sites, the first has a high affinity, the second site has a low affinity for the Prlr. These sites each interact with a Prlr forming a functional receptor dimer that activates a signalling cascade (Freeman *et al.*, 2000, Langenheim *et al.*, 2006). The pathways include the Ras/Raf/MAPK kinase/Erk (Das and Vonderhaar, 1996) and phosphatidylinositol 3-kinase/Akt signalling pathways. It is through these pathways that the Prlr induces cell differentiation, proliferation and survival (Shillingford *et al.*, 2002). Once the first site has bound it initiates the binding of the second site, when a disruptive mutation of prolactin binding site 2 was investigated, it stopped Prlr activation. This is because the Prlr only becomes active when the trimeric complex forms (Bole-Feysot *et al.*, 1998, Goffin *et al.*, 1996). This understanding has given rise to the idea that perhaps the gene expression profiles

exhibited in the cohorts may be a result of a "bounce back" effect. If this were the case it would give a reason for both the inverted U and U profiles. More explicitly this may be explained through promotor occupancy, when there is a low amount of Prls in the system then the Prlr is not activated as much. In contrast, as the levels of Prls increase, activation of the receptor increases. Theoretically therefore if levels reach a certain threshold then the Prlr's become occupied by the over abundance of Prls resulting in the inactivation of the Prlr's and mimicking the effect of low levels of the ligands.

A recent study by a group at Cardiff University investigating steroid sulfatase deficiency as a means to understand the brain pathway of PP identified the *CCN* family of genes as potential candidates (Humby *et al.*, 2016). *NOV/CCN3* was identified in their study and is interesting due to a number of factors that may warrant further research in this study. *NOV* when over expressed is associated with abnormal maternal behaviour which can be reversed through antipsychotic administration (Humby *et al.*, 2016). It is known that *NOV* is located on human chromosome 8, just below the linkage peak that has been implicated in bipolar affective disorder and PP (Jones, 2007). Disrupted calcium signalling is linked to bipolar disorder and PP (Riley and Watt, 1985), the protein encoded by *NOV* is involved in calcium signalling (Lombet *et al.*, 2003). It is also a regulator of angiogenesis in the placenta, as has been shown by its aberrant expression in cases of pre-eclampsia (Winterhager and Gellhaus, 2014). The findings of this study by this group looked at whole brain expression levels, which makes the significant up regulation ($p\text{-}value = 0.001$) of the gene in WT(TG) dams hippocampus interesting and may offer support to *NOV* being a key candidate gene in PP.

Through this study we discovered a decrease in BrdU labelled cells within the SVZ for both the WT(TG) and WT(KO) dams. This was contrasted with the cell counts in the SGZ for WT(KO) dams, where there was an increase in BrdU labelled cells. The decrease in positively labelled cells at this stage in the SVZ conflicted with our prediction that an increase in circulating *Prls* would cause an increase in maternal

neurogenesis. Shingo (2003) investigated the levels of neurogenesis at E7.5 and E14.5 followed by P0, P7 and P14. They chose these time points based on the reasoning that mammals experience a surge in prolactin concentrations during the first half of pregnancy before they steadily decrease until term, and then rise again postpartum during lactation (Freeman *et al.*, 2000). This led to Shingo (2003) hypothesising that due to the *Prlr*'s high expression adjacent to the SVZ and in the choroid plexus of the forebrain, that the *Prlr* expression could be involved with pregnancy mediated neurogenesis. Their evidence that neurogenesis decreased 50% at E7.5 in the forebrain of mice with a 50% decrease in the *Prlr* gene dosage supports the involvement of the receptor in mediating maternal neurogenesis (Shingo, 2003). They saw a gradual decrease in BrdU labelled cells onwards from this stage before they peaked again at P7. Based on there findings it was less surprising that at E16.5 we didn't see as higher numbers of BrdU labelled cells as Shingo (2003). The decision to chose E16.5 as our time point was because it is the period of maximal placental signalling, with relation to the SpT and the circulating Prls (Soares, 2004). Despite this E16.5 has failed to be previously regarded, therefore it would probably have been wise to perhaps assess 129 WT dams and pseudopregnant dams baseline levels of neurogenesis to gain a fuller characterisation of this phenomenon at this stage.

It is possible the decrease in BrdU cells seen in the WT(KO) dams may be due to promotor occupancy of the Prlr, thus the decrease in BrdU labelled cells could be explained through the reduction in signals to the Prlr's that are mediating neurogenesis. The next question is whether the cells labelled for BrdU are indeed new neurons or another type of brain cell such as an astrocyte or glial cell. DCX and Nestin labelling was performed in separate assays. In this case we saw a reduction in DCX and Nestin labelled cells in the WT(KO) dams SVZ but not in the WT(TG) SVZ. DCX labels migrating neurons and Nestin labels neural stem cells. The nearly 50% decrease in both of these cell types at E16.5 suggests that neurogenesis is reduced.

New neurons can take up to 4 weeks to become functionally active at which point

the doubling of olfactory interneurons can significantly enhance olfactory function in mice (Rochefort *et al.*, 2002). Consequently to truly determine the importance of these differences in neurogenesis would require a longitudinal study to look at the functional relevance of the new neurons. The use of the *Prlr* conditional KO could help with determining the ambiguity of the ELISAs and offer preliminary support to the hypothesis that the Prls are functionally relevant in mediating the changes in the maternal transcriptome via signalling through the Prlr in the maternal brain. A longitudinal study that utilises the *Prlr* conditional KO would allow for the assessment what the impact new neurons born during late gestation may have upon the second pregnancy of these dams.

7.4 Offspring Outcome

Key Findings (Chapter 6)	• The EPM, OF, PPI and ASR assays didn't uncover any behavioural differences between the groups. • Offspring from WT(TG) mothers showed a decreased hedonic response in the LCA that was partially independent of genotype. • The were no changes in gene expression of the opioid or dopamine receptors in the VS or hypothalamus of either group of offspring.

The final experimental chapter focussed upon the outcomes of the pups raised by dams that were exposed to TG placenta. The results showed that the underlying phenotypic responses were intact between the groups. The key finding was that both TG and NON-TG litter mates displayed a disturbance in their hedonic responses at 4% sucrose solution in the LCA assay. This was also apparent in the TG offspring at the 16% sucrose level. Both showed differences in the test using saccharin. This observation highlighted a distinct non-genetic mechanism to this phenotype, because NON-TG offspring are genetically WT. It is known that natural variations in maternal care are often observed between laboratory environments among inbred and outbred rodents. These natural variations are used as a predictors of offspring neurobiology and behaviour (Champagne *et al.*, 2007, 2003). This approach has been more thoroughly explored in rats, but emerging data from studies in mice illustrate that there are persistent effects on emotionality as a consequence from variations in maternal care (Coutellier *et al.*, 2009b). Use of systematic and methodological approaches to observe home cage maternal care toward offspring is essential in studies examining the direct impact of maternal care on offspring development (Franks *et al.*, 2011). Our model provided the perfect platform to assess this and try and decipher

the differential effects that maternal care, genotype and the *in utero* environment has upon the offspring.

The mothers in this chapter were not subjected to any behavioural assessments during this study. This was so that any outcomes displayed by the offspring could be attributed to the individual differences in baseline maternal nurturing given during the post partum period before weaning. It should be noted that despite no noticeable physiological differences between the pups, there is the potential that the behavioural phenotype displayed by the TG and NON-TG offspring could be a consequence of the altered genotypes of the TG offspring and not a direct result in maternal care. The programming effects of altered placental imprinted gene expression on later life adult behaviour have been demonstrated before for *Igf2* (Mikaelsson *et al.*, 2013). *Phlda2* is known to restrict fetal growth (Tunster, 2009, Tunster *et al.*, 2014), theoretically the TG pups physiological phenotype could have caused the mothers to react differently toward them and their NON-TG litter mates. This factor was not controlled for during this study, meaning that using cross fostering would be required to help determine whether the pup genotypes played a role in developmental outcomes. It would also be useful to assess levels of vocalisations by the offspring, as decreased calling has been linked to social isolation in pups (Takayanagi *et al.*, 2005). Similarly there is some evidence that litter composition can play a role on the development of the offspring (Crews *et al.*, 2004), therefore it is possible that litter composition could play a part in maternal development too.

It is possible that the developing fetal brain could have been primed *in utero*, in preparation for the external environment occupied by the mother. Prenatal adversity is a well known phenomenon that results in functional alterations within systems controlled by imprinted gene dosage, these include metabolic function (Entringer, 2013), fetal growth (Baibazarova *et al.*, 2013, Tunster *et al.*, 2016) and behaviour (Bale *et al.*, 2010b, McNamara and Isles, 2014). This has formally been reviewed by Bateson *et al.* (2004) and in chapter 1. In addition the TG pups suffer from IUGR, in humans IUGR preterm infants at 16 days display decreased hedonic responding

to a sucrose solution (Ayres *et al.*, 2012).

Although care was taken to maintain the in cage environments the same for the newly weaned offspring, there is the possibility that differences in in-cage environments post weaning could act as a causal factor to the identified phenotype (Tucci *et al.*, 2006). In particular, it is important to note that before testing and post weaning of the offspring, there were issues with the animal house at Cardiff University. Meaning that the mice had to be re-housed in a room that was formerly a rat room, which has been shown to increase stress responses in mice (Adamec *et al.*, 2004). Although initially thought not to be an issue there was an increase in so called "crunching" of food by several cages of mice, which is thought to be a behavioural response to stress as well as increased in cage fighting. This aggressive behaviour was particularly prevalent in the cages of TG mice. There has been much work that has linked stress to adverse behavioural responses in rodents (Champagne and Meaney, 2006, Meaney, 2001). Mice prefer to be housed alone as it reduces their stress levels, something which is opposite to rats Campos *et al.* (2013). These factors mean the findings reported in the LCA are valid, although along with the other behavioural assays the results may have been confounded by the environment, therefore they would need to be repeated in order to validate the results. Furthermore, it would be informative for a larger number of sucrose concentrations to be used and to measure serum levels of cortisol to help account for the affects of stress.

The actual lick cluster sizes performed by the mice were comparatively small in contrast to published rat data (Wright *et al.*, 2013), however evidence from an increasing number of mouse trials indicate this to be be normal in the 129 strain (Dwyer, personal communications). Despite these weaknesses it is important to note that Champagne *et al.* (2003) illustrated that maternal behaviour, particularly in licking/grooming, act to regulate the development of endocrine, emotional and cognitive responses to stress in pups. It is therefore not inconceivable that any stress responses observed in the TG and NON-TG mice may be due to a maladaptation of the pups to stress during development. The epigenetic profile of the pups that

are exposed to altered maternal care would be an exciting area to look at, as the multifunctional roles and the flexibility of epigenetic marks have led to the suggestion that imprinted genes may contribute to the fetal programming of adverse outcomes (Keverne, 2010). Experimental animal models have previously demonstrated that early-life stress can leave a mark on the offspring epigenome, with alterations reported in offspring exposed to prenatal stress (Mueller and Bale, 2008), maternal separation (Kundakovic and Champagne, 2015) and low levels of maternal postnatal care (Szyf *et al.*, 2005). It is therefore exciting to see behavioural changes in the offspring of WT dams exposed to TG placenta, it would be important to investigate whether this care results in epigenetic changes that lead to alter gene expression and account for the behavioural changes seen in the offspring.

The increased aggression that was observed in the TG mice cages was interesting and should be further assessed. There has recently been observations that variations in *PHLDA2* expression in the placenta of humans, is linked to an increased aggression in human infants scored using the CICS scoring system (Janssen, personal communications). This is another exciting area that needs further work. The fact that this model allows the multigenerational assessment of the potential impact that an aberrant placenta may have upon maternal care and the subsequent outcomes of the offspring highlight the importance of gaining a better understanding of this area of study.

7.5 Final Remarks

There are still questions regarding the mechanisms that underpin the phenotypes described for both the dams and the offspring, but this model provides a novel and intriguing system that combines the deficits seen in the maternal care with the direct consequences this care has upon the offsprings development. It is the first ever demonstration that a placental defect can alter maternal care behaviour in a

WT mother and that this behaviour can influence the future behaviour of the WT offspring exposed to the same environment. Meaning that this model is unique in that it gives the ability to understand a potential non-genetic mechanism behind the postnatal behavioural phenotypes of the dams that stems from a placental origin.

So how does this research translate to the human condition? Throughout this thesis the link between IUGR, LBW and maternal psychiatric conditions has been discussed. The origin of maternal mood disorders is still an enigma, however the co-occurrence of these disorders with IUGR and LBW provided a novel hypothesis that the switch that initiates them may not have a genetic origin. Recent research has implicated the imprinted gene *PEG3* with prenatal depression in humans, changes in the expression of *PHLDA2* didn't reach significance in this particular study by Janssen *et al.* (2016), the ever increasing knowledge of imprinted genes roles in the placenta mean that there are likely other genes that have similar roles to play. Similarly these imprinted genes are known to be influenced by the environment including low protein diet. Understanding the placental origins of these conditions and discovering whether environmental factors can play a role in their expression may help improve the clinical understanding of these disorders and ultimately lead to their prevention and treatment.

Developmental Biology 409 (2016) 251–260

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

The imprinted *Phlda2* gene modulates a major endocrine compartment of the placenta to regulate placental demands for maternal resources

S.J. Tunster, H.D.J. Creeth, R.M. John *

Cardiff School of Biosciences, Cardiff University, Cardiff, Wales CF10 3AX, UK

ARTICLE INFO

Article history:
Received 4 August 2015
Received in revised form
2 October 2015
Accepted 11 October 2015
Available online 23 October 2015

Keywords:
Phlda2
Placenta
Hormones
Epigenetics
Imprinting

ABSTRACT

Imprinted genes, which are expressed from a single parental allele in response to epigenetic marks first established in the germline, function in a myriad of processes to regulate mammalian development. Recent work suggests that imprinted genes may regulate the signalling function of the placenta by modulating the size of the endocrine compartment. Here we provide *in vivo* evidence that this hypothesis is well founded. Elevated expression of the imprinted *Pleckstrin homology-like domain, family a, member 2* (*Phlda2*) gene drives a reduction of the spongiotrophoblast endocrine compartment, diminished placental glycogen and asymmetric foetal growth restriction. Using both loss-of-function and gain-in-expression mouse models, here we further show that *Phlda2* exclusively modulates the spongiotrophoblast compartment of the placenta without significantly altering the composition of the trophoblast giant cell endocrine lineages that share a common progenitor with this lineage. Additionally, we show that *Phlda2* loss-of-function placentae contain nearly three times more placental glycogen than non-transgenic placentae. Remarkably, relative to a fully wild type scenario, wild type placentae also accumulate excessive glycogen. While loss-of-function of *Phlda2* increased both placental weight and placental glycogen, the weight of both mutant and non-transgenic fetuses was lower than that found in a fully wild type scenario indicating that excessive glycogen accumulation comes at the cost of foetal growth. This work firstly highlights a novel signalling function for the spongiotrophoblast in stimulating the global accumulation of placental glycogen. Furthermore, this work suggests that *Phlda2* manipulates the placenta's demands for maternal resources, a process that must be tightly regulated by epigenetic marks to ensure optimal foetal growth.
Crown Copyright © 2015 Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

Genomic imprinting is an epigenetic phenomenon which drives the preferential expression of certain genes from one parental allele (John and Surani, 1996). The finding that some paternally silenced genes restrain foetal growth while some maternally silenced genes are growth promoting led to the suggestion that imprinting may have evolved in response to the different energetic contributions that male and female mammals make to their offspring (Moore and Haig, 1991) and the effectively antagonistic selective pressure acting on the mother–offspring relationship (Moore, 2011). However, another conflict exists in mammals because maternal resources are utilised by both the growing foetus and the developing extra-embryonic tissues. In some mammals these supporting tissues can consume more than half the maternal resources allocated to a pregnancy (Fowden, 1997; Carter, 2000) introducing the potential for competition between their energetic requirements and that of the growing foetus.

In mice, a significant number of imprinted genes functionally converge to regulate placental growth and development (Tunster et al., 2013). The mature mouse placenta supports foetal growth from approximately embryonic day (E) 9.5 until birth and is organised into the histologically distinct labyrinth zone, junctional zone and maternal decidua interspersed with trophoblast giant cells (TGCs) lining the maternal tissues and vasculature at specific sites (Rai and Cross, 2014). While the function of the labyrinth in placental transport is well established, the function of the spongiotrophoblast and the glycogen cell lineages, which reside within the junctional zone, has yet to be fully determined experimentally. Placental glycogen, which accumulates in the glycogen cells of the junctional zone from mid-gestation, may provide a store of easily mobilisable energy late in gestation to support the final stages of foetal growth (Coan et al., 2006) supported by numerous mouse models in which limited glycogen stores are associated with foetal growth restriction (Lefebvre, 2012). The function of the

* Corresponding author.
E-mail address: JohnRM@cf.ac.uk (R.M. John).

http://dx.doi.org/10.1016/j.ydbio.2015.10.015
0012-1606/Crown Copyright © 2015 Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

spongiotrophoblast lineage is less well understood but several placental lactogens (Prls) and pregnancy-specific glycoproteins (Psgs) are expressed from this lineage some of which have been shown to act on the mother to induce the physiological changes required for a successful pregnancy (Samaan et al., 1968; Bhattacharyya et al., 2002; Muller et al., 1999; Kammerer and Zimmermann, 2010; Wu et al., 2008). While this lineage is required for foetal viability (Guillemot et al., 1994; Ono et al., 2006), fetuses can survive to term with very little spongiotrophoblast (Oh-McGinnis et al., 2011). A reduced spongiotrophoblast has been linked to foetal growth restriction in several studies (Oh-McGinnis et al., 2011; Hitz et al., 2005; Zheng-Fischhofer et al., 2007; Withington et al., 2006; Tunster et al., 2010) while an expanded spongiotrophoblast may drive delayed parturition associated with foetal and maternal death (Denda et al., 2011). Loss of function of several maternally expressed imprinted genes results in an expansion of the spongiotrophoblast lineage (John and Hemberger, 2012), most recently *Sirh7*/*Ldoc1* (Naruse et al., 2014), suggesting that the paternal genome has selectively silenced genes that limit spongiotrophoblast-specific functions. However, while there are a number of mouse mutants in which alterations in the spongiotrophoblast lineage have been reported, these defects commonly occur alongside alterations in additional placental lineages. In particular, the glycogen cell lineage and four of the six distinct TGC lineages which share a common progenitor with the spongiotrophoblast (Rai and Cross, 2014; Hu and Cross, 2010; Simmons et al., 2007; Gasperowicz et al., 2013) confounding their functional assessment.

Pleckstrin homology-like domain family A member 2 (Phlda2) is a maternally expressed gene that maps to the imprinted domain on mouse distal chromosome 7, which encodes a PH domain-only protein (Frank et al., 1999; Qian et al., 1997). Prior to the formation of the mature mouse placenta, *Phlda2* is expressed most strongly in the ectoplacental cone and the visceral endoderm of the yolk sac (Frank et al., 1999; Dunwoodie and Beddington, 2002; Takao et al., 2012). The ectoplacental cone contains the *trophoblast specific protein alpha* (*Tpbpa*)-positive progenitors that give rise to the spongiotrophoblast, the glycogen cells and four of the six TGC subtypes of the mature placenta (Rai and Cross, 2014; Simmons et al., 2007, 2008; Gasperowicz et al., 2013). Loss-of-function of *Phlda2* results in an enlarged placenta with an expanded junctional zone and more placental glycogen but without foetal overgrowth (Frank et al., 2002). Elevated expression, at two-fold the endogenous level, results in placental stunting, a loss of the spongiotrophoblast lineage and reduced placental glycogen accumulation but without an alteration in the representation of the glycogen cell lineage or the parietal trophoblast giant cells which line the maternal decidua (Tunster et al., 2010; Salas et al., 2004). Additionally, elevated *Phlda2* drives a late, asymmetric foetal growth restriction (Tunster et al., 2014). Taken together, these data suggested that *Phlda2* acts indirectly to restrain foetal growth by limiting the expansion of the spongiotrophoblast lineage, which is required to stimulate glycogen accumulation. However, the effects of elevated *Phlda2* gene dosage on all the TGCs lineages has not been reported. Moreover, a characterisation of the placental lineages in the context of loss-of-function has not been performed.

To further investigate the role of *Phlda2* in regulating the placental endocrine lineages, glycogen accumulation and foetal growth, we performed an examination of the placental lineages in the different *Phlda2* gene dosage mouse models and biochemically quantified placental glycogen at different stages of development in response to loss-of-function of *Phlda2*. Remarkably, in addition to the anticipated over accumulation of placental glycogen in response to loss-of-function of *Phlda2*, we noted a similar phenotypes in non-transgenic placenta sharing the uterine environment. Rather than supporting enhanced foetal growth, these stores came to the detriment of foetal growth identifying a novel role for *Phlda2* in regulating maternal resource allocation between the placenta and the foetus.

2. Materials and methods

2.1. Mouse strains and genotyping

Animal studies and breeding were approved by the Universities of Cardiff Ethical Committee and performed under a UK Home Office project license (RMJ). All mice were housed under standard conditions throughout the study on a 12 h light–dark cycle with lights coming on at 06.00 h with a temperature range of 21 °C ± 2 with free access to water (tap water) and standard chow. The *Phlda2* targeted allele (Frank et al., 2002) was crossed into the 129S2/SvHsd (Harlan, 129) strain background for +8 generations. The single copy *Phlda2* transgenic line $Phlda2^{+/+BACx1}$ (Tunster et al., 2014) was maintained on the 129 background by paternal transmission. *Phlda2* deficient fetuses were generated by crossing $Phlda2^{+/-}$ females with wild type males. $Phlda2^{+/-}$ females were crossed with $Phlda2^{+/+BACx1}$ males to generate four genotypes: $Phlda2^{+/+}$ (non-transgenic; 1X), $Phlda2^{-/+}$ (maternal KO; 0X), $Phlda2^{+/+BACx1}$ (single copy *Phlda2* transgene; 2X) and $Phlda2^{-/+BACx1}$ (single copy transgene plus maternal KO; 1X). 129 wild type colonies were maintained alongside the transgenic colonies. For recipient transfer experiments, 2-cell embryos were surgically transferred into E0.5 wild type 129 recipients mated with vasectomised males.

2.2. Quantitative RNA analysis

RNA was extracted from whole placenta following careful removal of membranes and umbilicus. Quantitative PCR of reverse transcribed RNA (RT-qPCR) was performed and analysed as described (Tunster et al., 2010; Schmittgen and Livak, 2008). RNA was hybridised to Affymetrix Mouse Gene 2.0 ST chips. Data was analysed essentially as described (Zhang et al., 2009) and using Partek Genomic suite. Genes significantly up in $Phlda2^{-/+)}$ (0X) and significantly down in $Phlda2^{+/+BACx1}$ (2X) E16.5 whole placenta were tested for enrichment of gene ontology molecular function and biological process using the Database for Annotation, Visualisation and Integrated Discovery (DAVID). Microarray data available in GEO repository accession *******.

2.3. In situ hybridisation and histological analyses

Placentas were fixed overnight in phosphate-buffered 4% paraformaldehyde, paraffin-embedded and 6 μm sections taken through the midline. Riboprobe preparation and *in situ* hybridisation were performed as previously described (Tunster et al., 2010, 2012).

2.4. Weighing studies and biochemical determination of placental glycogen concentration

Foetal and placental wet weights were taken at the stated time points after a discernable plug and normalised. Genotyping data was obtained from yolk sac DNA as previously described (Frank et al., 2002; John et al., 2001). Glycogen was extracted from whole placenta, and resuspended in 1 ml of H_2O and assayed according to the method of Lo et al. (1970) at a dilution of 1 in 2.

2.5. Statistical analyses

Statistical significance (probability values) was determined using the Student's *t*-Test (two tailed distribution and two sample unequal variance) for comparisons between knockout and control littermates. Comparisons with the fully wild type scenario were undertaken using ANOVA with Bonferroni correction.

3. Results

3.1. *Phlda2 supresses the expansion of the spongiotrophoblast without altering the representation of the TGC lineages*

A characterisation of placental lineages in response to loss of *Phlda2* function was undertaken after breeding the original line carrying a targeted deletion of *Phlda2* onto a 129S2/SvHsd (129) genetic background. *Phlda2*$^{-/+}$ (maternal KO; 0X) placenta expressed *Tpbpa*, a gene expressed by both the spongiotrophoblast and the glycogen cell lineage (Lescisin et al., 1988), at 2.20-fold ($\pm$0.26; $p=3.77\times10^{-4}$) the normal level (Fig. 1A, and Table S1). *Prl8a8*, a gene expressed exclusively in spongiotrophoblast cells (Simmons et al., 2008), was elevated by 2.10-fold ($\pm$0.15;

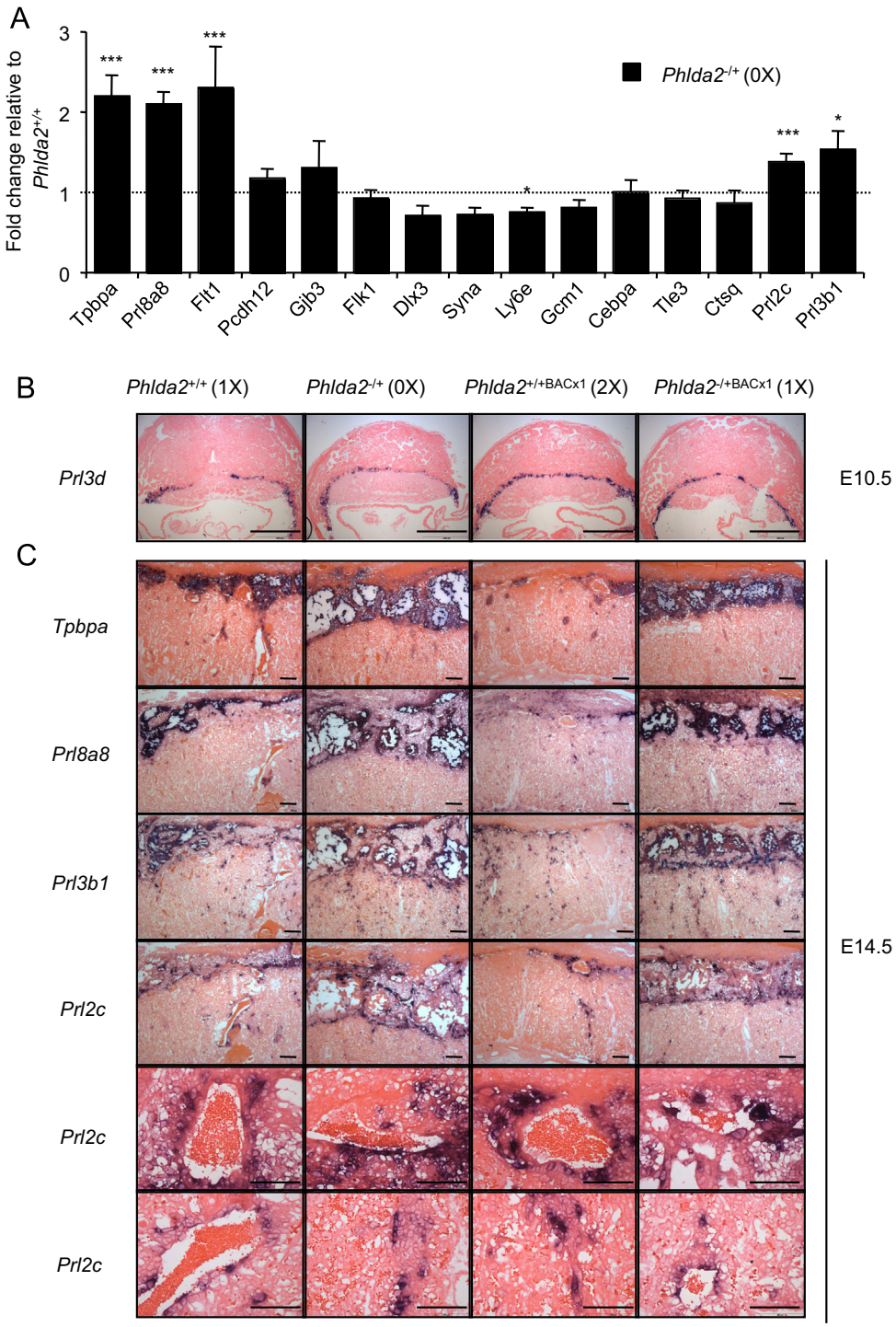


Fig. 1. *Phlda2* restrains the expansion of the spongiotrophoblast lineage without significantly altering other trophoblast cell lineages. A. RT-qPCR analysis of placental lineage markers mRNA levels between E14.5 *Phlda2*$^{+/+}$(non-transgenic; 1X) and *Phlda2*$^{-/+}$(0X) placentae from littermates examined on the 129 genetic background. B. *In situ* hybridisation with *Prl3d (Pl1)* of midline sections from E10.5 *Phlda2*$^{+/+}$(1X), *Phlda2*$^{-/+}$(0X), *Phlda2*$^{-/+BACx1}$(2X) and *Phlda2*$^{-/+BACx1}$ (1X) 129 placenta. C. *In situ* hybridisation of midline sections E14.5 placenta with *Tpbpa*, *Prl8a8*, *Prl3b1* (*Pl2*) and *Prl2c (Plf)* (scale bar=200 μm). *Prl2c* also shown at a higher magnification illustrating similar staining of the canal and the spiral artery TGC (scale bar=100 μm). For the RT-qPCR analysis, $N=4$ placenta per genotype (2 *vs* 2 from 2 independent litters); error bars represent SEM. Statistical significance calculated using *t*-test. $^{NS}P>0.05$, $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.005$.

$p=8.78\pm10^{-5}$) and *FMS-like tyrosine kinase 1* (*Flt1*), a marker of the junctional zone (Breier et al., 1995) was elevated by 2.31-fold (±0.51; $p=0.00551$). Expression of glycogen cell markers (*Gjb3*/*Cx31* and *Pcdh12*) and traditional markers of the labyrinth was comparable to wild type (Fig. 1A). These data were reciprocal to our findings in the gain-in-expression *Phlda2* model (Tunster et al., 2010, 2014) confirming the inverse relationship between *Phlda2* gene dosage and the spongiotrophoblast but not the glycogen cell lineage. Some markers of the TGC lineages, *Tle3* and *Ctsq*, were expressed normally whereas *Prl2c (Plf)* and *Prl3b1,* which are expressed in both the spongiotrophoblast and TGCs, were elevated in response to loss-of-function of *Phlda2* (Fig. 1A) suggesting that some TGC lineages may also be altered in this model. To further refine the analysis, four placental genotypes were generated from crosses between *Phlda2*$^{+/-}$ females and males carrying a single copy of a transgene spanning the *Phlda2* locus: *Phlda2*$^{+/+}$ (non-transgenic; 1X), *Phlda2*$^{-/+}$ (maternal KO; 0X), *Phlda2*$^{+/+BACx1}$ (single copy *Phlda2* transgene; 2X) and *Phlda2*$^{-/+BACx1}$ (single copy transgene plus maternal KO; 1X). *In situ* hybridisation performed with *Prl3d*, an exclusive marker of the parietal TGC lineage at E10.5 (Simmons et al., 2008), excluded any gross alterations in this lineage (Fig. 1B). Hybridisation with *Tpbpa* and *Prl8a8* at E14.5 further highlighted the inverse relationship between *Phlda2* gene dosage and the spongiotrophoblast with an expansion of this lineages occurring in response to the decreasing expression of *Phlda2*. Hybridisation with *Prl2b1* and *Prl2c*, which identify the canal, sinusoidal and spiral artery TGCs (Simmons et al., 2007), was similar irrespective of *Phlda2* gene dosage (Fig. 1C) indicating that these lineages were relatively unaffected by the loss-of-function of *Phlda2*. These data identified *Phlda2* as a gene that acts exclusively to constrain the expansion of the spongiotrophoblast lineage without significantly altering the cellular composition of other trophoblast lineages which share a common progenitor.

3.2. *Phlda2 regulates the expression of several placental hormones*

The spongiotrophoblast expresses a number of *Prls* (Simmons et al., 2008), genes that encode placental lactogens that act on the mother during pregnancy to induce changes required for a successful pregnancy (John, 2013). Several *Prl* members were elevated in the *Phlda2*$^{-/+}$ (maternal KO; 0X) placenta and reduced in the *Phlda2*$^{+/+BACx1}$ (2X) placenta (Fig. 2A,Table S1). The spongiotrophoblast is also a major source of pregnancy specific glycoproteins (*Psgs*), another large gene family important for pregnancy (McLellan et al., 2005). RT-qPCR analysis of the most abundantly expressed *Psgs* during mid gestation, *Psg17*, *Psg18*, *Psg19* and *Psg21* (Fig. 2B), and *in situ* hybridisation with *Psg17* (Fig. 2C) revealed a reciprocal relationship between *Phlda2* expression and the *Psgs*. *In situ* hybridisation (Figs. 1B, C and 2C) and RT-qPCR analyses (Table S1) on double transgenic placenta carrying both the transgene and the maternally inherited targeted *Phlda2* allele (1X) formally assigned these changes to *Phlda2*.

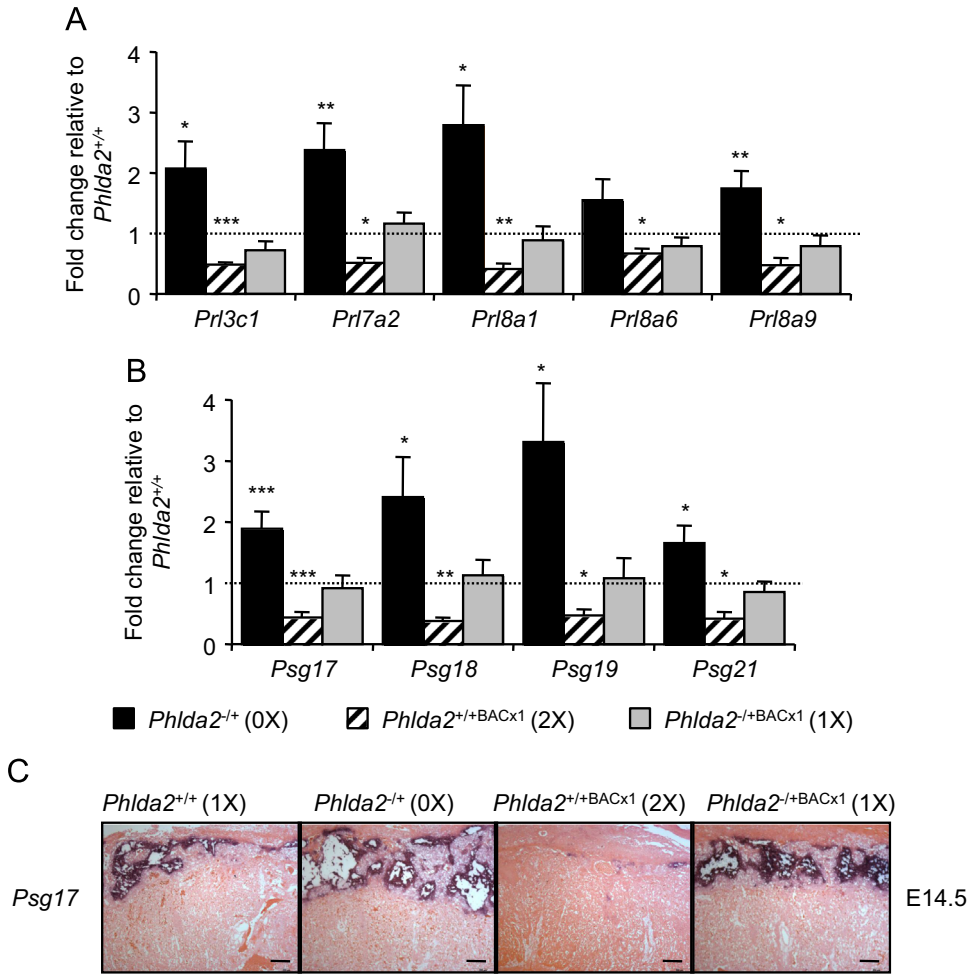
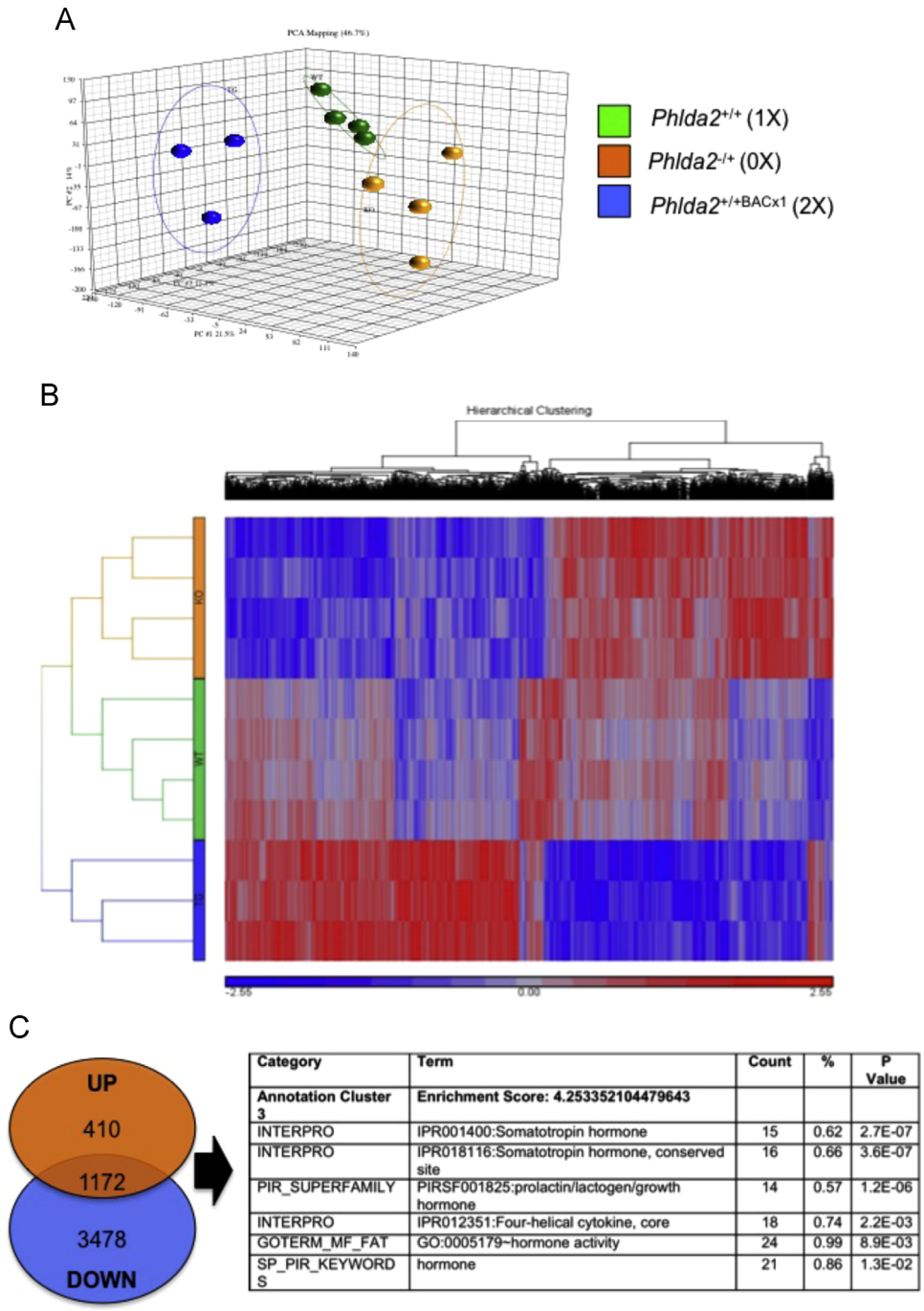


Fig. 2. *Phlda2* indirectly suppresses the expression of key placental hormones. A. RT-qPCR comparison of *Prls* at E14.5 between *Phlda2*$^{+/+}$(1X), *Phlda2*$^{-/+}$(0X), *Phlda2*$^{-/+BACx1}$(2X) and *Phlda2*$^{-/+BACx1}$(1X) 129 placentae. B. RT-qPCR comparison of *Psgs* at E14.5. C. *In situ* hybridisation of midline sections E14.5 placentae with *Psg17* (scale bar=200 μm). For the RT-qPCR analysis, $N=4$ placenta per genotype (2 *vs* 2 from 2 independent litters); error bars represent SEM. Statistical significance calculated using *t*-test. $^{NS}P>0.05$, $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.005$.



Category	Term	Count	%	P Value
Annotation Cluster 3	**Enrichment Score: 4.253352104479643**			
INTERPRO	IPR001400:Somatotropin hormone	15	0.62	2.7E-07
INTERPRO	IPR018116:Somatotropin hormone, conserved site	16	0.66	3.6E-07
PIR_SUPERFAMILY	PIRSF001825:prolactin/lactogen/growth hormone	14	0.57	1.2E-06
INTERPRO	IPR012351:Four-helical cytokine, core	18	0.74	2.2E-03
GOTERM_MF_FAT	GO:0005179~hormone activity	24	0.99	8.9E-03
SP_PIR_KEYWORDS	hormone	21	0.86	1.3E-02

Fig. 3. Microarray analysis identifies the transcriptomic signature of the spongiotrophoblast. A. Three-dimensional principle components analysis plot clustering placental gene expression with genotypes. B. Heat map showing genes significantly altered in relations to changes in *Phlda2* gene dosage. The colour represents the expression level of the gene. Red represents high expression, while green represents low expression. The expression levels are continuously mapped on the colour scale provided at the bottom of the figure. *Phlda2*$^{-/+}$(0X) and *Phlda2*$^{+/+BACx1}$(2X) placenta show a reciprocal pattern of expression. C. Enrichment for placental hormones after pathways analysis of genes significantly UP in *Phlda2*$^{-/+(129)}$(0X) and significantly DOWN in *Phlda2*$^{+/+BACx1}$(2X) placenta ($p \leq 0.05$) presumed to originate within the spongiotrophoblast. Supplemental Table 1 list the DAVID results and the UP DOWN annotated probe sets with a significant differences in gene expression at $P \geq 0.05$.

A more objective analysis of gene expression changes was performed using an Affymetrix mouse microarray to profile gene expression on E16.5 *Phlda2*$^{+/+}$ (1X), *Phlda2*$^{-/+}$ (0X) and *Phlda2*$^{+/+BACx1}$ (2X) placenta ($N=3$–4 of each genotype, two independent litters for each genotype). Data was initially analysed at the genome wide level using a Bioconductor package Limma (Linear Model for Microarray data Analysis) written in R statistical software to identify differentially expressed genes between all pair-wise comparisons of the groups (Fig. 3A and B). Database for Annotation, Visualisation and Integrated Discovery (DAVID) was used to generate molecular function and biological processes pathway data on the unselected dataset highlighting significant changes in genes involved in cell cycle, cytokine–cytokine interactions and sphingolipid metabolism (Table S2). Genes both significantly up in *Phlda2*$^{-/+}$ placenta and significantly down in *Phlda2*$^{+/+BACx1}$ E16.5 whole placenta likely represent the spongiotrophoblast transcriptome (Fig. 3C, and Table S3). A dataset of these UP DOWN genes was further analysed by DAVID giving a functional annotation clustering analysis output which highlighted cytoskeleton pathways, cell division pathways and the protein

superfamily prolactin/lactogen/growth hormone cluster (Cluster 3 Enrichment Score: 4.25) (Tables S4 and S5). Within cluster 3 were a number of *placental prolactins* known to be expressed within the spongiotrophoblast (Simmons et al., 2008) and *secretin*, also highly expressed within the spongiotrophoblast (Knox et al., 2011) (Table S6). This data, together with our RT-qPCR and *in situ* analyses (Figs. 1 and 2), confirmed *Phlda2* as a major rheostat for placental hormone gene expression.

3.3. Excessive accumulation of placental glycogen in Phlda2-deficient placenta

Phlda2-deficiency results in a transient foetal growth restriction in C57BL/6 (BL6) mice (Frank et al., 2002; Salas et al., 2004). Given the less favourable foetal:placental ratio in 129 mice (Tunster et al., 2012), we asked whether foetal overgrowth might manifest on this genetic background. *Phlda2*$^{-/+}$ fetuses were similar in weight to their 129 non-transgenic counterparts at E14.5, E16.5 and E18.5 (Fig. 4A). *Phlda2*$^{-/+}$ placenta weighed more than non-transgenic at each time point with a maximal increase of 50% at E16.5 ($p = 7.42 \times 10^{-8}$) (Fig. 4B). *Phlda2*-deficiency resulted in a lower F:P ratios (Fig. 4C) particularly evident at E16.5 with a change in ratio from 8.8 ± 0.26 to 6.4 ± 0.30 (73.1% of control; $p = 2.75 \times 10^{-6}$).

Phlda2-deficiency resulted in increased Periodic Acid Schiff (PAS) staining for glycogen (Tunster et al., 2010; Frank et al., 2002). When we biochemically determined the amount of glycogen stored in the 129 placenta, *Phlda2*-deficiency resulted in greater stores of placental glycogen, as anticipated, with a maximal difference in total glycogen at E16.5 of 2.3-fold (129; Fig. 4D). These

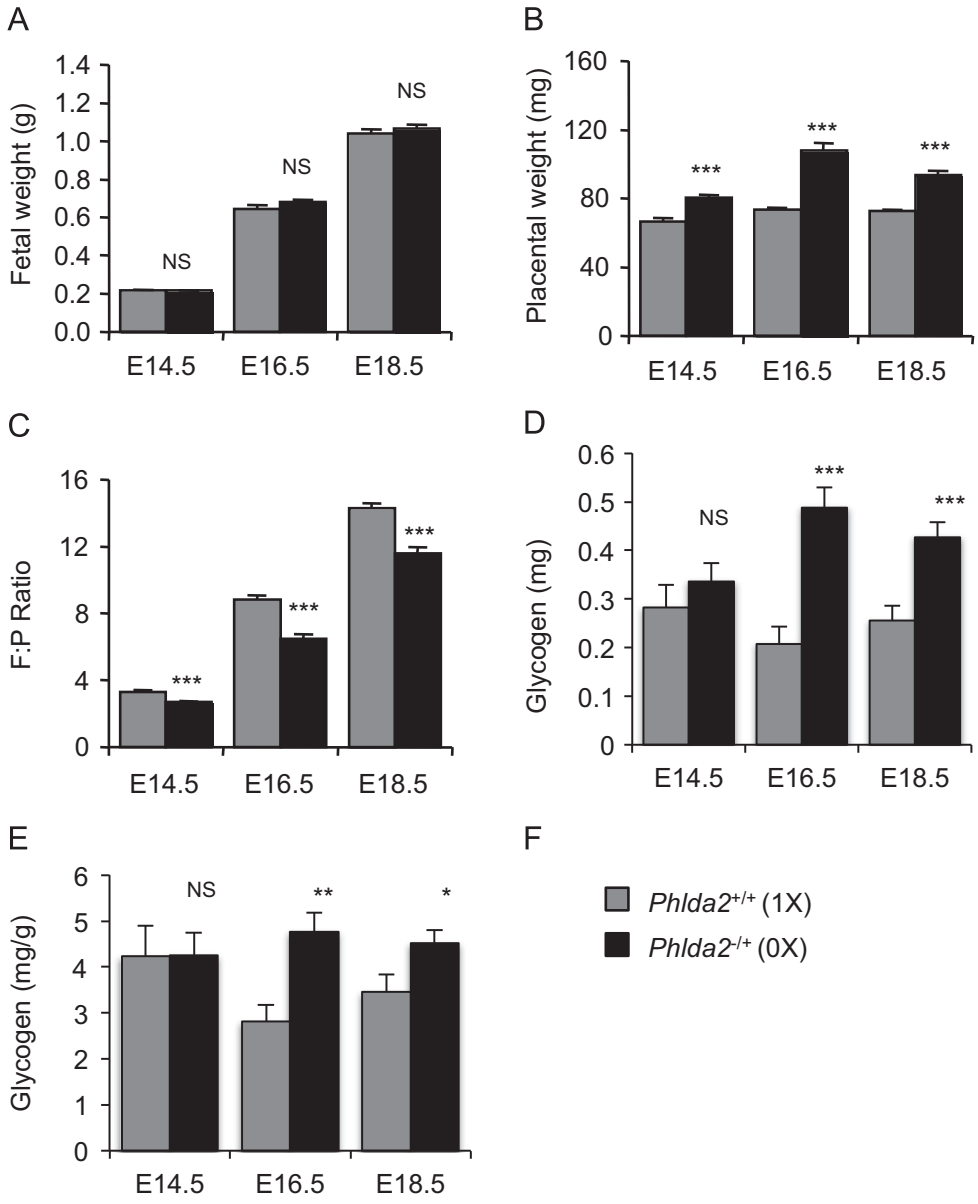


Fig. 4. Influence of *Phlda2* deficiency on foetal and placental weights, and glycogen accumulation. A. Weights of non-transgenic and *Phlda2*$^{-/+}$(0X) fetuses at the indicated gestational stages. *Phlda2*$^{-/+}$(0X) fetuses weigh the same as their non-transgenic counterparts at each time point. B. Weights of placentae at the indicated gestational stages. *Phlda2*$^{-/+}$(0X) placenta weigh more than their non-transgenic counterparts at each timepoint. C. Comparison of F:P ratios on the 129 background. D. Direct biochemical determination of total glycogen (mg) stored in *Phlda2*$^{+/+}$ and *Phlda2*$^{-/+}$(0X) placenta at E14.5, E16.5 and E18.5. *Phlda2*$^{-/+}$(0X) placenta contain significantly more glycogen than non-transgenic placenta at E16.5 and E18.5. E. Milligrams (mg) of glycogen stored per gram of placenta in *Phlda2*$^{+/+}$ and *Phlda2*$^{-/+}$(0X) placenta at E16.5 and E18.5. Numbers of samples given in Supplemental Table S7. Error bars represent SEM. Statistical significance calculated using *t*-test: $^{NS}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.005$.

differences were maintained when placental weights were taken into account (Fig. 4E).

3.4. Global foetal growth restriction

Given the function of *Phlda2* in restraining the spongiotrophoblast lineage and thus, indirectly, the production of placental hormones, loss-of-function of *Phlda2* might promote the accumulation of placental glycogen and potentially support the increased growth of both the loss-of-function fetuses and their non-transgenic counterparts sharing the intrauterine environment. The within litter comparison would not necessary exclude this possibility. To address this, data on *Phlda2*-deficient fetuses and their non-transgenic littermates were compared to those from fully wild type litters at E18.5 collected concurrently using the parent strain. Remarkably, rather than an anticipated increase in foetal weights, we observed a 15% reduction in weight of both $Phlda2^{-/+}$ and $Phlda2^{+/+}$ compared to fully wild type litters (Fig. 5A). Non-transgenic placentae were similar in weight to fully wild-type placentae (Fig. 5B). Consequently the F:P ratios for both the *Phlda2*-deficient scenario and the non-transgenic scenario were both significantly different to the fully wild-type scenario (Fig. 5C). Remarkably, while placental glycogen stores from non-transgenic placenta at E18.5 contained significantly less glycogen per gram of placenta than the *Phlda2*-deficient placenta, they contained more glycogen overall than the fully wild type placenta (Fig. 5D). Phenotypes in the non-transgenic samples sharing the intrauterine environment with the transgenic individuals highlighted a global effect on the entire litter as a result of localised loss-of-function of *Phlda2*.

The litters we examined were carried by dams inheriting the targeted *Phlda2* allele, albeit paternally and thus the inactive allele. While *Phlda2* is imprinted strongly in the placenta and extra-embryonic membranes, functional imprinting is less well maintained in embryonic and adult tissues (Qian et al., 1997). To formally exclude a haploinsufficiency phenotype in the dams, *Phlda2*-deficient embryos and their non-transgenic controls were transferred into wild-type recipient mothers. The weights of both $Phlda2^{-/+}$ (KO) and non-transgenic fetuses were significantly lighter when compared to fully wild-type litters (Fig. 6A). $Phlda2^{-/+}$ (KO) placental weights were significantly increased (Fig. 6B) and the F:P ratio was lower (Fig. 6C). Importantly, whether examined as total glycogen or as glycogen per gram of placenta, the $Phlda2^{-/+}$ (KO) and non-transgenic values were significantly higher than fully wild-type control values (Fig. 6D and E). These data excluded a maternal genotype-effect and identified a novel function for the spongiotrophoblast in promoting the global accumulation of placental glycogen.

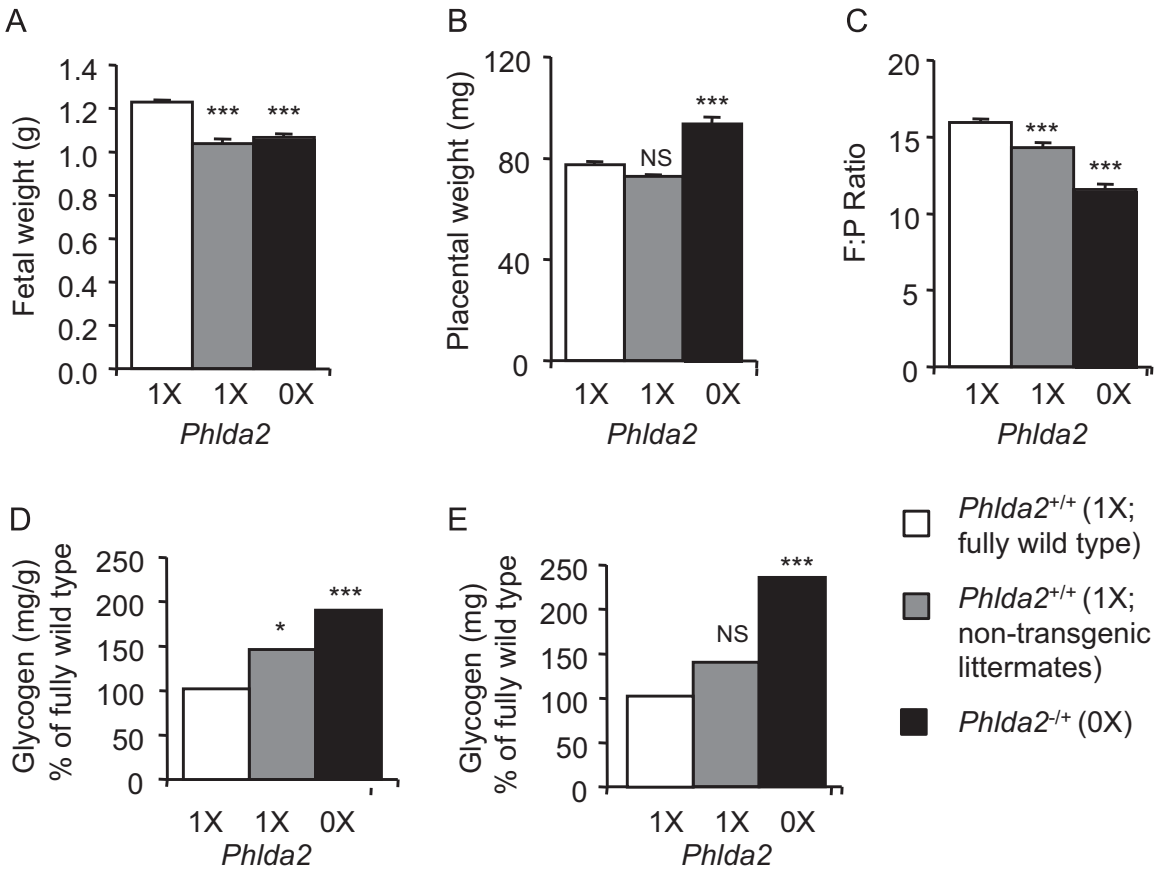


Fig. 5. *Phlda2* deficiency drives global foetal growth restriction. A. Weights of non-transgenic and $Phlda2^{-/+}$(0X) fetuses compared with true 129 wild type values (wild type fetuses carried by wild type females). Both genotypes weigh less than fully wild type fetuses. B. Weights of non-transgenic and $Phlda2^{-/+}$(0X) placenta compared with true 129 wild type values. $Phlda2^{-/+(129)}$(0X) placenta weigh considerably more than fully wild type placenta. C. Comparison of F:P ratios between non-transgenic, $Phlda2^{-/+(129)}$(0X) and true 129 wild type. Both the non-transgenic and the $Phlda2^{-/+}$ratios are significantly different to the fully wild type ratio. D. Direct biochemical determination of glycogen in $Phlda2^{+/+}$ and $Phlda2^{-/+}$(0X) placenta at E18.5 as a percentage of true 129 wild type values expressed as total amount (mg) and as a proportion of placental weight (mg/g placenta). $Phlda2^{-/+}$(0X) placenta and the non-transgenic placenta both contain significantly more glycogen than fully wild type placenta on the 129 genetic background when placental weights are taken into account. Numbers of samples given in Supplemental Table S8. Error bars represent SEM. Statistical significance calculated using one way ANOVA with Bonferroni correction for multiple comparisons: $^{NS}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.005$.

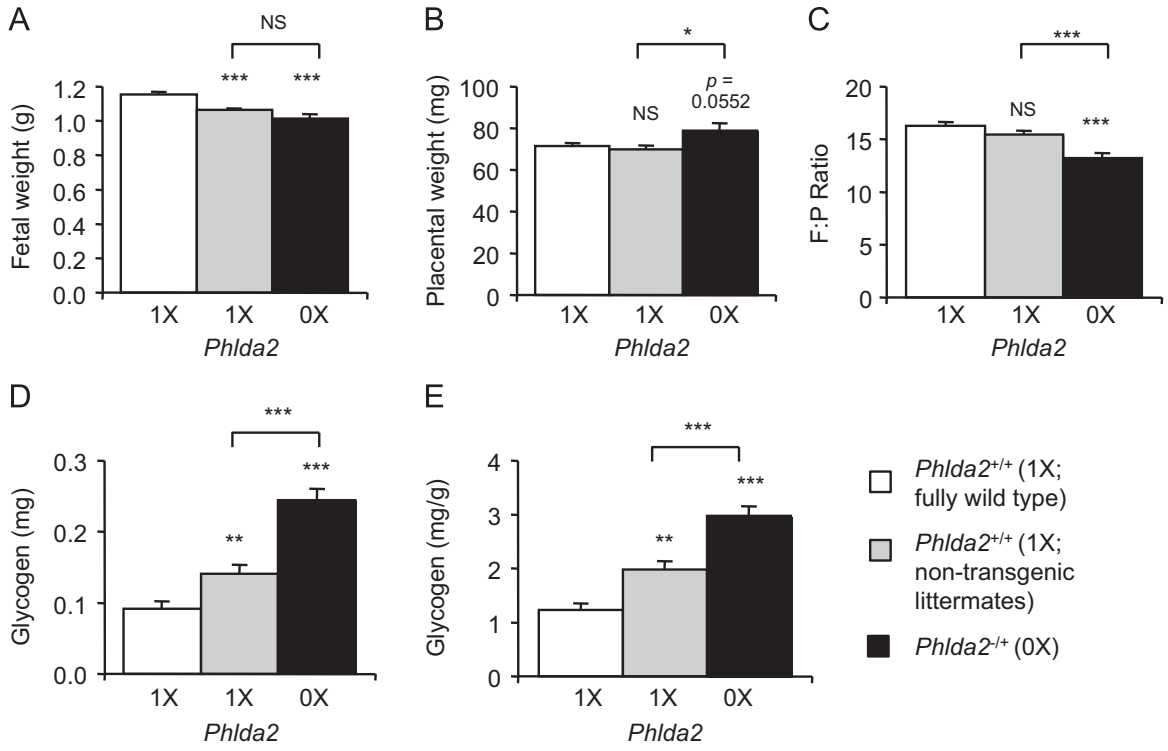


Fig. 6. Exclusion of a maternal genotype phenotype. A. Comparison of wet weights of *Phlda2*$^{-/+}$ (0X) and non-transgenic fetuses carried by wild type dams generated by recipient transfer compared with fully wild type foetal weights at E18.5. Both foetal genotypes weight less than fully wild type fetuses. B. Comparison of wet weights of *Phlda2*$^{-/+}$ (0X) and non-transgenic placenta carried by wild type dams generated by recipient transfer compared with fully wild type placental weights at E18.5. C. F:P ratios from data in A and B. D. Comparison of total placental glycogen (mg) at E18.5. Both placental genotypes contain more glycogen than fully wild type placenta. E. Comparison of placental glycogen per gram of placenta (mg/g) at E18.5. Both placental genotypes contain more glycogen when placental weight is taken into account than fully wild type placenta. Numbers of samples given in Supplemental Table S9. Error bars represent SEM. Statistical significance calculated using one way ANOVA with Bonferroni correction for multiple comparisons: $^{NS}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.005$.

4. Discussion

Here we show that the imprinted *Phlda2* gene acts in a lineage-specific manner to exclusively modulate the size of the spongiotrophoblast compartment of the mature mouse placenta. *Phlda2* is the first gene described which has this specific function allowing the *in vivo* assessment of the function of this lineage. Using both loss-of-function and gain-in-expression models we show that, via the spongiotrophoblast, *Phlda2* negatively regulates the expression of a number of key placental hormones. We show that an

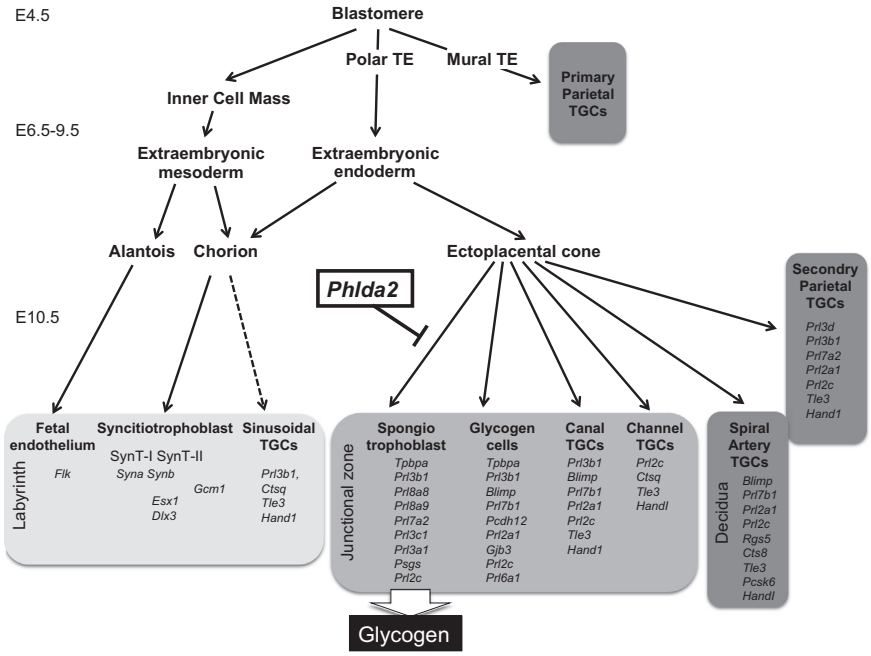


Fig. 7. Summary of the cell autonomous and non-cell autonomous functions of *Phlda2* in regulating placental glycogen accumulation. Trophoblast lineage allocation and lineage markers adapted from Rai and Cross (2014).

expanded spongiotrophoblast compartment, driven by loss-of-function of *Phlda2*, drives an inappropriate accumulation of placental glycogen both locally, in the genetically modified individuals, and also globally in individuals that were genetically wild type. This work identifies a novel signalling function for the spongiotrophoblast. The presence of foetal growth restriction in both the genetically modified individuals and their non-transgenic counterparts further suggests that *Phlda2* balances resource allocation between the foetus and the placenta, a process that must be precisely regulated for optimal foetal growth.

Phlda2 is the only gene to date which acts to limit the size of the spongiotrophoblast compartment of the mouse placenta without significantly altering the contribution of additional placental lineages that share a common progenitor (Rai and Cross, 2014). This suggests that *Phlda2* acts in placental development after these lineage decisions are made but before *Phlda2* expression subsides (Fig. 7). This unique specificity for the spongiotrophoblast allowed us to explore the function of the spongiotrophoblast. One key finding from this study was that an expanded spongiotrophoblast drove the excessive accumulation of placental glycogen both directly, within the loss-of-function placenta, and also indirectly in the non-transgenic placenta sharing the uterine environment. To our knowledge, this is the first example of a gene modification affecting the phenotype in wild type conceptuses of the same litter. However, very few studies make the comparison with fully wild type data. These data suggest that the spongiotrophoblast produces a signal that acts locally and at a distance to stimulate glycogen accumulation. The spongiotrophoblast expresses a number of placental prolactins (Simmons et al., 2008) and pregnancy specific glycoproteins (McLellan et al., 2005). Consequently, by changing the size of the spongiotrophoblast compartment, *Phlda2* indirectly and negatively regulates the expression of several placental hormones. Many of these placental hormones act on the maternal system to channel nutrients to the foetus and ensure a healthy and successful pregnancy (Samaan et al., 1968; Bhattacharyya et al., 2002; Muller et al., 1999; Kammerer and Zimmermann, 2010; Wu et al., 2008). One explanation for our findings is that an increased signal (*Phlda2* loss-of-function) leads to increased nutrient availability potentially explaining increased placental glycogen in both genotypes. However, we observed foetal growth restriction of both the *Phlda2* KO fetuses and their non-transgenic, genetically wild-type littermates. We could discount a haploinsufficiency phenotype in the dams, which carry the paternal targeted allele, because embryos transferred into wild-type recipients also displayed both foetal growth restriction and excessive placental glycogen of both genotypes (Fig. 6). Another explanation is that the defect lies at the level of foetal uptake of glucose or the ability to transport it across the placenta to the foetus such that placental glycogen accumulates because it is not utilised. A third explanation is that the spongiotrophoblast produces a signal demanding nutrients to support placental growth. When the signal is too high, as in the *Phlda2* loss-of-function model, it outcompetes the demand signal from the foetus resulting in the diversion of maternal resources away from supporting foetal growth and towards supporting placental growth. This latter explanation is plausible as the placenta is a highly metabolic organ consuming maternal energy in order to support the active transport of nutrients to the foetus and also the production and secretion of vast quantities of placental hormones (John, 2013). In mammals, more than half of the total uterine glucose and oxygen uptake is channelled to the uteroplacental tissues (Fowden, 1997; Carter, 2000). A larger placenta, observed in the *Phlda2* loss-of-function model, would require a greater proportion of maternal resources that might occur to the detriment of foetal growth. While further work is required to determine the precise mechanism driving foetal growth restriction in this model, this study has demonstrated that a precisely regulated dose of *Phlda2* is essential for optimal foetal growth with both loss-of-function and gain-in-expression driving foetal growth restriction in mice.

Phlda2 does not appear to follow the straightforward rules applied to a substantial number of imprinted genes whereby paternally expressed genes promote placental and foetal growth whereas maternally expressed genes restrain placental and foetal growth. *Phlda2* resides within a complex imprinted domain spanning several maternally expressed genes including *Cdkn1c*, a potent embryonic growth restriction gene (Andrews et al., 2007; Tunster et al., 2011). Neither loss-of-function of *Phlda2* nor loss-of-function of *Cdkn1c* alone in mice results in the increase in birth weight predicted by the genomic conflict theory. It may be that loss of function of such critically important genes has too severe an impact on foetal development. Alternatively, the combined alteration may be required to generate larger offspring. Our data from mouse models would predict an increase in foetal weight, due to reduced *Cdkn1c* expression, alongside an increase in the signalling function of the placenta, due to reduced *Phlda2* expression, thus retaining the balance between foetal growth and placental demands on maternal resources. Co-imprinting of these two genes, which occurred after marsupials diverged for Eutherian mammals (Suzuki et al., 2005, 2011), may consequently have played an important role in the evolution of modern day mammals that give birth to relatively mature offspring.

In conclusion, we have shown that *Phlda2* modulates the signalling function of the placenta to limit the accumulation of placental glycogen and placental growth. While this does not provide a facile explanation for paternal silencing of *Phlda2*, our work highlights the complex relationship between the mother, the foetus and the placenta whose requirements must be carefully balanced for an optimal reproductive success.

Competing interests statement

The authors declare that there is no conflict of interest financial or otherwise associated with this submission.

Author contributions

SJT performed the bulk of the experiments, analysed data and contributed to the writing of the manuscript. HDJC performed the microarray analysis and some of the animal work. RMJ conceived and designed the experiment, interpreted the data and wrote the paper.

Acknowledgements

We thank Mary Cleaton and Charlotte Taylor for help with glycogen extraction, Derek Scarborough for histological sections and Bridget Allen (Wales Gene Park) for recipient transfers and Louis Lefebvre for comments on the manuscript. SJT was supported by Biotechnology and Biological Sciences Research Council BBSRC) Grant BB/J015156 and HDJC was supported by an Ewen Maclean Fellowship.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.10.015.

Developmental Biology 418 (2016) 55–65

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Original research article

Increased dosage of the imprinted *Ascl2* gene restrains two key endocrine lineages of the mouse Placenta

S.J. Tunster, G.I. McNamara, H.D.J. Creeth, R.M. John *

Cardiff School of Biosciences, Cardiff University, Cardiff, Wales CF103AX, UK

ARTICLE INFO

Article history:
Received 19 May 2016
Received in revised form
26 July 2016
Accepted 15 August 2016
Available online 16 August 2016

Keywords:
Ascl2
Placenta
Phlda2
Epigenetics
Imprinting

ABSTRACT

Imprinted genes are expressed primarily from one parental allele by virtue of a germ line epigenetic process. *Achaete-scute complex homolog 2* (*Ascl2* aka *Mash2*) is a maternally expressed imprinted gene that plays a key role in placental and intestinal development. Loss-of-function of *Ascl2* results in an expansion of the parietal trophoblast giant cell (P-TGC) lineage, an almost complete loss of *Trophoblast specific protein alpha* (*Tpbpa*) positive cells in the ectoplacental cone and embryonic failure by E10.5. *Tpbpa* expression marks the progenitors of some P-TGCs, two additional trophoblast giant cell lineages (spiral artery and canal), the spongiotrophoblast and the glycogen cell lineage. Using a transgenic model, here we show that elevated expression of *Ascl2* reduced the number of P-TGC cells by 40%. Elevated *Ascl2* also resulted in a marked loss of the spongiotrophoblast and a substantial mislocalisation of glycogen cells into the labyrinth. In addition, Ascl2-Tg placenta contained considerably more placental glycogen than wild type. Glycogen cells are normally located within the junctional zone in close contact with spongiotrophoblast cells, before migrating through the P-TGC layer into the maternal decidua late in gestation where their stores of glycogen are released. The failure of glycogen cells to release their stores of glycogen may explain both the inappropriate accumulation of glycogen and fetal growth restriction observed late in gestation in this model. In addition, using in a genetic cross we provide evidence that *Ascl2* requires the activity of a second maternally expressed imprinted gene, *Pleckstrin homology-like domain, family a, member 2* (*Phlda2*) to limit the expansion of the spongiotrophoblast. This "belts and braces" approach demonstrates the importance of genomic imprinting in regulating the size of the placental endocrine compartment for optimal placental development and fetal growth.

© 2016 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

Genomic imprinting is a remarkable process whereby certain genes are preferentially silenced on one parental allele as a consequence of epigenetic events initiated in the germ line (Surani, 1998). Imprinted genes have been shown to functionally converge on biological processes that, while not exclusive to mammals, were likely important for the relative dominance of mammals on Earth today, including placentation, advanced maturity at birth and high maternal care (Kaneko-Ishino and Ishino, 2010; Keverne, 2013; Renfree et al., 2013; Moore, 2011; Cleaton et al., 2014; Peters, 2014). The majority of studies on imprinted genes rely on loss-of-expression models. However, imprinting is a mechanism that exclusively modulates gene dosage. Examining models in which gene dosage is increased may further contribute to our understanding of genomic imprinting.

Achaete-scute complex homolog 2 (*Ascl2* aka *Mash2*) was one of the first imprinted genes to be knocked out in mice (Guillemot et al., 1995). These studies demonstrated that fetal survival beyond E10.5 requires the maternal *Ascl2* allele (Guillemot et al., 1995, 1994). Loss of function restricted to the embryo had no overt consequence during gestation highlighting a requirement for placental *Ascl2* in the transition to the mature chorioallantoic placenta (Tanaka et al., 1997). The mature mouse placenta is organised into the histologically distinct labyrinth zone, junctional zone and maternal decidua, all of which are interspersed with trophoblast giant cells (TGCs) (John and Hemberger, 2012; Rai and Cross, 2014). Loss-of-function of *Ascl2* resulted in an expansion of one TGC type, the parietal (P-) TGCs. These normally form a single layer of cells located beneath the maternal decidua surrounding large lakes of maternal blood leading into the uterine veins, that becomes somewhat discontinuous as development proceeds (Rai and Cross, 2014). In *Ascl2*$^{-/+}$ placenta there were multiple layers of P-TGCs (Guillemot et al., 1994; Tanaka et al., 1997). Four other distinct TGC types exist which are defined by their characteristic

* Corresponding author.
E-mail address: JohnRM@cf.ac.uk (R.M. John).

http://dx.doi.org/10.1016/j.ydbio.2016.08.014
0012-1606/© 2016 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

gene expression profiles and their position with respect to maternal circulation (Rai and Cross, 2014; Simmons et al., 2007, 2008; Gasperowicz et al., 2013). The spiral artery (SpA-) TGCs are located within the maternal decidua lining the maternal blood system on entry to the placenta; the canal (C-) TGCs line the maternal blood canals as they pass through the junctional zone; the sinusoidal TGCs (S-TGCs) are in direct contact with the maternal blood spaces within the labyrinth and the recently discovered channel (Ch-) TGCs line the maternal blood spaces located just beneath the decidua where maternal blood drains into the venous sinuses to be returned to the maternal circulation (Rai and Cross, 2014; Simmons et al., 2007, 2008; Gasperowicz et al., 2013). These additional lineages were not examined in *Ascl2*$^{-/+}$ placenta as these fail too early in development, but the original report (Guillemot et al., 1994) noted an almost complete lack of expression of *Trophoblast specific protein alpha (Tpbpa)*. *Tpbpa* is expressed in the progenitor cells of 50% of the P-TGCs, all the SpA-TGCs and C-TGCs, as well as the glycogen cell and spongiotrophoblast lineages that form the bulk of the junctional zone (Hu and Cross, 2011). Another mouse model (*Del*$^{7A1/+}$) in which *Ascl2* levels were reduced by 50%, allowed survival to term and the assessment of a later placental phenotype (Oh-McGinnis et al., 2011). *Del*$^{7A1/+}$ placenta possessed a similarly expanded P-TGC layer alongside a reduced spongiotrophoblast and a complete loss of the glycogen cell lineage (Oh-McGinnis et al., 2011; Lefebvre et al., 2009). *Del*$^{7A1/+}$ involves maternal inheritance of a 280 kb deletion physically adjacent to *Ascl2* and spanning the maternally expressed *Tyrosine hydroxylase* (*Th*) gene. Consequently, these placenta lack *Th* expression after maternal transmission of this deletion. *Del*$^{7A1/+}$ placenta also express *Pleckstrin Homology-Like Domain, Family A, Member 2* (*Phlda2*) at two-fold the normal level (Data summarised in Supplemental Table 1). *Phlda2* maps to the same imprinted domain at *Ascl2* and *Th* (Fitzpatrick et al., 2002). We have previously shown that two-fold expression of *Phlda2* results in a 50% loss of the spongiotrophoblast lineage (Tunster et al., 2010, 2014, 2015). Consequently the loss of spongiotrophoblast in the *Del*$^{7A1/+}$ placenta may be a direct consequence of reduced *Ascl2* expression or increased *Phlda2* expression.

These *in vivo* studies highlighted a potential role for *Ascl2* as a dosage-sensitive cellular switch between the spongiotrophoblast/glycogen cell lineages and the P-TGC lineage. Consistent with a role in lineage choice, *Ascl2* is expressed in the ectoplacental cone and extra embryonic ectoderm at E7.5 where the placental progenitors reside with expression restricted to the diploid trophoblast cells, and later diploid trophoblast cells located in the labyrinthine and spongiotrophoblast layers (Guillemot et al., 1994., 1993). This model would be consistent with the finding that ectopic expression of *Ascl2* can inhibit the normal differentiation of Rcho-1 cells, a rat choriocarcinoma cell line, towards a TGC-like fate (Kraut et al., 1998; Cross et al., 1995; Hughes et al., 2004). However, ectopic expression of *Ascl2* in trophoblast stem (TS) cells, which can differentiate into a number of trophoblast lineages, resulted in decreased expression of both *Prl3d*, a marker of TGCs, and *Tpbpa*, a gene expressed in the progenitors of a number of lineages including the spongiotrophoblast, suggesting that *Ascl2* could function to repress more than one placental lineage (Hughes et al., 2004; Takao et al., 2012). The consequences of increased expression of *Ascl2 in vivo* on placental development have not been fully explored. A P1 transgene spanning the genomic locus expressing *Ascl2* at 4–7 fold the endogenous level was shown to rescue embryonic lethality associated with maternal inheritance of the *Ascl2* targeted allele, but no overt consequence for placental or fetal weight were reported (Rentsendorj et al., 2010). To investigate the effect of *Ascl2* overexpression on placental development and fetal growth, we made use of an existing line of mice carrying a bacterial artificial chromosome (BAC) spanning the *Ascl2* locus previously used to explore the relevance of elevated *Ascl2* in intestinal tumorigenesis (Reed et al., 2012). We observed a reciprocal relationship between *Ascl2* expression and P-TGC number as anticipated, but a marked loss of spongiotrophoblast arguing against a role for *Ascl2* as a simple cell fate switch.

2. Materials and methods

2.1. Mouse strains and genotyping

All animal studies and breeding was approved by the Universities of Cardiff ethical committee and performed under a UK Home Office project license (RMJ). Mice were housed in a conventional unit on a 12-h light–dark cycle with lights coming on at 06.00 h with a temperature range of 21 °C ±2 with free access to tap water and standard chow unless otherwise stated. The *Ascl2* BAC transgenic line (Ascl2-Tg; Genome Systems BAC225J16) described previously (Reed et al., 2012) was primarily studied on the C57BL/6 (BL6) strain background. Some experiments were performed when the transgene was bred into 129S2/SvHsd (G6; 129). The *Phlda2* targeted allele (Frank et al., 2002) was maintained by paternal transmission on the BL6 genetic background.

2.2. Weighing studies

Embryonic and placental wet weights were taken following careful dissection to remove the yolk sac, umbilicus and excess decidua prior to RNA extraction at the stated time points after a discernable plug. Genotyping data was obtained from yolk sac DNA using primers CACATACGTTCCGCCATTCC and CCACTTCAACGTAACACCGC to amplify from the BAC.

2.3. Quantitative RNA analysis

Quantitative PCR of reverse transcribed RNA (QRT-PCR) was performed as described (Tunster et al., 2010) with additional primers (Supplemental Table 2).

2.4. In situ hybridisation and histological analyses

Placentas were fixed overnight in phosphate-buffered 4% paraformaldehyde, paraffin-embedded and 10 μm sections taken through the midline. Haematoxylin and eosin (H&E) staining, riboprobe preparation, *in situ* hybridizations and PAS staining were performed as previously described (Tunster et al., 2010; John et al., 2001). Biochemical determination of glycogen was performed on fresh snap-frozen placenta as previously described (Tunster et al., 2010; John et al., 2001). Measurements were taken from midline sections obtained by cutting a number of serial sections and judging overall thickness as previously described (Tunster et al., 2010). For counting P-TGC, sections were analysed without knowing the genotype, with independent repeat blind counts.

2.5. Statistical analyses

Statistical significance (Probability values) was determined using the Student's *t*-Test (two tailed distribution and two sample unequal variance). The significance in the difference in observed over the expected appearance of a particular genotype was determined using the *Chi*-squared Test.

3. Results

The previously described transgenic line (Reed et al., 2012) carries a bacterial artificial chromosome physically encompassing

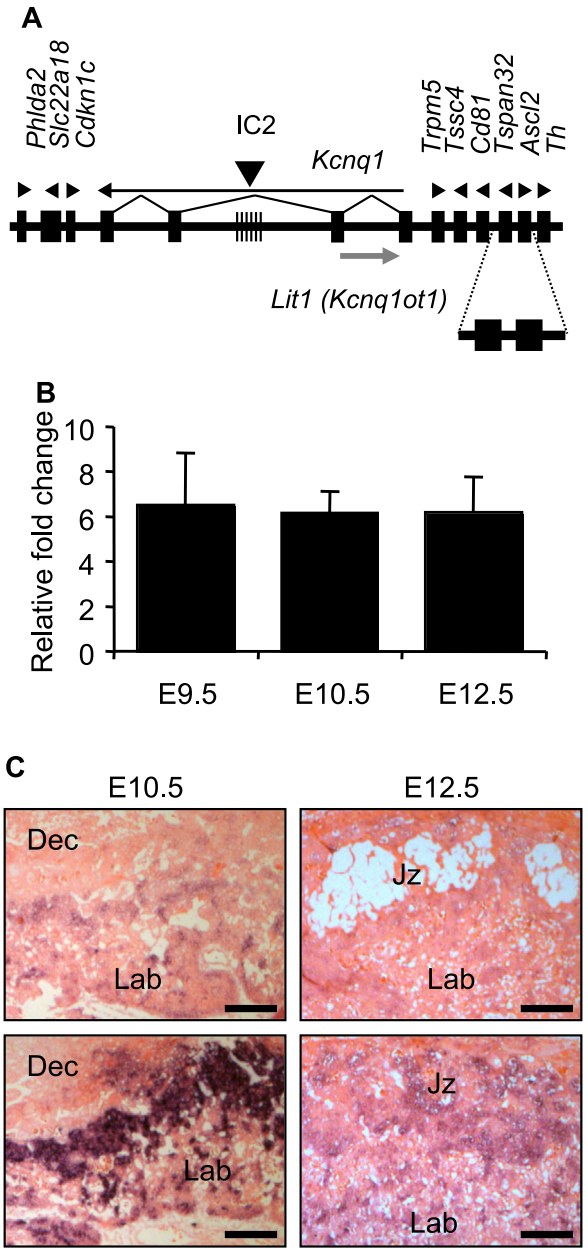


Fig. 1. Spatially and temporally accurate (entopic) overexpression of *Ascl2* in the mouse placenta. (A) Schematic of the BAC transgene. Top line shows a genomic map of the IC2 region on distal mouse chromosome 7. Hatched region marks the *KvDMR1* that is methylated only on the maternal allele. Arrows indicate direction of transcription. Below is a map of the BAC transgene containing the *Ascl2* gene. Filled boxes represent imprinted genes (not to scale). (B) Placental RT-QPCR results for *Ascl2* in transgenic placenta at E9.5, E10.5 and E12.5. N=4 placenta per genotype (2 *versus* 2 from 2 independent litters); error bars represent SEM. Statistical significance calculated using *t*-test. $^{**}P<0.01$, $^{***}P<0.005$. (C) *In situ* hybridisation of midline placental sections with *Ascl2* riboprobe reveals similar spatial localisation of mRNA in control and Ascl2-Tg placentas at E10.5 and E12.5. Scale bars=200 μm. Data for Fig. 2 in Supplemental Table 2.

the *Ascl2* locus (Fig. 1A). E9.5, E10.5 and E12.5 Ascl2-Tg transgenic placenta were found to express *Ascl2* at ~6-fold the wild type level (Fig. 1B, Supplemental Table 3). The transgene spanned a second gene, *Tspan32*, not expressed in the placenta (Gartlan et al., 2010; Nicholson et al., 2000) and not ectopically expressed from the transgene (Supplemental Fig. 1). *In situ* hybridisation with an *Ascl2* riboprobe demonstrated the correct localisation of the *Ascl2* signal within Ascl2-Tg placenta to a subset of diploid cells in the ectoplacental cone and chorion at E10.5 and restricted expression within a subset of cells within the junctional zone at E12.5 (Fig. 1C). Previously, adenovirus driven overexpression of *Ascl2* at 10-fold the normal level was found to down-regulate expression of both *Tpbpa* and *Prl3d1* in trophoblast stem cells cultured under stem cell conditions, alongside reduced expression of *Phlda2* (Takao et al., 2012) (Supplemental Table 1). The spatio-temporally accurate elevated expression of *Ascl2* in this animal model provided a tool to explore the consequences of similarly increased *Ascl2* dosage on placental development *in vivo*.

At E10.5, P-TGC form a distinct and histologically recognisable single cell layer between the maternal decidua and the developing chorio-allantoic placenta. Haematoxylin staining of E10.5 Ascl2-Tg midline placental sections revealed a marked loss of the giant, polyploid cells lining the maternal decidua (Fig. 2A). *In situ* hybridisation with *Prl3d* (*Pl1*), exclusively expressed in the P-TGC lineage at this time point (Jackson et al., 1986) identified these polyploid cells as P-TGCs (Fig. 2B). Counting of these cells in midline sections revealed a significant ~40% loss of P-TGCs at E10.5 (36.3 ± 7.1 *versus* 19.8 ± 1.9; 10 WT v 16 Ascl2-Tg placental sections from 5 litters; $p=0.0120$; Fig. 2C, Supplemental Table 4). Consistent with the loss of P-TGCs, RT-QPCR revealed a significant 30% reduction in expression of *Prl3d* at E9.5 and E10.5 (Fig. 2D). Expression of *Prl3b1* (aka *Pl2*), a second gene expressed in P-TGC at E9.5 and E10.5 (Simmons et al., 2008), was also markedly reduced, by 70% and 40% respectively (Fig. 2D, Supplemental Table 4). *Hand1* and *Prl2c*, genes expressed in all or most of the TGC lineages (Simmons et al., 2008; Scott et al., 2000), were not significantly altered suggesting the defect was not attributable to the loss of other emergent TGC lineages. The loss of P-TGC was reciprocal to the phenotype reported in response to reduced *Ascl2* expression (Guillemot et al., 1994; Tanaka et al., 1997). Markers associated with the emerging spongiotrophoblast and glycogen cell lineages were also measured (Fig. 2E). *Tpbpa*, expressed in the precursors of the spongiotrophoblast, the glycogen cells, S-TGC, SpA-TGC and 50% of the P-TGC (Hu and Cross, 2011), was expressed at near normal levels (Fig. 2E). *Blimp1, Rgs5, Pcsk6* and *Prl7b1*, expressed in the precursors of the glycogen cells, the SpA-TGC and the C-TGC (Mould et al., 2012) or exclusively in the SpA TGC (Mould et al., 2012) were expressed at near normal levels. *Protocadherin 12* (*Pcdh12*), one of the earliest markers of the glycogen cell lineage (Bouillot et al., 2006), was expressed at wild type levels at E9.5 but elevated at E10.5, by 1.5-fold (Fig. 2E, Supplemental Table 4).

To further investigate the effects of the *Ascl2* transgene on the placental lineages, Ascl2-Tg placenta were histologically examined at E14.5. H&E staining revealed an unexpected, distinct and dramatic loss of the junctional zone, where the glycogen and spongiotrophoblast cells normally reside (Fig. 3A). *In situ* hybridisation with *Tpbpa*, exclusively expressed in both the spongiotrophoblast and the glycogen cells (Lescisin et al., 1988), further highlighted the dramatic loss of cells from the junctional zone (Fig. 3B). *In situ* hybridisation with *Psg17*, an exclusive marker of the spongiotrophoblast (Kromer et al., 1996), suggested a substantial loss of this lineage from the junctional zone (Fig. 3C). *In situ hybridisation* with *Prl3b1*, expressed in the spongiotrophoblast, C-TGC, P-TGC and S-TGCs at E14.5 (Simmons et al., 2008), was also consistent with a significant loss of the spongiotrophoblast lineage from the junctional zone, and further identified a degradation of the interface between the junctional zone and maternal decidua consistent with the loss of P-TGC from this region (Fig. 3D). What little remained of the junctional zone stained for Periodic acid Schiff (PAS), which distinguishes glycogen cells (Adamson et al., 2002), suggesting that the junctional zone was primarily composed of this cell type (Fig. 3E). There was an overall 40% decrease in junctional zone area (Fig. 3F; Supplemental Table 5) and an increased number of junctional zone-like clusters of cells present in the labyrinth. While clusters were apparent in both WT and Ascl2-

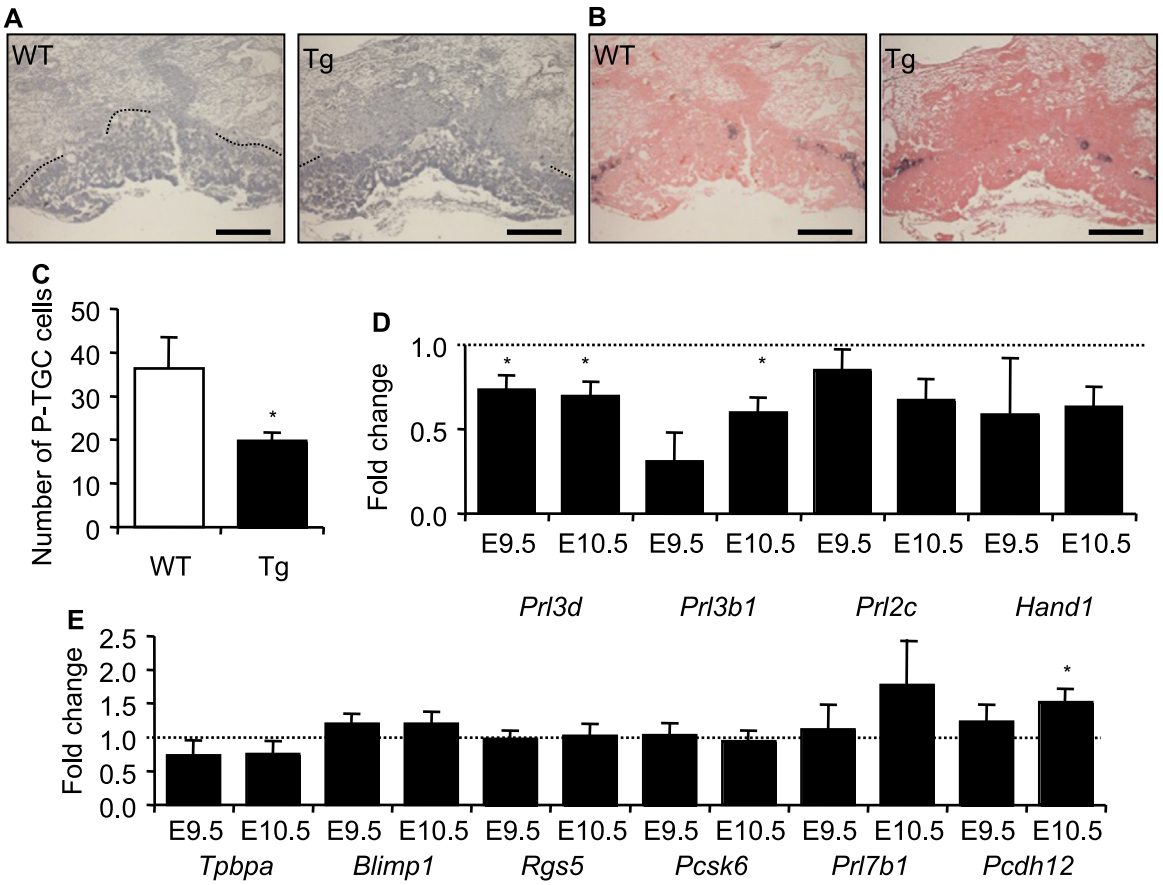


Fig. 2. A reduction in the parietal trophoblast giant cell lineage. (A) Haematoxylin staining of midline placental sections at E10.5. Parietal trophoblast giant cells (P-TGC) are indicated by dashed lines. Scale bars = 500 μm. (B) *In situ* hybridisation with a *Prl3d* (*Pl1*) riboprobe, which specifically marks P-TGCs at E10.5. Scale bars = 500 μm. (C) P-TGC number per section at E10.5. N = 26. (D) Relative expression levels of key markers of P-TGC at E9.5 and E10.5. *Prl3d1, Prl3b1* (aka *Pl2*), *Prl2c* (aka *Plf*) and *Hand1* are all expressed the P-TGC lineage at this time point. (E) Relative expression levels of markers of the emerging spongiotrophoblast (*Tpbpa*), glycogen cell (*Pcdh12, Prl7b1*), S-TGC, SpA-TGC and C-TGC (*Blimp1*, *Rgs5*, *Pcsk6*, *Prl7b1*) lineages at E9.5 and E10.5. For the RT-qPCR analysis, N = 4 placenta per genotype (2 *versus* 2 from 2 independent litters); error bars represent SEM. Statistical significance calculated using *t*-test. $^{NS}P > 0.05$, $^{*}P < 0.05$. Data for Fig. 2 in Supplemental Table 3.

Tg placenta, the number present in the labyrinth of Ascl2-Tg placenta was 7-fold greater than WT (Fig. 3G; Supplemental Table 5). The size of the clusters was similar between the two genotypes (Fig. 3H; Supplemental Table 5) and they were *Tpbpa*+ve/*Psg17*−ve/*Prl3b1*−ve/*Ascl2*+ve/PAS+ve (Fig. 3B–E, I; right panels). This analysis identified them as mislocalised glycogen cells.

At E18.5, just prior to term, the junctional zone was similarly disorganised with clusters of mislocalised glycogen cells observable in the labyrinth of Ascl2-Tg placentae (Fig. 4A–C). As quantified by area measurements of midline sections, the 12% decrease in junctional zone at E18.5 was not significantly different to WT and there was small (11%) but significant increase in labyrinth (Fig. 4D, Supplemental Table 6).

As a proxy for the representation of the different lineages, a RT-QPCR analysis was performed (Fig. 5, Supplemental Table 7). *Tpbpa, Tpbpb* and *FMS-like tyrosine kinase 1* (*Flt1*), markers of the junctional zone lineages at E14.5 (Henke et al., 2013; Hirashima et al., 2003), were expressed at considerably lower levels in Ascl2-Tg placenta compared to controls (Fig. 5A). *Prl8a8*, *Prl3a1*, *Prl3c1*, *Prl3a1, Prl7a2*, *Prl8a9*, *Psg17*, *Psg18*, *Psg19* and *Psg21*, all markers exclusively or predominantly expressed in the spongiotrophoblast (Simmons et al., 2008; McLellan et al., 2005), were expressed at markedly lower levels in Ascl2-Tg placenta (Fig. 5B). *Prl3b1,* which at E14.5 is expressed in P-TGC, C-TGC, S-TGC and spongiotrophoblast (Simmons et al., 2008), was reduced (Fig. 5B). However two other markers associated predominantly with the spongiotrophoblast, *Prl8a1* (SpT and P-TGCs) and *Prl8a6* (SpT and C-TGCs) (Simmons et al., 2008), were expressed at wild type levels. These data essentially supported a loss of the spongiotrophoblast lineage.

Markers of the glycogen cell lineage were also assessed (Fig. 5C). *Pcdh12* and *Gap junction protein, beta 3* (*Gjb3*/*Cx31*), a marker of glycogen cell maturation (Zheng-Fischhofer et al., 2007), were expressed at near normal levels, however *Prl7b1* and *Prl2a1*, expressed predominantly but not exclusively in glycogen cells (Simmons et al., 2008), were both significantly elevated (Fig. 5C). *Prl7b1* is a marker of migrating glycogen cells and is also expressed in SpA-TGC and C-TGC at E14.5, while *Prl2a1* is expressed in P-TGC, SpA-TGC and C-TGC, in addition to the glycogen cell lineage (Simmons et al., 2008). *Glucan (1,4-alpha-), branching enzyme 1* (*Gbe1*), involved in glycogen branching, was elevated. However, *Prl6a1*, expressed in glycogen cells and SpA-TGCs, and other glycogen metabolism enzymes, *glycogenin* (*Gyg*), *glycogen synthase 1* (*gys*) and *UDP-glucose pyrophosphorylase 2* (*Ugp2*), were not significantly elevated. These data supported a change in the nature of the glycogen cell lineage rather than a significant alteration in the cellular contribution of this lineage to the placenta.

An analysis of the markers *Hand1, Tle3* and *Prl2c,* genes expressed in all or most of the TGC lineages (Simmons et al., 2008; Gasperowicz et al., 2013; Scott et al., 2000), and *Ctsq,* expressed in just the S-TGC and Ch-TGC lineages (Rai and Cross, 2014), suggested a near normal representation of these lineages (Fig. 5D). *Flk1*, *Dlx3*, *Tfeb*, *Syna*, *Synb*, *Gcm1* and *Cebpa*, all genes primarily or exclusively expressed in the labyrinth at E14.5 (Simmons et al., 2008; Hirashima et al., 2003; Steingrimsson et al., 1998; Berghorn et al., 2005; Anson-Cartwright et al., 2000) were also expressed at

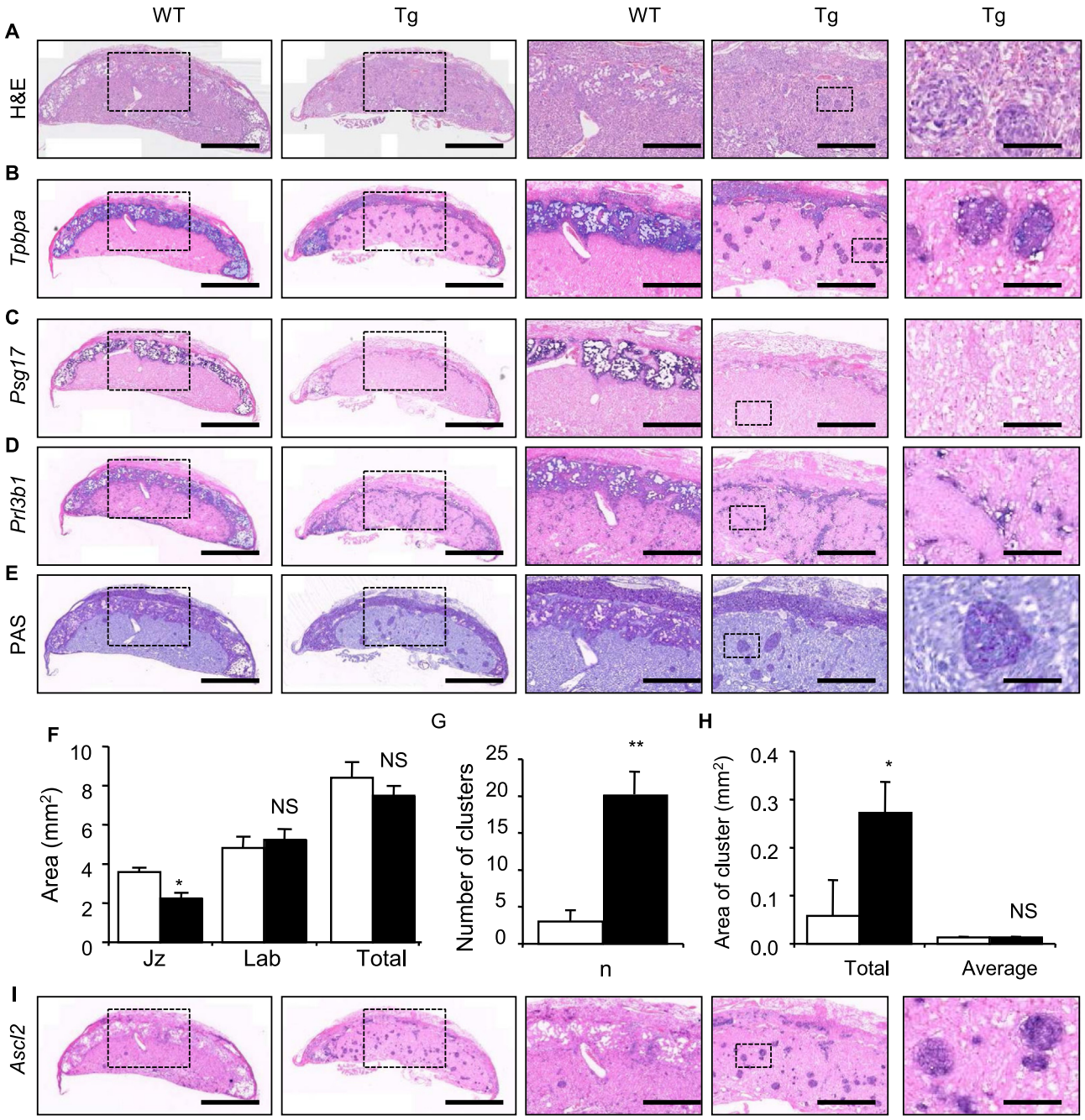


Fig. 3. A substantial loss of the spongiotrophoblast and mislocalisation of glycogen cells at E14.5. (A) H+E staining at E14.5 illustrating loss of the junctional zone. (B) *In situ* hybridisation with a *Tpbpa* riboprobe, which specifically marks the spongiotrophoblast and glycogen cell lineages in the junctional zone, at E14.5. (C) *In situ* hybridisation with a *Psg17* riboprobe, which is expressed in just the spongiotrophoblast at E14.5. (D) *In situ* hybridisation with a *Prl3b1* riboprobe, which is expressed in the P-TGC, C-TGC, S-TGC and the spongiotrophoblast at E14.5, showing a degraded interface between the junctional zone and maternal decidua. (E) PAS staining of glycogen cells in the junctional zone at E14.5. Three right hand images highlighting mislocalisation of glycogen cells in the labyrinth. (F) Midline section areas (mm^2) occupied by the junctional zone and labyrinth, and total area of both at E14.5. (G) Number of *Tpbpa*+ve/*Psg17*−ve/*Prl3b1*−ve/PAS+ve/*Ascl2*+ve clusters present in the labyrinth. (H) Total and average area (mm^2) occupied by *Tpbpa*+ve/*Psg17*−ve/*Prl3b1*−ve/PAS+ve/*Ascl2*+ve clusters in the labyrinth. (I) *In situ* hybridisation with an *Ascl2* riboprobe, demonstrating mislocalised cells express *Ascl2*. Scale bars=1000 μm (two images on left); 350 μm (middle and adjacent right image) and 150 μm (far right image).

levels similar to controls (Fig. 5E). Together, these data were consistent with a substantial loss of the spongiotrophoblast and P-TGCs with limited consequence for the other placental lineages.

The loss of spongiotrophoblast was very marked in the Ascl2-Tg placenta. Previously we have shown a direct relationship between the spongiotrophoblast and the amount of stored placental glycogen late in gestation, both of which were associated with fetal growth restriction (Tunster et al., 2010, 2014, 2015; Salas et al., 2004). To assess the consequences of elevated *Ascl2*, fetuses and placenta were collected at E12.5, E14.5, E16.5 and E18.5. The observed ratios between Ascl2-Tg and non-transgenic fetuses were not significantly different to the expected ratios indicating no loss of viability (Critical value 3.841 with $p=0.05$ and DoF=1; E12.5: 2.083, E14.5: 0.049, E16.5: 0.170 and E18.5: 0.865). Ascl2-Tg fetuses were not significantly different in weight to controls at E12.5, E14.5, and E16.5 but at E18.5 there was a 6% ($p=0.00521$) reduction in wet weight (Fig. 6A, Supplemental Table 8). Ascl2-Tg placentae were significantly lighter than controls at E12.5 and E14.5, by 17% ($p=0.00898$) and 11% ($p=9.29\times10^{-5}$) respectively, but weights had recovered at E16.5 and E18.5 (Fig. 6B). As a

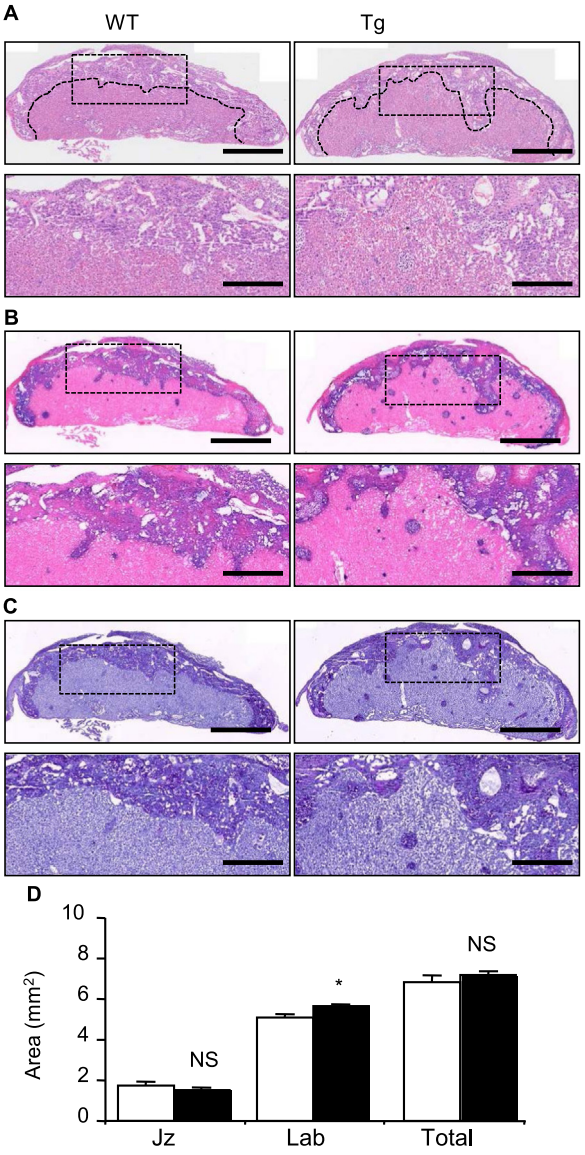


Fig. 4. Persistence of junctional zone phenotype at E18.5. (A) H+E staining at E18.5 illustrating loss of the junctional zone. Lower panels are higher magnification of the regions indicated. (B) *In situ* hybridisation with a *Tpbpa* riboprobe, which specifically marks the spongiotrophoblast and glycogen cell lineages, at E18.5. Lower panels are higher magnification of the regions indicated. (C) PAS staining for glycogen cells at E18.5. Lower panels are higher magnification of the regions indicated. (D) Midline section areas (mm^2) occupied by the junctional zone and labyrinth, and total area of both at E18.5. Scale bars A–C=1000 μm (upper panels); 400 μm (lower panels).

consequence, the Fetal:Placental (F:P) ratios were higher at E12.5 and E14.5 and lower at E18.5 (Fig. 6C). A biochemical determination of glycogen was performed at E14.5, E16.5 and E18.5. At E18.5, there was a substantial (+61%; mg) increase in the total amount of glycogen present in Ascl2-Tg placenta (Fig. 6D). When expressed relative to placental weight (mg/g), glycogen was significantly increased at both E16.5 (+25%; $p=0.0458$) and E18.5 (+58%; $p=0.00277$).

Fetal growth restriction was very modest (6%) in comparison to the placental defect. Our analysis of a different mouse model (transgenic overexpression of *Phlda2*) with a similar substantial loss of spongiotrophoblast revealed a fetal growth restriction phenotype apparent on a 129 strain background and absent on a BL6 background (Tunster et al., 2010, 2014, 2015) potentially explained by the less permissive F:P ratio in 129 mice (Tunster et al., 2012). Ascl2-Tg was examined after >6 generations of backcrossing into 129. In initial studies, few plugged females were pregnant when checked at E14.5 (Supplemental Table 9). When the line was switched to a more nutrient-rich diet in a barrier unit, pregnancy success rates improved (Supplemental Table 9) but there was no difference in fetal weight between Ascl2-Tg fetuses and WT littermates at E18.5 under these more favourable conditions (Supplemental Table 10).

The dramatic reduction of the spongiotrophoblast lineage was consistent with data demonstrating that ectopic expression of *Ascl2* in TS cells represses the expression of *Tpbpa* (Takao et al., 2012) (Supplemental Table 1). However, a significant loss of the spongiotrophoblast lineage *in vivo* has been reported in association with a 50% reduction in the expression of *Ascl2* in $Del^{7A1/+}$ placenta (Oh-McGinnis et al., 2011). In this complex model, *Phlda2* was expressed at two-fold higher than normal (Supplemental Table 1). We have previously shown that two-fold elevation in the expression of *Phlda2* results in a substantial (50%) reduction of the spongiotrophoblast (Tunster et al., 2010, 2014, 2015). Together these data suggest that *Ascl2* could regulate the spongiotrophoblast indirectly *via Phlda2*. *Phlda2* and *Ascl2* co-localise to a subset of cells with the developing chorio-allantoic placenta potentially marking progenitors of the spongiotrophoblast (Oh-McGinnis et al., 2011; Takao et al., 2012) (Fig. 7A). Ectopic expression of *Ascl2* in TS cells results in lower expression of *Phlda2* (Takao et al., 2012) (Supplemental Table 1). *In vivo*, a similar elevation in *Ascl2* did not result in significantly lower expression of *Phlda2* (Fig. 7B). To genetically test the relationship between *Ascl2* and *Phlda2*, double transgenic placenta carrying both the *Ascl2* transgene and a maternally inherited targeted *Phlda2* allele ($Phlda2^{-/+}$;Ascl2-Tg) were generated. Both the *Phlda2* loss-of-function placenta and the double transgenic placenta possessed a markedly expanded junctional zone, as evidenced by *Tpbpa* staining of E14.5 placental sections (Fig. 7C). In the absence of *Phlda2*, *Ascl2* was detectable by *in situ* indicating that *Ascl2* expression was not dependent on *Phlda2* (Fig. 7D). While is possible that the dominance of the *Phlda2* phenotype is a consequence of earlier events, these data are consistent with *Ascl2* acting *via Phlda2* to suppress the expansion of the spongiotrophoblast lineage (Fig. 8).

4. Discussion

Ascl2 is a gene expressed from the maternal allele in the placenta. Previous studies in mice examining the consequences of reduced expression of *Ascl2* have suggested a pivotal role for this gene in repressing the expansion of the parietal trophoblast giant cell lineage (Guillemot et al., 1995, 1994; Tanaka et al., 1997; Oh-McGinnis et al., 2011). Here, we have confirmed this directly in an *Ascl2* over expression model. We also highlight a novel role for the *Ascl2* in repressing the expansion of the spongiotrophoblast, a function that depends on expression of a second maternally expressed imprinted gene, *Phlda2*. We previously reported that two-fold expression of *Phlda2* resulted in a loss of the spongiotrophoblast associated with a significant reduction in placental glycogen, which led us to hypothesise a role for the spongiotrophoblast in driving the accumulation of these stores (Tunster et al., 2010). Here, we observed a similar loss of spongiotrophoblast but coincident with increased stores of placental glycogen late in gestation. Moreover, glycogen cells were markedly mislocalised to the labyrinth. One interpretation of this data is that the mislocalisation of the glycogen cells, in response to the loss of both the P-TGC and the spongiotrophoblast, precludes the utilisation of these stores resulting in late fetal growth restriction.

We have now identified three maternally expressed genes located in a single, mechanistically distinct imprinted domain (Fitzpatrick et al., 2002) that all act on the spongiotrophoblast

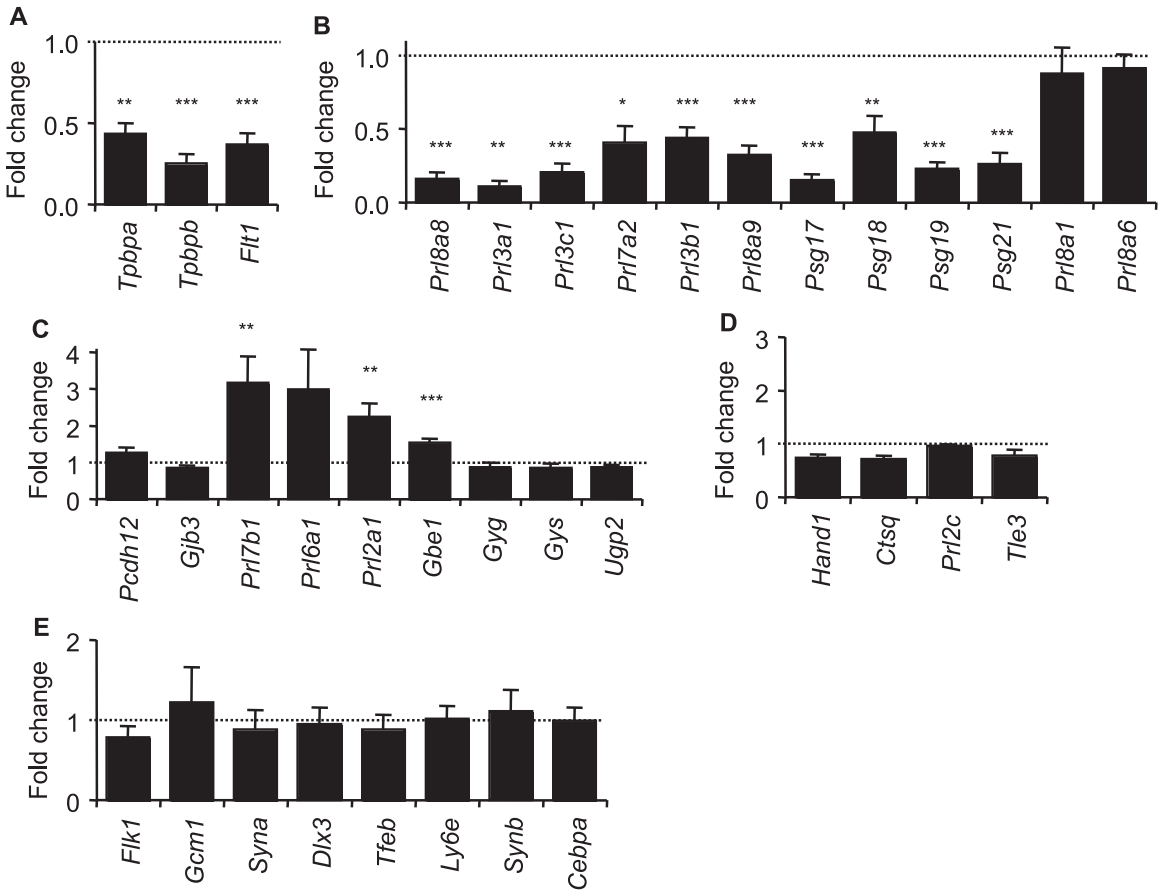


Fig. 5. Lineage analysis reveals a marked loss of the spongiotrophoblast and an increased representation of the glycogen cell lineage. (A) Quantitation of junctional zone markers *Tpbpa, Tpbpb* and *Flt1* at E14.5. (B) Quantitation of genes exclusively or predominantly expressed in the spongiotrophoblast at E14.5. (C) Quantitation of genes exclusively or predominantly expressed in the glycogen cell lineage at E14.5. (D) Quantitation of genes exclusively or predominantly expressed in the TGC lineages at E14.5. (E) Quantitation of genes exclusively or predominantly expressed in the labyrinth at E14.5. For the RT-qPCR analysis, N=4 placenta per genotype (2 vs. 2 from 2 independent litters); error bars represent SEM. Statistical significance calculated using *t*-test. $^{NS}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$. Data for Fig. 5 in Supplemental Table 4.

lineage of the mouse placenta. *Ascl2* and *Phlda2* repress the expansion of this lineage while *Cdkn1c* is required for this lineage to develop normally (Tunster et al., 2010, 2014, 2015, 2011; Salas et al., 2004). Additionally, *Ascl2* and *Cdkn1c* are functionally important for the P-TGC and S-TGCs lineages, respectively. There is some evidence that the IC2 imprinted domain encompassing these

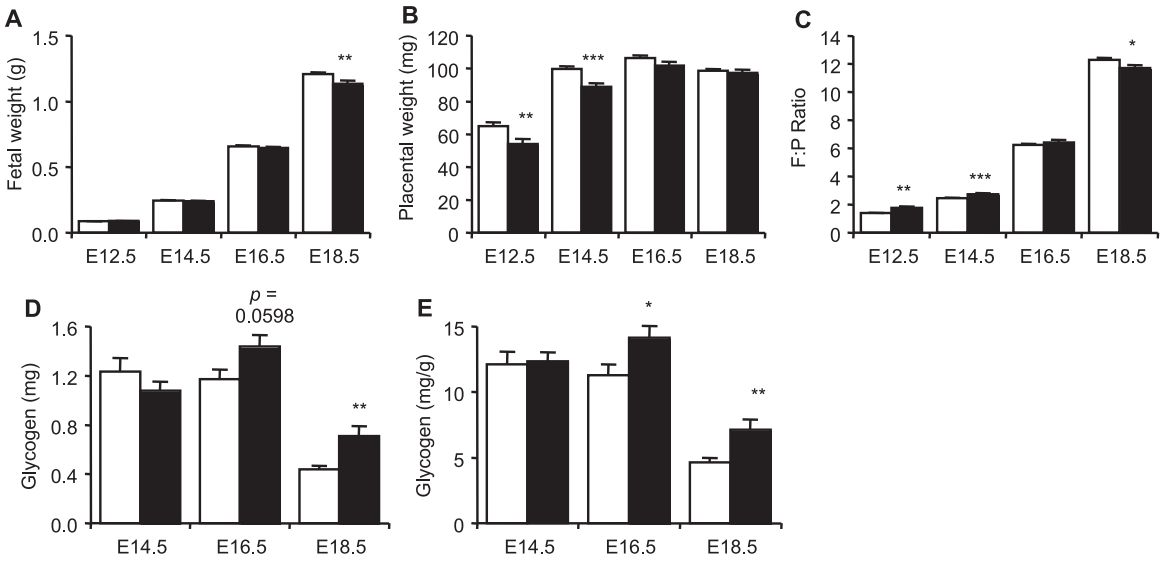


Fig. 6. Placental and fetal growth restriction but increased placental glycogen. (A) Transgenic *versus* non-transgenic fetal wet weights at E12.5 (WT N=29; Ascl2-Tg N=19), E14.5 (WT N=42; Ascl2-Tg N=40), E16.5 (WT N =25; Ascl2-Tg N=28) and E18.5 (WT N=41; Ascl2-Tg N =33). (B) Transgenic *versus* wild type placental wet weights at the same time points. (C) Fetal:Placental (F:P) ratios at the same time points. (D) Biochemical determination of placental glycogen at E14.5, E16.5 and E18.5 expressed as the total amount of glycogen (mg) E. Biochemical determination of placental glycogen at E14.5, E16.5 and E18.5 expressed in relation to placental weight (mg/g). Data for Fig. 6 in Supplemental Table 5.

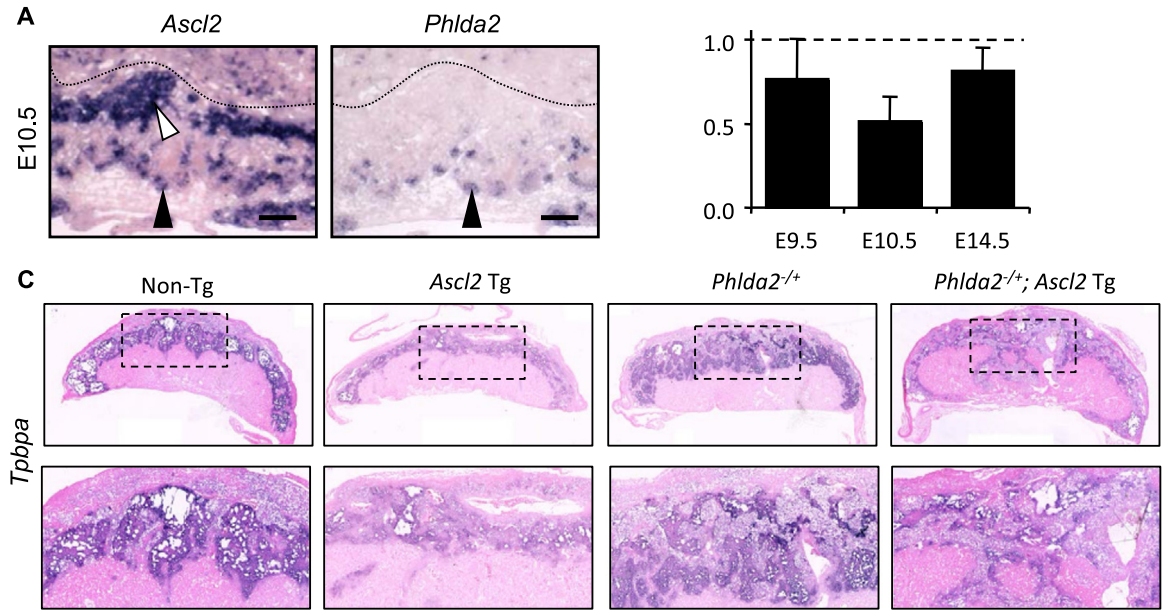


Fig. 7. *Ascl2* requires *Phlda2* to repress the expansion of the spongiotrophoblast lineage. (A) *In situ* hybridisation of sequential midline sections of wild type placenta at E10.5 with *Ascl2* and *Phlda2* riboprobes. The two genes are co-expressed in subset of cells at the base of the developing junctional zone (filled arrowheads) but not those located near the P-TGC layer (white arrow). Scale bars 100 μm. (B) Quantitation of *Phlda2* mRNA at E9.5, E10.5 and E14.5. (C) *In situ* hybridisation of midline sections of non-transgenic, Ascl2-Tg, *Phlda2*$^{-/+}$ and *Phlda2*$^{-/+}$; Ascl2-Tg double transgenic placenta with the junctional zone marker *Tpbpa* at E14.5. D. *In situ* hybridisation of midline sections of non-transgenic, Ascl2-Tg, *Phlda2*$^{-/+}$ and *Phlda2*$^{-/+}$; Ascl2-Tg double transgenic placenta with an *Ascl2* riboprobe at E14.5. Scale bars in C and D = 1000 μm (upper panels); 400 μm (lower panels).

genes became imprinted after marsupials diverged from Eutherian mammals (Suzuki et al., 2005, 2011.). It may be significant that a key difference between marsupials and Eutherians is the extent to which extra embryonic tissues support growth *in utero* with the Eutherian newborn being substantially larger at term, relative to the size of the mother, and distinctly more mature than the

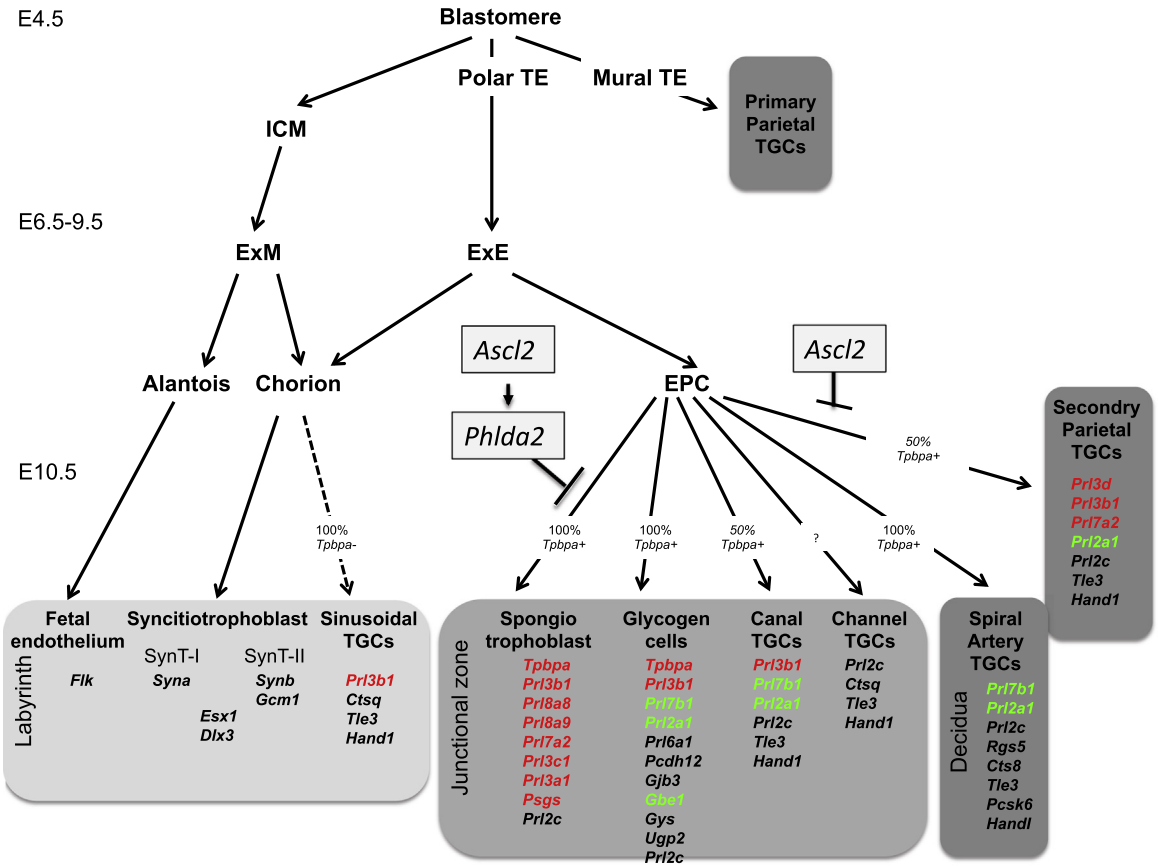


Fig. 8. Summary of gene expression data in relation to placental lineages. Genes indicated in red are significantly down and genes indicated in green are significantly elevated in Ascl2-Tg placenta. *Ascl2* requires *Phlda2* to repress the expansion of the spongiotrophoblast lineage.

marsupial newborn. There are a number of maternally expressed imprinted genes, including *Esx1*, *Cited1, Plac1* and *Nrk* (maternally expressed by virtue of their location on the paternally inactive X-chromosome), that repress the spongiotrophoblast lineage and at least two paternally expressed imprinted genes, *Peg3* and *Peg10*, predicted to increase the size of this compartment (John, 2013). This convergence suggests that this lineage is a major site of parental genomic conflict. The spongiotrophoblast is a major site of production of placental lactogens (Simmons et al., 2008). Some members of this extensive gene family (*Prl3d* and *Prl3b1*) have been shown to induce the changes in the mother required for a successful pregnancy (Bhattacharyya et al., 2002; Muller et al., 1999). The spongiotrophoblast also manufactures pregnancy-specific glycoproteins (PSGs) which are another family of highly similar secreted proteins thought to contribute in the protection of the semiallotypic fetus from the maternal immune system, and which also remodel placental and maternal vasculature (Kammerer et al., 2010; Wu et al., 2008). Essentially the spongiotrophoblast is a hormone factory and the considerable drain on maternal resources required to sustain the production of these placental hormones may be why this lineage is so tightly regulated by imprinting. Alternatively, this regulation may reflect the function of these hormones in pregnancy. Placental hormones are manufactured in large quantities during pregnancy and act on the maternal system to direct resources to support fetal growth. While it remains to be determined whether such changes in gene expression in the placenta have a consequence for maternal physiology in this model, this data provides further evidence that the maternal and paternal genomes are involved in a continuing battle over the endocrine function of the mouse placenta.

We have previously shown that the maternally expressed *Phlda2* gene limits the expansion of the spongiotrophoblast alongside placental stunting, a loss of placental glycogen and fetal growth restriction (Tunster et al., 2010, 2014, 2015). Similarly, elevated *Ascl2* also resulted in fetal growth restriction but, in contrast to the *Phlda2* transgenic model, placental glycogen stores were markedly increased in the near term placenta when fetal growth restriction was apparent. Moreover, there was a marked appearance of clusters of glycogen cells in the labyrinth at a time when these cells are normally migrating into the decidua. Expression of some glycogen cell markers, such as the marker of migrating glycogen cells *Prl7b1*, was altered but the majority of markers were expressed at similar levels to WT suggesting mislocalisation from the junctional zone. A milder mislocalisation of junctional zone cells was also apparent in our *Phlda2* overexpression model where there was also a loss of spongiotrophoblast (Tunster et al., 2010) suggesting that the spongiotrophoblast is important for maintaining the glycogen cells within the junctional zone. Mislocalisation of glycogen cells in the Ascl2-Tg model was considerably more severe than in our *Phlda2* model suggesting that the loss of P-TGC may further contribute to this phenotype. The presence of excess glycogen and fetal growth restriction is not consistent with the suggestion that placental glycogen is required to support late fetal growth (Coan et al., 2006). However, these data can be reconciled if the release of glycogen into the maternal system was prevented as a consequence of the mislocalisation of glycogen cells into the labyrinth.

Fetal growth restriction was very modest (6%) on the BL6 background despite the rather dramatic loss of both P-TGC and spongiotrophoblast cells. BL6 placenta have a higher F:P ratio and are thought to have a greater reserve capacity to support fetal growth compared to the 129 strain. We had great difficulty breeding Ascl2-Tg into 129. Under standard conditions in a conventional unit few plugged females were pregnant at E14.5. When the line was rederived into a barrier unit where the mice were maintained on an enriched diet, the pregnancy success rate improved sufficiently to assess fetal growth but under these conditions we observed no fetal growth restriction. The use of different diets under a different health status confounds the interpretation of this data. Notably, targeted loss of function of the placental lactogens *Prl4a1* and *Prl7b1* had no overt phenotypic consequence under normal husbandry conditions but pregnancies failed under stressed conditions (Ain et al., 2004; Bu et al., 2016). There are 22 members of the *Prl* gene family (Simmons et al., 2008). These data suggests an inherent redundancy in the functions of members of the *Prl* family such that overt fetal complications manifest only when adverse conditions are combined with reduced expression.

Both loss-of-function (Guillemot et al., 1995) and overexpression of *Ascl2 in vivo* (Figs. 2D, and 5A) results in fewer cells expressing *Tpbpa*. Ectopic overexpression of *Ascl2* in TS cells also resulted in low *Tpbpa* (Takao et al., 2012). These data suggest that *Ascl2* is both required for the development of *Tpbpa*+ve lineages and restrains their proliferation. In the $Del^{7A1/+}$ model, in which placenta express *Ascl2* at 50% normal, at E9.5 placenta initially appeared to have an increased number of cells expressing *Tpbpa*. By E15.5 very few cells expressed this marker (Oh-McGinnis et al., 2011). The $Del^{7A1/+}$ model involves a 280 kb deletion of the IC1–IC2 interval directly or indirectly disrupting the expression of three imprinted genes in this region: *Ascl2* (50%), *Phlda2* (200%) and *Th* (0%) (Oh-McGinnis et al., 2011). We have previously shown that just two-fold expression of *Phlda2* alone can reduce the size of the spongiotrophoblast lineage by 50% (Tunster et al., 2010, 2014, 2015). The findings in these different models can be reconciled if the spongiotrophoblast phenotype in the $Del^{7A1/+}$ model is due to elevated *Phlda2* rather than a direct consequence of elevated *Ascl2*.

Previous studies have suggested a direct relationship between *Ascl2* and *Phlda2* (Data summarised in Supplemental Table 1). *Ascl2* is co-expressed with *Phlda2* in a subset of cells in the ectoplacental cone at E7.5, E9.5 (Oh-McGinnis et al., 2011; Takao et al., 2012) and E10.5 (Fig. 7A) where progenitors of the spongiotrophoblast reside. Adenoviral-driven overexpression of *Ascl2* in trophoblast stem cells resulted in a 60% reduction in the expression of *Phlda2* under stem cell culture conditions (Takao et al., 2012). Conversely, knockdown of *Ascl2* expression in TS cells (Takao et al., 2012) or reduced expression *in vivo* (Oh-McGinnis et al., 2011) resulted in increased *Phlda2* expression. In this current study, we did not observe a statistically significant reduction in *Phlda2* expression *in vivo* in response to overexpression of *Ascl2*. When we genetically tested the relationship between *Ascl2* and *Phlda2* by combining overexpression of *Ascl2* with loss-of-expression of *Phlda2,* this resulted in a markedly expanded spongiotrophoblast similar to loss of expression of *Phlda2* alone. This tells us that, while both elevated *Phlda2* (Tunster et al., 2010, 2014, 2015) and elevated *Ascl2* repress the spongiotrophoblast, *Ascl2* can only do so in the presence of *Phlda2* (Fig. 7C). These data are consistent with *Ascl2* functioning upstream of *Phlda2* to control the expansion of the spongiotrophoblast in a progenitor cell type (Fig. 8).

In conclusion, we have demonstrated that overexpression of the imprinted *Ascl2* gene has considerable consequences for placental development, specifically for the P-TGC and spongiotrophoblast lineages both of which express pregnancy-related hormones. Either as a consequence of the reduced function of the endocrine compartment or the failure in the appropriate migration of glycogen cells, elevated *Ascl2* resulted in a late fetal growth restriction. The presence of three imprinted genes within a single mechanistically distinct imprinted domain that all act to regulate placental lineages critical for the endocrine function of the placenta suggest that the imprinting of this domain was key to the switch to prolonged gestation and greater maturity at birth observed in Eutherian mammals.

Bibliography

Abrous, D. N. and Wojtowicz, J. M. (2008). 21 Neurogenesis and Hippocampal Memory System. *Cold Spring Harbor Monograph Archive*, 52:445–461.

Adamec, R., Walling, S., and Burton, P. (2004). Long-lasting, selective, anxiogenic effects of feline predator stress in mice. *Physiol Behav*, 83(3):401–10.

Adamson, S. L., Lu, Y., Whiteley, K. J., Holmyard, D., Hemberger, M., Pfarrer, C., and Cross, J. C. (2002). Interactions between trophoblast cells and the maternal and fetal circulation in the mouse placenta. *Dev Biol*, 250(2):358–73.

Agid, O., Shapira, B., Zislin, J., Ritsner, M., Hanin, B., Murad, H., Troudart, T., Bloch, M., Heresco-Levy, U., and Lerer, B. (1999). Environment and vulnerability to major psychiatric illness: a case control study of early parental loss in major depression, bipolar disorder and schizophrenia. *Molecular psychiatry*, 4(2):163–172.

Alexander, B. T., Henry Dasinger, J., and Intapad, S. (2014). Effect of Low Birth Weight on Women's Health. *Clin Ther*.

Altman, J. (1969). Autoradiographic and histological studies of postnatal neurogenesis. IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb. *J Comp Neurol*, 137(4):433–57.

Alvarez-Buylla, A. and Garcia-Verdugo, J. M. (2002). Neurogenesis in adult subventricular zone. *J Neurosci*, 22(3):629–34.

Alvarez-Buylla, A. and Lim, D. A. (2004). For the long run: maintaining germinal niches in the adult brain. *Neuron*, 41(5):683–6.

American Psychiatric Association, American Psychiatric Association, and DSM-5 Task Force (2013). *Diagnostic and statistical manual of mental disorders: DSM-5.* American Psychiatric Association, Arlington, Va.

Apter-Levi, Y., Zagoory-Sharon, O., and Feldman, R. (2014). Oxytocin and vasopressin support distinct configurations of social synchrony. *Brain Res*, 1580:124–32.

Arboleda, V. A., Lee, H., Parnaik, R., Fleming, A., Banerjee, A., Ferraz-de Souza, B., Délot, E. C., Rodriguez-Fernandez, I. A., Braslavsky, D., Bergadá, I., Dell'Angelica, E. C., Nelson, S. F., Martinez-Agosto, J. A., Achermann, J. C., and Vilain, E. (2012). Mutations in the PCNA-binding domain of CDKN1C cause IMAGe syndrome. *Nat Genet*, 44(7):788–92.

Arima, T., Kamikihara, T., Hayashida, T., Kato, K., Inoue, T., Shirayoshi, Y., Oshimura, M., Soejima, H., Mukai, T., and Wake, N. (2005). ZAC, LIT1 (KCNQ1OT1) and p57KIP2 (CDKN1C) are in an imprinted gene network that may play a role in Beckwith-Wiedemann syndrome. *Nucleic Acids Res*, 33(8):2650–60.

Atif, N., Lovell, K., and Rahman, A. (2015). Maternal mental health: The missing "m" in the global maternal and child health agenda. *Semin Perinatol*, 39(5):345–52.

Aubert, A., Goodall, G., Dantzer, R., and Gheusi, G. (1997). Differential effects of lipopolysaccharide on pup retrieving and nest building in lactating mice. *Brain Behav Immun*, 11(2):107–18.

Ayres, C., Agranonik, M., Portella, A. K., Filion, F., Johnston, C. C., and Silveira, P. P. (2012). Intrauterine growth restriction and the fetal programming of the hedonic response to sweet taste in newborn infants. *Int J Pediatr*, 2012:657379.

Bachner-Melman, R. and Ebstein, R. P. (2014). The role of oxytocin and vasopressin in emotional and social behaviors. *Handb Clin Neurol*, 124:53–68.

Baibazarova, E., van de Beek, C., Cohen-Kettenis, P. T., Buitelaar, J., Shelton, K. H., and van Goozen, S. H. M. (2013). Influence of prenatal maternal stress, maternal plasma cortisol and cortisol in the amniotic fluid on birth outcomes and child temperament at 3 months. *Psychoneuroendocrinology*, 38(6):907–15.

Baik, J.-H. (2013). Dopamine signaling in food addiction: role of dopamine D2 receptors. *BMB Rep*, 46(11):519–26.

Bailey, K. R. and Crawley, J. N. (2009). Anxiety-related behaviors in mice.

Bale, T. L., Baram, T. Z., Brown, A. S., Goldstein, J. M., Insel, T. R., McCarthy, M. M., Nemeroff, C. B., Reyes, T. M., Simerly, R. B., Susser, E. S., *et al.* (2010a). Early life programming and neurodevelopmental disorders. *Biological psychiatry*, 68(4):314–319.

Bale, T. L., Baram, T. Z., Brown, A. S., Goldstein, J. M., Insel, T. R., McCarthy, M. M., Nemeroff, C. B., Reyes, T. M., Simerly, R. B., Susser, E. S., and Nestler, E. J. (2010b). Early life programming and neurodevelopmental disorders. *Biol Psychiatry*, 68(4):314–9.

Barker, D. J. (1995). Fetal origins of coronary heart disease. *BMJ*, 311(6998):171–4.

Barker, D. J. (2006). Adult consequences of fetal growth restriction. *Clinical obstetrics and gynecology*, 49(2):270–283.

Barlow, D. P., Stöger, R., Herrmann, B. G., Saito, K., and Schweifer, N. (1991). The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the Tme locus. *Nature*, 349(6304):84–7.

Bartolomei, M. S. and Ferguson-Smith, A. C. (2011). Mammalian genomic imprinting. *Cold Spring Harb Perspect Biol*, 3(7).

Barton, S. C., Surani, M. A., and Norris, M. L. (1984). Role of paternal and maternal genomes in mouse development. *Nature*, 311(5984):374–6.

Bateson, P., Barker, D., Clutton-Brock, T., Deb, D., D'Udine, B., Foley, R. A., Gluckman, P., Godfrey, K., Kirkwood, T., Lahr, M. M., McNamara, J., Metcalfe, N. B., Monaghan, P., Spencer, H. G., and Sultan, S. E. (2004). Developmental plasticity and human health. *Nature*, 430(6998):419–21.

Bauer, A., Knapp, M., and Parsonage, M. (2016). Lifetime costs of perinatal anxiety and depression. *J Affect Disord*, 192:83–90.

Bazer, F. (1998). *Endocrinology of pregnancy*. Humana Press, Totowa, N.J.

Bellinger, L., Sculley, D. V., and Langley-Evans, S. C. (2006). Exposure to undernutrition in fetal life determines fat distribution, locomotor activity and food intake in ageing rats. *Int J Obes (Lond)*, 30(5):729–38.

Berridge, K. C. (2009). 'Liking' and 'wanting' food rewards: brain substrates and roles in eating disorders. *Physiol Behav*, 97(5):537–50.

Bhardwaj, R. D., Curtis, M. A., Spalding, K. L., Buchholz, B. A., Fink, D., Björk-Eriksson, T., Nordborg, C., Gage, F. H., Druid, H., Eriksson, P. S., and Frisén, J. (2006). Neocortical neurogenesis in humans is restricted to development. *Proc Natl Acad Sci U S A*, 103(33):12564–8.

Bhattacharyya, S., Lin, J., and Linzer, D. I. (2002). Reactivation of a hematopoietic endocrine program of pregnancy contributes to recovery from thrombocytopenia. *Molecular Endocrinology*, 16(6):1386–1393.

Bick, J. and Dozier, M. (2010). Mothers' and Children's Concentrations of Oxytocin Following Close, Physical Interactions with Biological and Non-biological Children. *Dev Psychobiol*, 52(1):100–107.

Blagitko, N., Mergenthaler, S., Schulz, U., Wollmann, H. A., Craigen, W., Eggermann, T., Ropers, H. H., and Kalscheuer, V. M. (2000). Human GRB10 is imprinted and expressed from the paternal and maternal allele in a highly tissue- and isoform-specific fashion. *Hum Mol Genet*, 9(11):1587–95.

Blakeley, P. M., Capron, L. E., Jensen, A. B., O'Donnell, K. J., and Glover, V. (2013). Maternal prenatal symptoms of depression and down regulation of placental monoamine oxidase A expression. *J Psychosom Res*, 75(4):341–5.

Bole-Feysot, C., Goffin, V., Edery, M., Binart, N., and Kelly, P. A. (1998). Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocr Rev*, 19(3):225–68.

Bond, T. L. Y., Neumann, P. E., Mathieson, W. B., and Brown, R. E. (2002). Nest building in nulligravid, primigravid and primiparous C57BL/6J and DBA/2J mice (Mus musculus). *Physiol Behav*, 75(4):551–5.

Bourke, C. H., Capello, C. F., Rogers, S. M., Yu, M. L., Boss-Williams, K. A., Weiss, J. M., Stowe, Z. N., and Owens, M. J. (2013). Prenatal exposure to escitalopram and/or stress in rats : A prenatal stress model of maternal depression and its treatment. *Psychopharmacology*. PMID: 23436130.

Braff, D. L., Geyer, M. A., Light, G. A., Sprock, J., Perry, W., Cadenhead, K. S., and Swerdlow, N. R. (2001). Impact of prepulse characteristics on the detection of sensorimotor gating deficits in schizophrenia. *Schizophrenia research*, 49(1):171–178.

Braun, A. A., Skelton, M. R., Vorhees, C. V., and Williams, M. T. (2011). Comparison of the elevated plus and elevated zero mazes in treated and untreated male Sprague-Dawley rats: effects of anxiolytic and anxiogenic agents. *Pharmacol Biochem Behav*, 97(3):406–15.

Bressan, F. F., De Bem, T. H. C., Perecin, F., Lopes, F. L., Ambrosio, C. E., Meirelles, F. V., and Miglino, M. A. (2009). Unearthing the roles of imprinted genes in the placenta. *Placenta*, 30(10):823–34.

Bridges, R. S. (1994). The role of lactogenic hormones in maternal behavior in female rats. *Acta paediatrica (Oslo, Norway: 1992). Supplement*, 397:33–39. PMID: 7981472.

Bridges, R. S. (2016). Long-term alterations in neural and endocrine processes induced by motherhood in mammals. *Horm Behav*, 77:193–203.

Bridges, R. S. and Freemark, M. S. (1995). Human placental lactogen infusions into the medial preoptic area stimulate maternal behavior in steroid-primed, nulliparous female rats. *Hormones and behavior*, 29(2):216–226. PMID: 7557924.

Bridges, R. S. and Grattan, D. R. (2003). Prolactin-induced neurogenesis in the maternal brain. *Trends in Endocrinology & Metabolism*, 14(5):199–201.

Bridges, R. S., Numan, M., Ronsheim, P. M., Mann, P. E., and Lupini, C. E. (1990). Central prolactin infusions stimulate maternal behavior in steroid-treated, nulliparous female rats. *Proc Natl Acad Sci U S A*, 87(20):8003–7.

Brisken, C. and O'Malley, B. (2010). Hormone action in the mammary gland. *Cold Spring Harb Perspect Biol*, 2(12):a003178.

Broad, K. D., Curley, J. P., and Keverne, E. B. (2009). Increased apoptosis during neonatal brain development underlies the adult behavioral deficits seen in mice lacking a functional paternally expressed gene 3 (Peg3). *Dev Neurobiol*, 69(5):314–25.

Brockington, I. (2004). Postpartum psychiatric disorders. *The Lancet*, 363(9405):303–310.

Brown, A. S., van Os, J., Driessens, C., Hoek, H. W., and Susser, E. S. (2000). Further evidence of relation between prenatal famine and major affective disorder. *American Journal of Psychiatry*, 157(2):190–195.

Brown, J. P., Couillard-Després, S., Cooper-Kuhn, C. M., Winkler, J., Aigner, L., and Kuhn, H. G. (2003). Transient expression of doublecortin during adult neurogenesis. *J Comp Neurol*, 467(1):1–10.

Brown, R. S., Kokay, I. C., Phillipps, H. R., Yip, S. H., Gustafson, P., Wyatt, A., Larsen, C. M., Knowles, P., Ladyman, S. R., LeTissier, P., *et al.* (2016). Conditional Deletion of the Prolactin Receptor Reveals Functional Subpopulations of Dopamine Neurons in the Arcuate Nucleus of the Hypothalamus. *The Journal of Neuroscience*, 36(35):9173–9185.

Bruce, H. M. (1959). An exteroceptive block to pregnancy in the mouse. *Nature*, 184:105.

Bruce, H. M. and M, P. D. (1960). Role of olfactory sense in pregnancy block by strange males. *Science*, 131(3412):1526.

Brunton, P. J. and Russell, J. A. (2008). The expectant brain: adapting for motherhood. *Nature Reviews Neuroscience*, 9(1):11–25.

Bu, P., Alam, S. M. K., Dhakal, P., Vivian, J. L., and Soares, M. J. (2016). A Prolactin Family Paralog Regulates Placental Adaptations to a Physiological Stressor. *Biol Reprod*, 94(5):107.

Buettner, V. L., Walker, A. M., and Singer-Sam, J. (2005). Novel paternally expressed intergenic transcripts at the mouse Prader-Willi/Angelman Syndrome locus. *Mamm Genome*, 16(4):219–27.

Burton, G. (2011). *The placenta and human developmental programming*. Cambridge University Press, Cambridge New York.

Butler, M. G. (2009). Genomic imprinting disorders in humans: a mini-review. *J Assist Reprod Genet*, 26(9-10):477–86.

Cahill, L., Haier, R. J., Fallon, J., Alkire, M. T., Tang, C., Keator, D., Wu, J., and McGaugh, J. L. (1996). Amygdala activity at encoding correlated with long-term, free recall of emotional information. *Proc Natl Acad Sci U S A*, 93(15):8016–21.

Cameron, H. A. and McKay, R. D. (1999). Restoring production of hippocampal neurons in old age. *Nat Neurosci*, 2(10):894–7.

Campos, A. C., Fogaça, M. V., Aguiar, D. C., and Guimarães, F. S. (2013). Animal models of anxiety disorders and stress. *Rev Bras Psiquiatr*, 35 Suppl 2:S101–11.

Capron, L. E., Glover, V., Pearson, R. M., Evans, J., O'Connor, T. G., Stein, A., Murphy, S. E., and Ramchandani, P. G. (2015). Associations of maternal and paternal antenatal mood with offspring anxiety disorder at age 18 years. *J Affect Disord*, 187:20–6.

Carlin, J., George, R., and Reyes, T. M. (2013). Methyl donor supplementation blocks the adverse effects of maternal high fat diet on offspring physiology. *PLoS One*, 8(5):e63549.

Carr, G. V., Chen, J., Yang, F., Ren, M., Yuan, P., Tian, Q., Bebensee, A., Zhang, G. Y., Du, J., Glineburg, P., Xun, R., Akhile, O., Akuma, D., Pickel, J., Barrow, J. C., Papaleo, F., and Weinberger, D. R. (2016). KCNH2-3.1 expression impairs cognition and alters neuronal function in a model of molecular pathology associated with schizophrenia. *Mol Psychiatry*.

Carter, A. M. (2007). Animal models of human placentation–a review. *Placenta*, 28 Suppl A:S41–7.

Cecchi, G. A., Petreanu, L. T., Alvarez-Buylla, A., and Magnasco, M. O. (2001). Unsupervised learning and adaptation in a model of adult neurogenesis. *J Comput Neurosci*, 11(2):175–82.

Chamberlain, S. J. and Brannan, C. I. (2001). The Prader-Willi syndrome imprinting center activates the paternally expressed murine Ube3a antisense transcript but represses paternal Ube3a. *Genomics*, 73(3):316–22.

Champagne, F., Diorio, J., Sharma, S., and Meaney, M. J. (2001). Naturally occurring variations in maternal behavior in the rat are associated with differences in estrogen-inducible central oxytocin receptors. *Proc Natl Acad Sci U S A*, 98(22):12736–41.

Champagne, F. A. (2011). Maternal imprints and the origins of variation. *Horm Behav*, 60(1):4–11.

Champagne, F. A. and Curley, J. P. (2009). Epigenetic mechanisms mediating the long-term effects of maternal care on development. *Neuroscience and biobehavioral reviews*, 33(4):593–600. PMID: 18430469.

Champagne, F. A., Curley, J. P., Keverne, E. B., and Bateson, P. P. G. (2007). Natural variations in postpartum maternal care in inbred and outbred mice. *Physiol Behav*, 91(2-3):325–34.

Champagne, F. A., Francis, D. D., Mar, A., and Meaney, M. J. (2003). Variations in maternal care in the rat as a mediating influence for the effects of environment on development. *Physiol Behav*, 79(3):359–71.

Champagne, F. A. and Meaney, M. J. (2006). Stress during gestation alters postpartum maternal care and the development of the offspring in a rodent model. *Biol Psychiatry*, 59(12):1227–35.

Chao, W. and D'Amore, P. A. (2008). IGF2: epigenetic regulation and role in development and disease. *Cytokine Growth Factor Rev*, 19(2):111–20.

Chapman, D. D., Shivji, M. S., Louis, E., Sommer, J., Fletcher, H., and Prodöhl, P. A. (2007). Virgin birth in a hammerhead shark. *Biol Lett*, 3(4):425–7.

Chourbaji, S., Hoyer, C., Richter, S. H., Brandwein, C., Pfeiffer, N., Vogt, M. A., Vollmayr, B., and Gass, P. (2011). Differences in Mouse Maternal Care Behavior–Is There a Genetic Impact of the Glucocorticoid Receptor? *PloS one*, 6(4):e19218.

Chu, K. M., Emasealu, O. V., Hu, Z., O'Donnell, F. L., and Clark, L. L. (2015). Risk of Mental Health Disorders Following an Initial Diagnosis of Postpartum Depression, Active Component, U.S. Armed Forces, 1998-2010. *MSMR*, 22(6):6–12.

Chu, S.-H., Ma, Y.-B., Feng, D.-F., Zhang, H., Qiu, J.-H., and Zhu, Z.-A. (2011). Elevated expression of solute carrier family 22 member 18 increases the sensitivity of U251 glioma cells to BCNU. *Oncol Lett*, 2(6):1139–1142.

Chuaqui, R. F., Bonner, R. F., Best, C. J. M., Gillespie, J. W., Flaig, M. J., Hewitt, S. M., Phillips, J. L., Krizman, D. B., Tangrea, M. A., Ahram, M., Linehan, W. M., Knezevic, V., and Emmert-Buck, M. R. (2002). Post-analysis follow-up and validation of microarray experiments. *Nat Genet*, 32 Suppl:509–14.

Coan, P. M., Burton, G. J., and Ferguson-Smith, A. C. (2005). Imprinted genes in the placenta – A review. *Placenta*, 26:S10–S20.

Coan, P. M., Conroy, N., Burton, G. J., and Ferguson-Smith, A. C. (2006). Origin and characteristics of glycogen cells in the developing murine placenta. *Dev Dyn*, 235(12):3280–94.

Constância, M., Hemberger, M., Hughes, J., Dean, W., Ferguson-Smith, A., Fundele, R., Stewart, F., Kelsey, G., Fowden, A., Sibley, C., and Reik, W. (2002). Placental-specific IGF-II is a major modulator of placental and fetal growth. *Nature*, 417(6892):945–8.

Coutellier, L., Friedrich, A.-C., Failing, K., Marashi, V., and Würbel, H. (2009a). Effects of foraging demand on maternal behaviour and adult offspring anxiety and stress response in C57BL/6 mice. *Behavioural brain research*, 196(2):192–199.

Coutellier, L., Friedrich, A.-C., Failing, K., Marashi, V., and Würbel, H. (2009b). Effects of foraging demand on maternal behaviour and adult offspring anxiety and stress response in C57BL/6 mice. *Behav Brain Res*, 196(2):192–9.

Cox, J. L., Murray, D., and Chapman, G. (1993). A controlled study of the onset, duration and prevalence of postnatal depression. *The British Journal of Psychiatry*, 163(1):27–31.

Crews, D., Fuller, T., Mirasol, E. G., Pfaff, D. W., and Ogawa, S. (2004). Postnatal environment affects behavior of adult transgenic mice. *Exp Biol Med (Maywood)*, 229(9):935–9.

Cross, J., Baczyk, D., Dobric, N., Hemberger, M., Hughes, M., Simmons, D., Yamamoto, H., and Kingdom, J. (2003). Genes, development and evolution of the placenta. *Placenta*, 24(2):123–130.

Cross, J. C. (2005). How to make a placenta: mechanisms of trophoblast cell differentiation in mice–a review. *Placenta*, 26 Suppl A:S3–9.

Crossey, P. A., Pillai, C. C., and Miell, J. P. (2002). Altered placental development and intrauterine growth restriction in IGF binding protein-1 transgenic mice. *J Clin Invest*, 110(3):411–8.

Curley, J. P. (2011). The mu-opioid receptor and the evolution of mother-infant attachment: theoretical comment on Higham et al. (2011). *Behav Neurosci*, 125(2):273–8.

Curley, J. P., Barton, S., Surani, A., and Keverne, E. B. (2004). Coadaptation in mother and infant regulated by a paternally expressed imprinted gene. *Proc Biol Sci*, 271(1545):1303–9.

Curley, J. P., Jordan, E. R., Swaney, W. T., Izraelit, A., Kammel, S., and Champagne, F. A. (2009). The meaning of weaning: influence of the weaning period on behavioral development in mice. *Dev Neurosci*, 31(4):318–31.

Curtis, M. A., Low, V. F., and Faull, R. L. M. (2012). Neurogenesis and progenitor cells in the adult human brain: a comparison between hippocampal and subventricular progenitor proliferation. *Dev Neurobiol*, 72(7):990–1005.

Dale, H. H. (1906). On some physiological actions of ergot. *J Physiol*, 34(3):163–206.

Das, R. and Vonderhaar, B. K. (1996). Involvement of SHC, GRB2, SOS and RAS in prolactin signal transduction in mammary epithelial cells. *Oncogene*, 13(6):1139–45.

Davies, J. R., Humby, T., Dwyer, D. M., Garfield, A. S., Furby, H., Wilkinson, L. S., Wells, T., and Isles, A. R. (2015). Calorie seeking, but not hedonic response, contributes to hyperphagia in a mouse model for Prader-Willi syndrome. *Eur J Neurosci*, 42(4):2105–13.

Deacon, R. M. J. (2006). Assessing nest building in mice. *Nat Protoc*, 1(3):1117–9.

Dearry, A., Gingrich, J. A., Falardeau, P., Fremeau, Jr, R. T., Bates, M. D., and Caron, M. G. (1990). Molecular cloning and expression of the gene for a human D1 dopamine receptor. *Nature*, 347(6288):72–6.

DeChiara, T. M., Efstratiadis, A., and Robertson, E. J. (1990). A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature*, 345(6270):78–80.

DeChiara, T. M., Robertson, E. J., and Efstratiadis, A. (1991). Parental imprinting of the mouse insulin-like growth factor II gene. *Cell*, 64(4):849–59.

Del Punta, K., Leinders-Zufall, T., Rodriguez, I., Jukam, D., Wysocki, C. J., Ogawa, S., Zufall, F., and Mombaerts, P. (2002). Deficient pheromone responses in mice lacking a cluster of vomeronasal receptor genes. *Nature*, 419(6902):70–4.

Dennis, R. L., Fahey, A. G., and Cheng, H. W. (2013). Alterations to embryonic serotonin change aggression and fearfulness. *Aggressive behavior*, 39(2):91–98. PMID: 23386480.

Dent, C. L. and Isles, A. R. (2014). Brain-expressed imprinted genes and adult behaviour: the example of Nesp and Grb10. *Mamm Genome*, 25(1-2):87–93.

Diaz-Meyer, N. (2003). Silencing of CDKN1C (p57KIP2) is associated with hypomethylation at KvDMR1 in Beckwith-Wiedemann syndrome. *Journal of Medical Genetics*, 40(11):797–801.

Dickenson, A. H. (1991). Mechanisms of the analgesic actions of opiates and opioids. *Br Med Bull*, 47(3):690–702.

Doetsch, F. and Alvarez-Buylla, A. (1996). Network of tangential pathways for neuronal migration in adult mammalian brain. *Proc Natl Acad Sci U S A*, 93(25):14895–900.

Dotson, C. D. and Spector, A. C. (2005). Drinking spout orifice size affects licking behavior in inbred mice. *Physiol Behav*, 85(5):655–61.

Drake, A. J. (2014). Glucocorticoids, Programming and the Transmission of Effects Across Generations. In *Hormones, Intrauterine Health and Programming*, pages 27–40. Springer.

Drew, M. R., Simpson, E. H., Kellendonk, C., Herzberg, W. G., Lipatova, O., Fairhurst, S., Kandel, E. R., Malapani, C., and Balsam, P. D. (2007). Transient overexpression of striatal D2 receptors impairs operant motivation and interval timing. *J Neurosci*, 27(29):7731–9.

Dubber, S., Reck, C., Müller, M., and Gawlik, S. (2015). Postpartum bonding: the role of perinatal depression, anxiety and maternal–fetal bonding during pregnancy. *Archives of women's mental health*, 18(2):187–195.

Dudley, C. A., Rajendren, G., and Moss, R. L. (1996). Signal processing in the vomeronasal system: modulation of sexual behavior in the female rat. *Crit Rev Neurobiol*, 10(3-4):265–90.

Duque, A., Coman, D., Carlyle, B. C., Bordner, K. A., George, E. D., Papademetris, X., Hyder, F., and Simen, A. A. (2012). Neuroanatomical changes in a mouse model of early life neglect. *Brain Struct Funct*, 217(2):459–72.

Dwyer, D. M. (2012). Licking and liking: The assessment of hedonic responses in rodents. *The Quarterly Journal of Experimental Psychology*, 65(3):371–394. PMID: 22404646.

Dwyer, D. M., Pincham, H. L., Thein, T., and Harris, J. A. (2009). A learned flavor preference persists despite the extinction of conditioned hedonic reactions to the cue flavors. *Learn Behav*, 37(4):305–10.

Elford, J., Whincup, P., and Shaper, A. G. (1991). Early life experience and adult cardiovascular disease: longitudinal and case-control studies. *Int J Epidemiol*, 20(4):833–44.

Entringer, S. (2013). Impact of stress and stress physiology during pregnancy on child metabolic function and obesity risk. *Curr Opin Clin Nutr Metab Care*, 16(3):320–7.

Eriksson, P. S., Perfilieva, E., Björk-Eriksson, T., Alborn, A. M., Nordborg, C., Peterson, D. A., and Gage, F. H. (1998). Neurogenesis in the adult human hippocampus. *Nat Med*, 4(11):1313–7.

Espina, V., Wulfkuhle, J. D., Calvert, V. S., VanMeter, A., Zhou, W., Coukos, G., Geho, D. H., Petricoin, E. F., and Liotta, L. A. (2006). Laser-capture microdissection. *Nature protocols*, 1(2):586–603.

Evans, J., Heron, J., Francomb, H., Oke, S., and Golding, J. (2001). Cohort study of depressed mood during pregnancy and after childbirth. *BMJ*, 323(7307):257–60.

Falls, J. G., Pulford, D. J., Wylie, A. A., and Jirtle, R. L. (1999). Genomic imprinting: implications for human disease. *Am J Pathol*, 154(3):635–47.

Famularo, R., Kinscherff, R., and Fenton, T. (1992). Psychiatric diagnoses of maltreated children: preliminary findings. *Journal of the American Academy of Child & Adolescent Psychiatry*, 31(5):863–867.

Farnsworth, R. L. and Talamantes, F. (1998). Calcyclin in the mouse decidua: expression and effects on placental lactogen secretion. *Biol Reprod*, 59(3):546–52.

Farr, S. L., Dietz, P. M., O'Hara, M. W., Burley, K., and Ko, J. Y. (2014). Postpartum anxiety and comorbid depression in a population-based sample of women. *J Womens Health (Larchmt)*, 23(2):120–8.

Faure, A., Richard, J. M., and Berridge, K. C. (2010). Desire and dread from the nucleus accumbens: cortical glutamate and subcortical GABA differentially generate motivation and hedonic impact in the rat. *PLoS One*, 5(6):e11223.

Feil, R. and Berger, F. (2007). Convergent evolution of genomic imprinting in plants and mammals. *Trends Genet*, 23(4):192–9.

Fiander, A. (2015). *Your essential revision guide : MRCOG part one : the official companion to the Royal College of Obstetricians and Gynaecologists revision course*. Cambridge University Press, London.

Filliol, D., Ghozland, S., Chluba, J., Martin, M., Matthes, H. W., Simonin, F., Befort, K., Gavériaux-Ruff, C., Dierich, A., LeMeur, M., Valverde, O., Maldonado, R., and Kieffer, B. L. (2000). Mice deficient for delta- and mu-opioid receptors exhibit opposing alterations of emotional responses. *Nat Genet*, 25(2):195–200.

Fitzpatrick, G. V., Soloway, P. D., and Higgins, M. J. (2002). Regional loss of imprinting and growth deficiency in mice with a targeted deletion of KvDMR1. *Nat Genet*, 32(3):426–31.

Flietstra, R. J. and Voogt, J. L. (1996). Rat placental lactogens initiate and maintain lactation yet inhibit suckling-induced prolactin release. *Endocrine*, 5(1):103–10.

Forsyth, I. A. and Wallis, M. (2002). Growth hormone and prolactin–molecular and functional evolution. *J Mammary Gland Biol Neoplasia*, 7(3):291–312.

Fowden, A. L., Coan, P. M., Angiolini, E., Burton, G. J., and Constancia, M. (2011). Imprinted genes and the epigenetic regulation of placental phenotype. *Prog Biophys Mol Biol*, 106(1):281–8.

Fowden, A. L., Sibley, C., Reik, W., and Constancia, M. (2006). Imprinted genes, placental development and fetal growth. *Horm Res*, 65 Suppl 3:50–8.

Fowler, C. D., Liu, Y., Ouimet, C., and Wang, Z. (2002). The effects of social environment on adult neurogenesis in the female prairie vole. *J Neurobiol*, 51(2):115–28.

Fowler, C. D., Liu, Y., and Wang, Z. (2008). Estrogen and adult neurogenesis in the amygdala and hypothalamus. *Brain Res Rev*, 57(2):342–51.

Frank, D., Fortino, W., Clark, L., Musalo, R., Wang, W., Saxena, A., Li, C.-M., Reik, W., Ludwig, T., and Tycko, B. (2002). Placental overgrowth in mice lacking the imprinted gene Ipl. *Proceedings of the National Academy of Sciences*, 99(11):7490–7495.

Frank, D., Mendelsohn, C. L., Ciccone, E., Svensson, K., Ohlsson, R., and Tycko, B. (1999). A novel pleckstrin homology-related gene family defined by Ipl/Tssc3, TDAG51, and Tih1: tissue-specific expression, chromosomal location, and parental imprinting. *Mamm Genome*, 10(12):1150–9.

Franks, B., Champagne, F. A., and Curley, J. P. (2015). Postnatal maternal care predicts divergent weaning strategies and the development of social behavior. *Dev Psychobiol*, 57(7):809–17.

Franks, B., Curley, J. P., and Champagne, F. A. (2011). Measuring variations in maternal behavior: relevance for studies of mood and anxiety. *Mood and Anxiety Related Phenotypes in Mice: Characterization Using Behavioral Tests, Volume II*, pages 209–224.

Freeman, M. E., Kanyicska, B., Lerant, A., and Nagy, G. (2000). Prolactin: structure, function, and regulation of secretion. *Physiol Rev*, 80(4):1523–631.

Freeman, W. M., Walker, S. J., and Vrana, K. E. (1999). Quantitative RT-PCR: pitfalls and potential. *Biotechniques*, 26(1):112–22, 124–5.

Frost, J. M. and Moore, G. E. (2010). The importance of imprinting in the human placenta. *PLoS Genet*, 6(7):e1001015.

Fulda, K. G., Kurian, A. K., Balyakina, E., and Moerbe, M. M. (2014). Paternal race/ethnicity and very low birth weight. *BMC Pregnancy Childbirth*, 14:385.

Gammie, S. C. (2005). Current models and future directions for understanding the neural circuitries of maternal behaviors in rodents. *Behavioral and cognitive neuroscience reviews*, 4(2):119–135.

Gandelman, R. (1973). Induction of maternal nest building in virgin female mice by the presentation of young. *Horm Behav*, 4(3):191–7.

Gandelman, R. (1975). Maternal nest-building performance and fetal number in Rockland-Swiss albino mice. *J Reprod Fertil*, 44(3):551–4.

Gandelman, R., McDermott, N. J., Kleinman, M., and DeJianne, D. (1979). Maternal nest building by pseudopregnant mice. *J Reprod Fertil*, 56(2):697–9.

Gangestad, S. W. and Grebe, N. M. (2016). Hormonal systems, human social bonding, and affiliation. *Horm Behav*.

Garland, M. and Svare, B. (1988). Suckling stimulation modulates the maintenance of postpartum aggression in mice. *Physiol Behav*, 44(3):301–5.

Gascuel, J., Lemoine, A., Rigault, C., Datiche, F., Benani, A., Penicaud, L., and Lopez-Mascaraque, L. (2012). Hypothalamus-olfactory system crosstalk: orexin a immunostaining in mice. *Front Neuroanat*, 6:44.

Gaynes, B. N., Gavin, N., Meltzer-Brody, S., Lohr, K. N., Swinson, T., Gartlehner, G., Brody, S., and Miller, W. C. (2005). Perinatal depression: prevalence, screening accuracy, and screening outcomes. *Evid Rep Technol Assess (Summ)*, (119):1–8.

George, E. D., Bordner, K. A., Elwafi, H. M., and Simen, A. A. (2010). Maternal separation with early weaning: a novel mouse model of early life neglect. *BMC Neurosci*, 11:123.

Georgiades, P., Ferguson-Smith, A. C., and Burton, G. J. (2002). Comparative developmental anatomy of the murine and human definitive placentae. *Placenta*, 23(1):3–19.

Gidley-Baird, A. A. (1981). Endocrine control of implantation and delayed implantation in rats and mice. *J Reprod Fertil Suppl*, 29:97–109.

Gleeson, J. G., Lin, P. T., Flanagan, L. A., and Walsh, C. A. (1999). Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. *Neuron*, 23(2):257–71.

Glynn, L. M. and Sandman, C. A. (2011). Prenatal Origins of Neurological Development: A Critical Period for Fetus and Mother. *Current Directions in Psychological Science*, 20(6):384–389.

Goergen, E. M., Bagay, L. A., Rehm, K., Benton, J. L., and Beltz, B. S. (2002). Circadian control of neurogenesis. *J Neurobiol*, 53(1):90–5.

Goffin, V., Kinet, S., Ferrag, F., Binart, N., Martial, J. A., and Kelly, P. A. (1996). Antagonistic properties of human prolactin analogs that show paradoxical agonistic activity in the Nb2 bioassay. *Journal of Biological Chemistry*, 271(28):16573–16579.

Goland, R. S., Wardlaw, S. L., MacCarter, G., Warren, W. B., and Stark, R. I. (1991). Adrenocorticotropin and cortisol responses to vasopressin during pregnancy. *J Clin Endocrinol Metab*, 73(2):257–61.

Golub, Y., Canneva, F., Funke, R., Frey, S., Distler, J., von Hörsten, S., Freitag, C., Kratz, O., Moll, G., and Solati, J. (2016). Effects of In utero environment and maternal behavior on neuroendocrine and behavioral alterations in a mouse model of prenatal trauma. *Developmental neurobiology*.

Grattan, D. (2011). A mother's brain knows. *Journal of neuroendocrinology*, 23(11):1188–1189. PMID: 22004569.

Grattan, D. R. and Kokay, I. C. (2008). Prolactin: A pleiotropic neuroendocrine hormone. *Journal of Neuroendocrinology*, 20(6):752–763. WOS:000256582900019.

Grattan, D. R., Ladyman, S. R., and Augustine, R. A. (2007). Hormonal induction of leptin resistance during pregnancy. *Physiol Behav*, 91(4):366–74.

Green, B. B., Kappil, M., Lambertini, L., Armstrong, D. A., Guerin, D. J., Sharp, A. J., Lester, B. M., Chen, J., and Marsit, C. J. (2015). Expression of imprinted genes in placenta is associated with infant neurobehavioral development. *Epigenetics*, 10(9):834–41.

Grissom, N. M. and Reyes, T. M. (2013). Gestational overgrowth and undergrowth affect neurodevelopment: similarities and differences from behavior to epigenetics. *Int J Dev Neurosci*, 31(6):406–14.

Grote, N. K., Bridge, J. A., Gavin, A. R., Melville, J. L., Iyengar, S., and Katon, W. J. (2010). A meta-analysis of depression during pregnancy and the risk of preterm birth, low birth weight, and intrauterine growth restriction. *Arch Gen Psychiatry*, 67(10):1012–24.

Groves, P. M., Boyle, R. D., Welker, R. L., and Miller, S. W. (1974). On the mechanism of prepulse inhibition. *Physiol Behav*, 12(3):367–75.

Hahn-Holbrook, J., Holt-Lunstad, J., Holbrook, C., Coyne, S. M., and Lawson, E. T. (2011). Maternal defense breast feeding increases aggression by reducing stress. *Psychological science*.

Haig, D. (1993). Genetic conflicts in human pregnancy. *Q Rev Biol*, 68(4):495–532.

Haig, D. (1994). Refusing the ovarian time bomb. *Trends Genet*, 10(10):346–7; author reply 348–9.

Haig, D. (1996). Placental hormones, genomic imprinting, and maternal—fetal communication. *Journal of Evolutionary Biology*, 9(3):357–380.

Hajnal, A., Smith, G. P., and Norgren, R. (2004). Oral sucrose stimulation increases accumbens dopamine in the rat. *Am J Physiol Regul Integr Comp Physiol*, 286(1):R31–7.

Halbreich, U. (2005). The association between pregnancy processes, preterm delivery, low birth weight, and postpartum depressions—The need for interdisciplinary integration. *American Journal of Obstetrics and Gynecology*, 193(4):1312–1322.

Hales, C. N. and Barker, D. J. (1992). Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia*, 35(7):595–601.

Haller, J., Harold, G., Sandi, C., and Neumann, I. D. (2014). Effects of adverse early-life events on aggression and anti-social behaviours in animals and humans. *J Neuroendocrinol*, 26(10):724–38.

HANDWERGER, S. (1991). CLINICAL COUNTERPOINT: The Physiology of Placental Lactogen in Human Pregnancy*. *Endocrine reviews*, 12(4):329–336.

Hanley, G. E., Brain, U., and Oberlander, T. F. (2013). Infant developmental outcomes following prenatal exposure to antidepressants, and maternal depressed mood and positive affect. *Early human development*. PMID: 23384962.

Harris, B., Lovett, L., Newcombe, R. G., Read, G. F., Walker, R., and Riad-Fahmy, D. (1994). Maternity blues and major endocrine changes: Cardiff puerperal mood and hormone study II. *BMJ*, 308(6934):949–53.

Hashimoto, R., Ohi, K., Yasuda, Y., Fukumoto, M., Yamamori, H., Kamino, K., Morihara, T., Iwase, M., Kazui, H., and Takeda, M. (2013). The KCNH2 gene is associated with neurocognition and the risk of schizophrenia. *World J Biol Psychiatry*, 14(2):114–20.

Hausknecht, K., Haj-Dahmane, S., and Shen, R.-Y. (2013). Prenatal stress exposure increases the excitation of dopamine neurons in the ventral tegmental area and alters their reponses to psychostimulants. *Neuropsychopharmacology*, 38(2):293–301.

Hazell, G. G. J., Hindmarch, C. C., Pope, G. R., Roper, J. A., Lightman, S. L., Murphy, D., O'Carroll, A.-M., and Lolait, S. J. (2012). G protein-coupled receptors in the hypothalamic paraventricular and supraoptic nuclei–serpentine gateways to neuroendocrine homeostasis. *Front Neuroendocrinol*, 33(1):45–66.

Heazell, A. E. P., Bernatavicius, G., Roberts, S. A., Garrod, A., Whitworth, M. K., Johnstone, E. D., Gillham, J. C., and Lavender, T. (2013). A randomised controlled trial comparing standard or intensive management of reduced fetal movements after 36 weeks gestation–a feasibility study. *BMC Pregnancy Childbirth*, 13:95.

Heide, J., Zhang, F., Bigos, K. L., Mann, S. A., Carr, V. J., Shannon Weickert, C., Green, M. J., Weinberger, D. R., and Vandenberg, J. I. (2016). Differential Response to Risperidone in Schizophrenia Patients by KCNH2 Genotype and Drug Metabolizer Status. *Am J Psychiatry*, 173(1):53–9.

Heinrichs, M., von Dawans, B., and Domes, G. (2009). Oxytocin, vasopressin, and human social behavior. *Frontiers in neuroendocrinology*, 30(4):548–557.

Henshaw, C. (2003). Mood disturbance in the early puerperium: a review. *Archives of women's mental health*, 6 Suppl 2:S33–42. PMID: 14615921.

Hess, W. R. and Akert, K. (1955). Experimental data on role of hypothalamus in mechanism of emotional behavior. *AMA Arch Neurol Psychiatry*, 73(2):127–9.

Higham, J. P., Barr, C. S., Hoffman, C. L., Mandalaywala, T. M., Parker, K. J., and Maestripieri, D. (2011). Mu-opioid receptor (OPRM1) variation, oxytocin levels and maternal attachment in free-ranging rhesus macaques Macaca mulatta. *Behav Neurosci*, 125(2):131–6.

Hockly, E., Cordery, P. M., Woodman, B., Mahal, A., van Dellen, A., Blakemore, C., Lewis, C. M., Hannan, A. J., and Bates, G. P. (2002). Environmental enrichment slows disease progression in R6/2 Huntington's disease mice. *Ann Neurol*, 51(2):235–42.

Hoheisel, J. D. (2006). Microarray technology: beyond transcript profiling and genotype analysis. *Nat Rev Genet*, 7(3):200–10.

Hu, D. and Cross, J. C. (2010). Development and function of trophoblast giant cells in the rodent placenta. *Int J Dev Biol*, 54(2-3):341–54.

Huang, D. W., Sherman, B. T., and Lempicki, R. A. (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc*, 4(1):44–57.

Hulshoff Pol, H. E., Hoek, H. W., Susser, E., Brown, A. S., Dingemans, A., Schnack, H. G., van Haren, N. E., Pereira Ramos, L. M., Gispen-de Wied, C. C., and Kahn, R. S. (2000). Prenatal exposure to famine and brain morphology in schizophrenia. *American Journal of Psychiatry*, 157(7):1170–1172.

Humby, T., Cross, E. S., Messer, L., Guerrero, S., and Davies, W. (2016). A pharmacological mouse model suggests a novel risk pathway for postpartum psychosis. *Psychoneuroendocrinology*, 74:363–370.

Hurley, M. J. and Jenner, P. (2006). What has been learnt from study of dopamine receptors in Parkinson's disease? *Pharmacol Ther*, 111(3):715–28.

Hutchison, K. E. and Swift, R. (1999). Effect of d-amphetamine on prepulse inhibition of the startle reflex in humans. *Psychopharmacology (Berl)*, 143(4):394–400.

Imai, S., Tokumoto, M., Fujiwara, Y., Honda, A., Hasegawa, T., Seko, Y., Lee, J.-Y., Nagase, H., and Satoh, M. (2014). Gene expression differences in the duodenum of 129/Sv and DBA/2 mice compared with that of C57BL/6J mice. *J Toxicol Sci*, 39(1):173–7.

Isles, A. and Wilkinson, L. (2011a). *Genomic imprinting effects on brain and behavior: Future directions.*

Isles, A. and Wilkinson, L. (2011b). Genomic Imprinting Effects on Brain and Behavior: Future Directions. In Petronis, A. and Mill, J., editors, *Brain, Behavior and Epigenetics*, Epigenetics and Human Health, pages 169–184. Springer Berlin Heidelberg.

Isles, A. R. and Holland, A. J. (2005). Imprinted genes and mother-offspring interactions. *Early Hum Dev*, 81(1):73–7.

Jahn, O., Tenzer, S., and Werner, H. B. (2009). Myelin proteomics: molecular anatomy of an insulating sheath. *Mol Neurobiol*, 40(1):55–72.

Janssen, A. B., Capron, L. E., O'Donnell, K., Tunster, S. J., Heazell, A. E. P., Ramchandani, P. G., Glover, V., and John, R. M. (2015). Prenatal maternal depression and aberrant placental imprinting. *Psychoneuroendocrinology*, 61:4.

Janssen, A. B., Kertes, D. A., McNamara, G. I., Braithwaite, E. C., Creeth, H. D. J., Glover, V. I., and John, R. M. (2016). A role for the placenta in programming maternal mood and childhood behavioural disorders. *J Neuroendocrinol*.

Jauniaux, E., Van Oppenraaij, R. H. F., and Burton, G. J. (2010). Obstetric outcome after early placental complications. *Curr Opin Obstet Gynecol*, 22(6):452–7.

Jelinic, P. and Shaw, P. (2007). Loss of imprinting and cancer. *The Journal of pathology*, 211(3):261–268. PMID: 17177177.

Jensen Peña, C., Monk, C., and Champagne, F. A. (2012). Epigenetic effects of prenatal stress on 11Îš-hydroxysteroid dehydrogenase-2 in the placenta and fetal brain. *PLoS One*, 7(6):e39791.

Johansson, C. B., Momma, S., Clarke, D. L., Risling, M., Lendahl, U., and Frisén, J. (1999). Identification of a neural stem cell in the adult mammalian central nervous system. *Cell*, 96(1):25–34.

John, R. and Hemberger, M. (2012). A placenta for life. *Reproductive BioMedicine Online*, 25(1):5–11.

John, R. M. (2013). Epigenetic regulation of placental endocrine lineages and complications of pregnancy. *Biochem Soc Trans*, 41(3):701–9.

John, R. M. and Surani, M. A. (2000). Genomic imprinting, mammalian evolution, and the mystery of egg-laying mammals. *Cell*, 101(6):585–8.

Jones, I. (2007). Psychiatric Disorders and Pregnancy. *The British Journal of Psychiatry*, 190(2):184–185.

Jones, I. and Craddock, N. (2001). Familiality of the puerperal trigger in bipolar disorder: results of a family study. *Am J Psychiatry*, 158(6):913–7.

Kajii, T. and Ohama, K. (1977). Androgenetic origin of hydatidiform mole. *Nature*, 268(5621):633–4.

Kaminsky, Z., Jones, I., Verma, R., Saleh, L., Trivedi, H., Guintivano, J., Akman, R., Zandi, P., Lee, R. S., and Potash, J. B. (2015). DNA methylation and expression of KCNQ3 in bipolar disorder. *Bipolar Disord*, 17(2):150–9.

Kanellopoulos-Langevin, C., Caucheteux, S. M., Verbeke, P., and Ojcius, D. M. (2003). Tolerance of the fetus by the maternal immune system: role of inflammatory mediators at the feto-maternal interface. *Reproductive Biology and Endocrinology*, 1(1):1.

Kawase, T., Ohki, R., Shibata, T., Tsutsumi, S., Kamimura, N., Inazawa, J., Ohta, T., Ichikawa, H., Aburatani, H., Tashiro, F., and Taya, Y. (2009). PH domain-only protein PHLDA3 is a p53-regulated repressor of Akt. *Cell*, 136(3):535–50.

Kee, N., Sivalingam, S., Boonstra, R., and Wojtowicz, J. (2002). The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis. *Journal of neuroscience methods*, 115(1):97–105.

Kempermann, G. and Gage, F. H. (1999). New nerve cells for the adult brain. *Sci Am*, 280(5):48–53.

Kempermann, G. and Gage, F. H. (2000). Neurogenesis in the adult hippocampus. *Novartis Found Symp*, 231:220–35; discussion 235–41, 302–6.

Kendler, K. S., Bulik, C. M., Silberg, J., Hettema, J. M., Myers, J., and Prescott, C. A. (2000). Childhood sexual abuse and adult psychiatric and substance use disorders in women: an epidemiological and cotwin control analysis. *Archives of general psychiatry*, 57(10):953–959.

Kendrick, K. M. (2000). Oxytocin, motherhood and bonding. *Exp Physiol*, 85 Spec No:111S–124S.

Kent, L., Bowdin, S., Kirby, G. A., Cooper, W. N., and Maher, E. R. (2008). Beckwith Weidemann syndrome: a behavioral phenotype-genotype study. *Am J Med Genet B Neuropsychiatr Genet*, 147B(7):1295–7.

Kersting, A., Dorsch, M., Wesselmann, U., Lüdorff, K., Witthaut, J., Ohrmann, P., Hörnig-Franz, I., Klockenbusch, W., Harms, E., and Arolt, V. (2004). Maternal posttraumatic stress response after the birth of a very low-birth-weight infant. *Journal of psychosomatic research*, 57(5):473–476.

Keverne, E. B. (1997). Genomic imprinting in the brain. *Curr Opin Neurobiol*, 7(4):463–8.

Keverne, E. B. (2010). Epigenetically regulated imprinted genes and foetal programming. *Neurotox Res*, 18(3-4):386–92.

Keverne, E. B. and Kendrick, K. M. (1994). Maternal behaviour in sheep and its neuroendocrine regulation. *Acta Paediatr Suppl*, 397:47–56.

Kim, H., Toyofuku, Y., Lynn, F. C., Chak, E., Uchida, T., Mizukami, H., Fujitani, Y., Kawamori, R., Miyatsuka, T., Kosaka, Y., Yang, K., Honig, G., van der Hart, M., Kishimoto, N., Wang, J., Yagihashi, S., Tecott, L. H., Watada, H., and German, M. S. (2010). Serotonin regulates pancreatic beta cell mass during pregnancy. *Nature Medicine*, 16(7):804–808.

Kinoshita, T., Yadegari, R., Harada, J. J., Goldberg, R. B., and Fischer, R. L. (1999). Imprinting of the MEDEA polycomb gene in the Arabidopsis endosperm. *Plant Cell*, 11(10):1945–52.

Kinsley, C. H., Meyer, E., and Rafferty, K. A. (2012). Sex Steroid Hormone Determination of the Maternal Brain: Effects Beyond Reproduction. *Mini-Reviews in Medicinal Chemistry*, 12(11):1063–1070. WOS:000309057700005.

Kinsley, C. H., Trainer, R., Stafisso-Sandoz, G., Quadros, P., Marcus, L. K., Hearon, C., Meyer, E. A. A., Hester, N., Morgan, M., Kozub, F. J., and Lambert, K. G. (2006). Motherhood and the hormones of pregnancy modify concentrations of hippocampal neuronal dendritic spines. *Hormones and Behavior*, 49(2):131–142.

Kirschenbaum, B., Doetsch, F., Lois, C., and Alvarez-Buylla, A. (1999). Adult subventricular zone neuronal precursors continue to proliferate and migrate in the absence of the olfactory bulb. *J Neurosci*, 19(6):2171–80.

Klampfl, S. M., Neumann, I. D., and Bosch, O. J. (2013). Reduced brain corticotropin-releasing factor receptor activation is required for adequate maternal care and maternal aggression in lactating rats. *Eur J Neurosci*, 38(5):2742–50.

Klempin, F., Kronenberg, G., Cheung, G., Kettenmann, H., and Kempermann, G. (2011). Properties of doublecortin-(DCX)-expressing cells in the piriform cortex compared to the neurogenic dentate gyrus of adult mice. *PLoS One*, 6(10):e25760.

Knox, K., Leuenberger, D., Penn, A. A., and Baker, J. C. (2011). Global hormone profiling of murine placenta reveals Secretin expression. *Placenta*, 32(11):811–6.

Kokay, I. C., Bull, P. M., Davis, R. L., Ludwig, M., and Grattan, D. R. (2006). Expression of the long form of the prolactin receptor in magnocellular oxytocin neurons is associated with specific prolactin regulation of oxytocin neurons. *Am J Physiol Regul Integr Comp Physiol*, 290(5):R1216–25.

Koketsu, D., Mikami, A., Miyamoto, Y., and Hisatsune, T. (2003). Nonrenewal of neurons in the cerebral neocortex of adult macaque monkeys. *J Neurosci*, 23(3):937–42.

Komada, M., Takao, K., and Miyakawa, T. (2008). Elevated Plus Maze for Mice. *Journal of Visualized Experiments*, (22).

Kompagne, H., Bárdos, G., Szénási, G., Gacsályi, I., Hársing, L. G., and Lévay, G. (2008). Chronic mild stress generates clear depressive but ambiguous anxiety-like behaviour in rats. *Behav Brain Res*, 193(2):311–4.

Kornack, D. R. and Rakic, P. (2001). The generation, migration, and differentiation of olfactory neurons in the adult primate brain. *Proc Natl Acad Sci U S A*, 98(8):4752–7.

Kristal, M. B. (2009). The biopsychology of maternal behavior in nonhuman mammals. *ILAR J*, 50(1):51–63.

Kuhn, H. G., Winkler, J., Kempermann, G., Thal, L. J., and Gage, F. H. (1997). Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. *J Neurosci*, 17(15):5820–9.

Kulkarni, S. K., Singh, K., and Bishnoi, M. (2007). Elevated zero maze: a paradigm to evaluate antianxiety effects of drugs. *Methods Find Exp Clin Pharmacol*, 29(5):343–8.

Kumar, P. and Magon, N. (2012). Hormones in pregnancy. *Niger Med J*, 53(4):179–83.

Kundakovic, M. and Champagne, F. A. (2015). Early-life experience, epigenetics, and the developing brain. *Neuropsychopharmacology*, 40(1):141–53.

Langenheim, J. F., Tan, D., Walker, A. M., and Chen, W. Y. (2006). Two wrongs can make a right: dimers of prolactin and growth hormone receptor antagonists behave as agonists. *Mol Endocrinol*, 20(3):661–74.

Larsen, C. and Grattan, D. (2012). Prolactin, neurogenesis, and maternal behaviors. *Brain, Behavior, and Immunity*, 26(2):201–209.

Larsen, C. M. and Grattan, D. R. (2010). Prolactin-Induced Mitogenesis in the Subventricular Zone of the Maternal Brain during Early Pregnancy Is Essential for Normal Postpartum Behavioral Responses in the Mother. *Endocrinology*, 151(8):3805–3814.

Larsen, C. M., Kokay, I. C., and Grattan, D. R. (2008). Male pheromones initiate prolactin-induced neurogenesis and advance maternal behavior in female mice. *Hormones and Behavior*, 53(4):509–517.

Lee, Y. and Voogt, J. L. (1999). Feedback effects of placental lactogens on prolactin levels and Fos-related antigen immunoreactivity of tuberoinfundibular dopaminergic neurons in the arcuate nucleus during pregnancy in the rat. *Endocrinology*, 140(5):2159–66.

Lehmann, J., Pryce, C. R., Bettschen, D., and Feldon, J. (1999). The maternal separation paradigm and adult emotionality and cognition in male and female Wistar rats. *Pharmacol Biochem Behav*, 64(4):705–15.

Leiser, R. and Dantzer, V. (1994). Initial vascularisation in the pig placenta: II. Demonstration of gland and areola-gland subunits by histology and corrosion casts. *The Anatomical Record*, 238(3):326–334.

Lewis, D. A. and Lieberman, J. A. (2000). Catching up on schizophrenia: natural history and neurobiology. *Neuron*, 28(2):325–34.

Lewis, K., Li, C., Perrin, M. H., Blount, A., Kunitake, K., Donaldson, C., Vaughan, J., Reyes, T. M., Gulyas, J., Fischer, W., Bilezikjian, L., Rivier, J., Sawchenko, P. E., and Vale, W. W. (2001). Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. *Proc Natl Acad Sci U S A*, 98(13):7570–5.

Li, E., Beard, C., Forster, A., Bestor, T., and Jaenisch, R. (1993). DNA methylation, genomic imprinting, and mammalian development. In *Cold Spring Harbor Symposia on Quantitative Biology*, volume 58, pages 297–305. Cold Spring Harbor Laboratory Press.

Li, L., Keverne, E. B., Aparicio, S. A., Ishino, F., Barton, S. C., and Surani, M. A. (1999). Regulation of maternal behavior and offspring growth by paternally expressed Peg3. *Science*, 284(5412):330–3.

Li, T., Vu, T. H., Lee, K.-O., Yang, Y., Nguyen, C. V., Bui, H. Q., Zeng, Z.-L., Nguyen, B. T., Hu, J.-F., Murphy, S. K., Jirtle, R. L., and Hoffman, A. R. (2002). An imprinted PEG1/MEST antisense expressed predominantly in human testis and in mature spermatozoa. *The Journal of biological chemistry*, 277(16):13518–13527. PMID: 11821432.

Lieberwirth, C., Liu, Y., Jia, X., and Wang, Z. (2012). Social isolation impairs adult neurogenesis in the limbic system and alters behaviors in female prairie voles. *Horm Behav*, 62(4):357–66.

Lin, X. B., Pierce, D. R., Light, K. E., and Hayar, A. (2013). The fine temporal structure of the rat licking pattern: what causes the variabiliy in the interlick intervals and how is it affected by the drinking solution? *Chem Senses*, 38(8):685–704.

Lindblom, R., Ververis, K., Tortorella, S. M., and Karagiannis, T. C. (2015). The early life origin theory in the development of cardiovascular disease and type 2 diabetes. *Mol Biol Rep*, 42(4):791–7.

Linzer, D. I. and Fisher, S. J. (1999). The placenta and the prolactin family of hormones: regulation of the physiology of pregnancy. *Mol Endocrinol*, 13(6):837–40.

Lisk, R. D., Pretlow, 3rd, R. A., and Friedman, S. M. (1969). Hormonal stimulation necessary for elicitation of maternal nest-building in the mouse (Mus musculus). *Anim Behav*, 17(4):730–7.

Liu, Y., Murphy, S. K., Murtha, A. P., Fuemmeler, B. F., Schildkraut, J., Huang, Z., Overcash, F., Kurtzberg, J., Jirtle, R., Iversen, E. S., Forman, M. R., and Hoyo, C. (2012). Depression in pregnancy, infant birth weight and DNA methylation of imprint regulatory elements. *Epigenetics*, 7(7):735–746. WOS:000306291900010.

Lombet, A., Planque, N., Bleau, A.-M., Li, C. L., and Perbal, B. (2003). CCN3 and calcium signaling. *Cell Commun Signal*, 1(1):1.

López-Fontana, C. M., Maselli, M. E., Salicioni, A. M., and Carón, R. W. (2012). The inhibitory effect of progesterone on lactogenesis during pregnancy is already evident by mid- to late gestation in rodents. *Reprod Fertil Dev*, 24(5):704–14.

Loret de Mola, C., Araújo de França, G. V., Quevedo, L. d. A., and Horta, B. L. (2014). Low birth weight, preterm birth and small for gestational age association with adult depression: systematic review and meta-analysis. *Br J Psychiatry*, 205(5):340–347.

Lucas, B. K., Ormandy, C. J., Binart, N., Bridges, R. S., and Kelly, P. A. (1998). Null mutation of the prolactin receptor gene produces a defect in maternal behavior. *Endocrinology*, 139(10):4102–4107. PMID: 9751488.

Lucassen, P. J., Meerlo, P., Naylor, A. S., van Dam, A. M., Dayer, A. G., Fuchs, E., Oomen, C. A., and Czéh, B. (2010). Regulation of adult neurogenesis by stress, sleep disruption, exercise and inflammation: Implications for depression and antidepressant action. *Eur Neuropsychopharmacol*, 20(1):1–17.

Lydall, E. S., Gilmour, G., and Dwyer, D. M. (2010). Analysis of licking microstructure provides no evidence for a reduction in reward value following acute or sub-chronic phencyclidine administration. *Psychopharmacology (Berl)*, 209(2):153–62.

Ma, S., Shipston, M. J., Morilak, D., and Russell, J. A. (2005). Reduced hypothalamic vasopressin secretion underlies attenuated adrenocorticotropin stress responses in pregnant rats. *Endocrinology*, 146(3):1626–37.

Macias, H. and Hinck, L. (2012). Mammary gland development. *Wiley Interdiscip Rev Dev Biol*, 1(4):533–57.

Madras, B. K., Miller, G. M., and Fischman, A. J. (2005). The dopamine transporter and attention-deficit/hyperactivity disorder. *Biol Psychiatry*, 57(11):1397–409.

Magavi, S. S., Leavitt, B. R., and Macklis, J. D. (2000). Induction of neurogenesis in the neocortex of adult mice. *Nature*, 405(6789):951–5.

Mairesse, J., Lesage, J., Breton, C., Bréant, B., Hahn, T., Darnaudéry, M., Dickson, S. L., Seckl, J., Blondeau, B., Vieau, D., Maccari, S., and Viltart, O. (2007). Maternal stress alters endocrine function of the feto-placental unit in rats. *Am J Physiol Endocrinol Metab*, 292(6):E1526–33.

Malassiné, A., Frendo, J. L., and Evain-Brion, D. (2003). A comparison of placental development and endocrine functions between the human and mouse model. *Hum Reprod Update*, 9(6):531–9.

Malberg, J. E. and Duman, R. S. (2003). Cell proliferation in adult hippocampus is decreased by inescapable stress: reversal by fluoxetine treatment. *Neuropsychopharmacology*, 28(9):1562–71.

Mancini-Dinardo, D., Steele, S. J. S., Levorse, J. M., Ingram, R. S., and Tilghman, S. M. (2006). Elongation of the Kcnq1ot1 transcript is required for genomic imprinting of neighboring genes. *Genes Dev*, 20(10):1268–82.

Mann, J. R. and Lovell-Badge, R. H. (1984). Inviability of parthenogenones is determined by pronuclei, not egg cytoplasm. *Nature*, 310(5972):66–7.

Marsit, C. J., Lambertini, L., Maccani, M. A., Koestler, D. C., Houseman, E. A., Padbury, J. F., Lester, B. M., and Chen, J. (2012). Placenta-imprinted gene expression association of infant neurobehavior. *J Pediatr*, 160(5):854–860.e2.

Mastorakos, G. and Ilias, I. (2003). Maternal and fetal hypothalamic-pituitary-adrenal axes during pregnancy and postpartum. *Ann N Y Acad Sci*, 997:136–49.

Matthes, H. W., Maldonado, R., Simonin, F., Valverde, O., Slowe, S., Kitchen, I., Befort, K., Dierich, A., Le Meur, M., Dollé, P., Tzavara, E., Hanoune, J., Roques, B. P., and Kieffer, B. L. (1996). Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. *Nature*, 383(6603):819–23.

Matthey, S., Barnett, B., Howie, P., and Kavanagh, D. J. (2003). Diagnosing postpartum depression in mothers and fathers: whatever happened to anxiety? *J Affect Disord*, 74(2):139–47.

McArthur, R. and Borsini, F. (2006). Animal models of depression in drug discovery: a historical perspective. *Pharmacol Biochem Behav*, 84(3):436–52.

McCormick, M. C., Gortmaker, S. L., and Sobol, A. M. (1990). Very low birth weight children: Behavior problems and school difficulty in a national sample. *The Journal of pediatrics*, 117(5):687–693.

McEwen, B. S. (2008). Understanding the potency of stressful early life experiences on brain and body function. *Metabolism*, 57 Suppl 2:S11–5.

McNamara, G. I. and Isles, A. R. (2013). Dosage-sensitivity of imprinted genes expressed in the brain: 15q11-q13 and neuropsychiatric illness. *Biochem Soc Trans*, 41(3):721–6.

McNamara, G. I. and Isles, A. R. (2014). Influencing the social group: the role of imprinted genes. *Adv Genet*, 86:107–34.

Meaney, M. J. (2001). Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. *Annu Rev Neurosci*, 24:1161–92.

Meaney, M. J. and Szyf, M. (2005). Maternal care as a model for experience-dependent chromatin plasticity? *Trends Neurosci*, 28(9):456–63.

Meek, L. R., Dittel, P. L., Sheehan, M. C., Chan, J. Y., and Kjolhaug, S. R. (2001). Effects of stress during pregnancy on maternal behavior in mice. *Physiol Behav*, 72(4):473–9.

Melo, A. I., Pérez-Ledezma, M., Clapp, C., Arnold, E., Rivera, J. C., and Fleming, A. S. (2009). Effects of prolactin deficiency during the early postnatal period on the development of maternal behavior in female rats: mother's milk makes the difference. *Horm Behav*, 56(3):281–91.

Meredith, M. (1991). Sensory processing in the main and accessory olfactory systems: comparisons and contrasts. *J Steroid Biochem Mol Biol*, 39(4B):601–14.

Mikaelsson, M. A., Constância, M., Dent, C. L., Wilkinson, L. S., and Humby, T. (2013). Placental programming of anxiety in adulthood revealed by Igf2-null models. *Nat Commun*, 4:2311.

Mill, J. and Petronis, A. (2008). Pre- and peri-natal environmental risks for attention-deficit hyperactivity disorder (ADHD): the potential role of epigenetic processes in mediating susceptibility. *J Child Psychol Psychiatry*, 49(10):1020–30.

Miller, R. L., Pallant, J. F., and Negri, L. M. (2006). Anxiety and stress in the postpartum: is there more to postnatal distress than depression? *BMC Psychiatry*, 6:12.

Millstein, R. A. and Holmes, A. (2007). Effects of repeated maternal separation on anxiety- and depression-related phenotypes in different mouse strains. *Neurosci Biobehav Rev*, 31(1):3–17.

Ming, G.-l. and Song, H. (2005). Adult neurogenesis in the mammalian central nervous system. *Annu Rev Neurosci*, 28:223–50.

Miozzo, M. and Simoni, G. (2002). The role of imprinted genes in fetal growth. *Biol Neonate*, 81(4):217–28.

Miyamoto, T., Hasuike, S., Jinno, Y., Soejima, H., Yun, K., Miura, K., Ishikawa, M., and Niikawa, N. (2002). The human ASCL2 gene escaping genomic imprinting and its expression pattern. *J Assist Reprod Genet*, 19(5):240–4.

Modney, B. K., Yang, Q. Z., and Hatton, G. I. (1990). Activation of excitatory amino acid inputs to supraoptic neurons. II. Increased dye-coupling in maternally behaving virgin rats. *Brain Res*, 513(2):270–3.

Moles, A., Kieffer, B. L., and D'Amato, F. R. (2004). Deficit in attachment behavior in mice lacking the mu-opioid receptor gene. *Science*, 304(5679):1983–6.

Moll, W. (1985). Physiological aspects of placental ontogeny and phylogeny. *Placenta*, 6(2):141–54.

Monk, C., Spicer, J., and Champagne, F. A. (2012). Linking prenatal maternal adversity to developmental outcomes in infants: The role of epigenetic pathways. *Development and Psychopathology*, 24(4):1361–1376. WOS:000309925800016.

Moore, G. E., Ishida, M., Demetriou, C., Al-Olabi, L., Leon, L. J., Thomas, A. C., Abu-Amero, S., Frost, J. M., Stafford, J. L., Chaoqun, Y., Duncan, A. J., Baigel, R., Brimioulle, M., Iglesias-Platas, I., Apostolidou, S., Aggarwal, R., Whittaker, J. C., Syngelaki, A., Nicolaides, K. H., Regan, L., Monk, D., and Stanier, P. (2015). The role and interaction of imprinted genes in human fetal growth. *Philos Trans R Soc Lond B Biol Sci*, 370(1663):20140074.

Moore, T. (2001). Genetic conflict, genomic imprinting and establishment of the epigenotype in relation to growth. *Reproduction*, 122(2):185–93.

Moore, T. and Dveksler, G. S. (2014). Pregnancy-specific glycoproteins: complex gene families regulating maternal-fetal interactions. *Int J Dev Biol*, 58(2-4):273–80.

Moore, T. and Haig, D. (1991). Genomic imprinting in mammalian development: a parental tug-of-war. *Trends Genet*, 7(2):45–9.

Morag, A., Pasmanik-Chor, M., Oron-Karni, V., Rehavi, M., Stingl, J. C., and Gurwitz, D. (2011). Genome-wide expression profiling of human lymphoblastoid cell lines identifies CHL1 as a putative SSRI antidepressant response biomarker. *Pharmacogenomics*, 12(2):171–84.

Morey, J. S., Ryan, J. C., and Van Dolah, F. M. (2006). Microarray validation: factors influencing correlation between oligonucleotide microarrays and real-time PCR. *Biol Proced Online*, 8:175–93.

Mueller, B. R. and Bale, T. L. (2008). Sex-specific programming of offspring emotionality after stress early in pregnancy. *J Neurosci*, 28(36):9055–65.

Munk-Olsen, T., Laursen, T. M., Meltzer-Brody, S., Mortensen, P. B., and Jones, I. (2012). Psychiatric disorders with postpartum onset: possible early manifestations of bipolar affective disorders. *Archives of general psychiatry*, 69(4):428–434. PMID: 22147807.

Murray, L. (1992). The Impact of Postnatal Depression on Infant Development. *Journal of Child Psychology and Psychiatry*, 33(3):543–561.

Musters, C., McDonald, E., and Jones, I. (2008). Management of postnatal depression. *BMJ*, 337(aug08 1):a736–a736.

Nemeroff, C. B. (2002). Recent advances in the neurobiology of depression. *Psychopharmacol Bull*, 36 Suppl 2:6–23.

Nephew, B. and Murgatroyd, C. (2013). The role of maternal care in shaping CNS function. *Neuropeptides*, 47(6):371–8.

Newbern, D. and Freemark, M. (2011). Placental hormones and the control of maternal metabolism and fetal growth. *Curr Opin Endocrinol Diabetes Obes*, 18(6):409–16.

Nishi, M., Horii-Hayashi, N., and Sasagawa, T. (2014). Effects of early life adverse experiences on the brain: implications from maternal separation models in rodents. *Frontiers in neuroscience*, 8:166.

Numan, M. (1994). Maternal behavior. *The physiology of reproduction*, 2:221–302.

Numan, M. (2006). Hypothalamic neural circuits regulating maternal responsiveness toward infants. *Behav Cogn Neurosci Rev*, 5(4):163–90.

Numan, M., Numan, M. J., Schwarz, J. M., Neuner, C. M., Flood, T. F., and Smith, C. D. (2005). Medial preoptic area interactions with the nucleus accumbens-ventral pallidum circuit and maternal behavior in rats. *Behav Brain Res*, 158(1):53–68.

Nutt, J. G., Carter, J. H., and Sexton, G. J. (2004). The dopamine transporter: importance in Parkinson's disease. *Ann Neurol*, 55(6):766–73.

O'Donnell, K., O'Connor, T. G., and Glover, V. (2009). Prenatal stress and neurodevelopment of the child: focus on the HPA axis and role of the placenta. *Dev Neurosci*, 31(4):285–92.

O'Donnell, K. J., Bugge Jensen, A., Freeman, L., Khalife, N., O'Connor, T. G., and Glover, V. (2012). Maternal prenatal anxiety and downregulation of placental 11Îš-HSD2. *Psychoneuroendocrinology*, 37(6):818–26.

O'Hara, M. W. and Wisner, K. L. (2014). Perinatal mental illness: definition, description and aetiology. *Best Pract Res Clin Obstet Gynaecol*, 28(1):3–12.

O'Keefe, J. and Nadel, L. (1978). *The hippocampus as a cognitive map*. Clarendon Press.

Okuda, H., Tatsumi, K., Makinodan, M., Yamauchi, T., Kishimoto, T., and Wanaka, A. (2009). Environmental enrichment stimulates progenitor cell proliferation in the amygdala. *J Neurosci Res*, 87(16):3546–53.

Olivier, J. D. A., Åkerud, H., Skalkidou, A., Kaihola, H., and Sundström-Poromaa, I. (2014). The effects of antenatal depression and antidepressant treatment on placental gene expression. *Front Cell Neurosci*, 8:465.

Ono, R., Kobayashi, S., Wagatsuma, H., Aisaka, K., Kohda, T., Kaneko-Ishino, T., and Ishino, F. (2001). A retrotransposon-derived gene, PEG10, is a novel imprinted gene located on human chromosome 7q21. *Genomics*, 73(2):232–7.

Otto, S. P. and Goldstein, D. B. (1992). Recombination and the evolution of diploidy. *Genetics*, 131(3):745–51.

Paksarian, D., Eaton, W. W., Mortensen, P., Merikangas, K., and Pedersen, C. (2015). A population-based study of the risk of schizophrenia and bipolar disorder associated with parent–child separation during development. *Psychological medicine*, 45(13):2825–2837.

Palazzo, A. F. and Gregory, T. R. (2014). The case for junk DNA. *PLoS Genet*, 10(5):e1004351.

Pan, Y.-W., Wang, W., and Xia, Z. (2013). Assessment of adult neurogenesis in mice. *Curr Protoc Toxicol*, Chapter 12:Unit12.20.

Patten, M. M., Ross, L., Curley, J. P., Queller, D. C., Bonduriansky, R., and Wolf, J. B. (2014). The evolution of genomic imprinting: theories, predictions and empirical tests. *Heredity (Edinb)*, 113(2):119–28.

Pearlstein, T., Howard, M., Salisbury, A., and Zlotnick, C. (2009). Postpartum depression. *Am J Obstet Gynecol*, 200(4):357–64.

Peciña, S., Cagniard, B., Berridge, K. C., Aldridge, J. W., and Zhuang, X. (2003). Hyperdopaminergic mutant mice have higher "wanting" but not "liking" for sweet rewards. *J Neurosci*, 23(28):9395–402.

Pedersen, C. A., Caldwell, J. D., McGuire, M., and Evans, D. L. (1991). Corticotropin-releasing hormone inhibits maternal behavior and induces pup-killing. *Life Sci*, 48(16):1537–46.

Pelcovitz, D., Kaplan, S., Goldenberg, B., Mandel, F., Lehane, J., and Guarrera, J. (1994). Post-traumatic stress disorder in physically abused adolescents. *Journal of the American Academy of Child & Adolescent Psychiatry*, 33(3):305–312.

Pencea, V., Bingaman, K. D., Freedman, L. J., and Luskin, M. B. (2001). Neurogenesis in the subventricular zone and rostral migratory stream of the neonatal and adult primate forebrain. *Exp Neurol*, 172(1):1–16.

Perani, C. V. and Slattery, D. A. (2014). Using animal models to study post-partum psychiatric disorders. *Br J Pharmacol*, 171(20):4539–55.

Perry, J. (1981). The mammalian fetal membranes. *Journal of Reproduction and Fertility*, 62(2):321–335.

Peters, J. (2014). The role of genomic imprinting in biology and disease: an expanding view. *Nat Rev Genet*, 15(8):517–30.

Pi, X., Zhang, B., Li, J., and Voogt, J. L. (2003). Promoter usage and estrogen regulation of prolactin receptor gene in the brain of the female rat. *Neuroendocrinology*, 77(3):187–197.

Pietropaolo, S. (2010). Mood and Anxiety-related Phenotypes in Mice: Characterization Using Behavioral Tests - Edited by T. D. Gould. *Genes, Brain and Behavior*, 9(5):544–544.

Plante, I., Stewart, M. K. G., and Laird, D. W. (2011). Evaluation of mammary gland development and function in mouse models. *J Vis Exp*, (53).

Powell, S. B., Zhou, X., and Geyer, M. A. (2009). Prepulse inhibition and genetic mouse models of schizophrenia. *Behav Brain Res*, 204(2):282–94.

Power, M. (2012). *The evolution of the human placenta*. Johns Hopkins University Press, Baltimore.

Pryce, C. (1992). A comparative systems model of the regulation of maternal motivation in mammals. *Animal Behaviour*, 43(3):417–441.

Qian, N., Frank, D., O'Keefe, D., Dao, D., Zhao, L., Yuan, L., Wang, Q., Keating, M., Walsh, C., and Tycko, B. (1997). The IPL gene on chromosome 11p15.5 is imprinted in humans and mice and is similar to TDAG51, implicated in Fas expression and apoptosis. *Hum Mol Genet*, 6(12):2021–9.

Rai, A. and Cross, J. C. (2014). Development of the hemochorial maternal vascular spaces in the placenta through endothelial and vasculogenic mimicry. *Dev Biol*, 387(2):131–41.

Rai, R., Cohen, H., Dave, M., and Regan, L. (1997). Randomised controlled trial of aspirin and aspirin plus heparin in pregnant women with recurrent miscarriage associated with phospholipid antibodies (or antiphospholipid antibodies). *BMJ*, 314(7076):253–7.

Räikkönen, K., Pesonen, A.-K., Heinonen, K., Kajantie, E., Hovi, P., Järvenpää, A.-L., Eriksson, J. G., and Andersson, S. (2008). Depression in young adults with very low birth weight: the Helsinki study of very low-birth-weight adults. *Archives of General Psychiatry*, 65(3):290–296.

Rainier, S., Johnson, L. A., Dobry, C. J., Ping, A. J., Grundy, P. E., and Feinberg, A. P. (1993). Relaxation of imprinted genes in human cancer.

Ralph, R. J., Paulus, M. P., Fumagalli, F., Caron, M. G., and Geyer, M. A. (2001). Prepulse inhibition deficits and perseverative motor patterns in dopamine transporter knock-out mice: differential effects of D1 and D2 receptor antagonists. *J Neurosci*, 21(1):305–13.

Ramos, E., Castilla, A., Navarro, N., Monasterio, N., Mena, F., and Morales, T. (2008). Suckling-induced oxytocin increase in the spinal cord of the rat. *Brain Res*, 1236:85–92.

Rao, M. S. and Shetty, A. K. (2004). Efficacy of doublecortin as a marker to analyse the absolute number anddendritic growth of newly generated neurons in the adult dentate gyrus. *European Journal of Neuroscience*, 19(2):234–246.

Rasika, S., Nottebohm, F., and Alvarez-Buylla, A. (1994). Testosterone increases the recruitment and/or survival of new high vocal center neurons in adult female canaries. *Proc Natl Acad Sci U S A*, 91(17):7854–8.

Reilly, J. J., Armstrong, J., Dorosty, A. R., Emmett, P. M., Ness, A., Rogers, I., Steer, C., and Sherriff, A. (2005). Early life risk factors for obesity in childhood: cohort study. *Bmj*, 330(7504):1357.

Reyes, T. M., Lewis, K., Perrin, M. H., Kunitake, K. S., Vaughan, J., Arias, C. A., Hogenesch, J. B., Gulyas, J., Rivier, J., Vale, W. W., and Sawchenko, P. E. (2001). Urocortin II: a member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. *Proc Natl Acad Sci U S A*, 98(5):2843–8.

Rheingold, H. L. and Eckerman, C. O. (1970). The infant separates himself from his mother. *Science*, 168(3927):78–83.

Rheingold, H. L. *et al.* (1963). Maternal behavior in mammals. *Maternal behavior in mammals.*

Rice, F., Jones, I., and Thapar, A. (2007). The impact of gestational stress and prenatal growth on emotional problems in offspring: a review. *Acta Psychiatrica Scandinavica*, 115(3):171–183.

Riley, D. M. and Watt, D. C. (1985). Hypercalcemia in the etiology of puerperal psychosis. *Biol Psychiatry*, 20(5):479–88.

Robertson, G. L. (1983). Thirst and vasopressin function in normal and disordered states of water balance. *The Journal of laboratory and clinical medicine*, 101(3):351–371.

Rochefort, C., Gheusi, G., Vincent, J.-D., and Lledo, P.-M. (2002). Enriched odor exposure increases the number of newborn neurons in the adult olfactory bulb and improves odor memory. *The Journal of neuroscience*, 22(7):2679–2689.

Rolls, A., Shechter, R., London, A., Ziv, Y., Ronen, A., Levy, R., and Schwartz, M. (2007). Toll-like receptors modulate adult hippocampal neurogenesis. *Nat Cell Biol*, 9(9):1081–8.

Rosenfeld, C. S. (2015). Sex-Specific Placental Responses in Fetal Development. *Endocrinology*, 156(10):3422–34.

Rossant, J. and Cross, J. C. (2001). Placental development: lessons from mouse mutants. *Nat Rev Genet*, 2(7):538–48.

Rosser, A. E., Remfry, C. J., and Keverne, E. B. (1989). Restricted exposure of mice to primer pheromones coincident with prolactin surges blocks pregnancy by changing hypothalamic dopamine release. *J Reprod Fertil*, 87(2):553–9.

Rougier, N. and Werb, Z. (2001). Minireview: Parthenogenesis in mammals. *Mol Reprod Dev*, 59(4):468–74.

Russell, J. A., Leng, G., and Douglas, A. J. (2003). The magnocellular oxytocin system, the fount of maternity: adaptations in pregnancy. *Frontiers in neuroendocrinology*, 24(1):27–61.

Rygula, R., Abumaria, N., Flügge, G., Fuchs, E., Rüther, E., and Havemann-Reinecke, U. (2005). Anhedonia and motivational deficits in rats: impact of chronic social stress. *Behav Brain Res*, 162(1):127–34.

Salas, M., John, R., Saxena, A., Barton, S., Frank, D., Fitzpatrick, G., Higgins, M. J., and Tycko, B. (2004). Placental growth retardation due to loss of imprinting of Phlda2. *Mech Dev*, 121(10):1199–210.

Saltzman, W. and Maestripieri, D. (2011). The neuroendocrinology of primate maternal behavior. *Prog Neuropsychopharmacol Biol Psychiatry*, 35(5):1192–204.

Samaan, N., Yen, S. C., Friesen, H., and Pearson, O. H. (1966). Serum placental lactogen levels during pregnancy and in trophoblastic disease. *J Clin Endocrinol Metab*, 26(12):1303–8.

Sandeman, R. and Sandeman, D. (2000). "Impoverished" and "enriched" living conditions influence the proliferation and survival of neurons in crayfish brain. *J Neurobiol*, 45(4):215–26.

Sandman, C. A., Davis, E. P., Buss, C., and Glynn, L. M. (2011). Exposure to prenatal psychobiological stress exerts programming influences on the mother and her fetus. *Neuroendocrinology*, 95(1):8–21.

Schaller, F., Watrin, F., Sturny, R., Massacrier, A., Szepetowski, P., and Muscatelli, F. (2010). A single postnatal injection of oxytocin rescues the lethal feeding behaviour in mouse newborns deficient for the imprinted Magel2 gene. *Hum Mol Genet*, 19(24):4895–905.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., and Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. *Nat Methods*, 9(7):676–82.

Schmittgen, T. D. and Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc*, 3(6):1101–8.

Schroeder, A., Mueller, O., Stocker, S., Salowsky, R., Leiber, M., Gassmann, M., Lightfoot, S., Menzel, W., Granzow, M., and Ragg, T. (2006). The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Mol Biol*, 7:3.

Shapiro, L. A., Ng, K., Zhou, Q.-Y., and Ribak, C. E. (2009). Subventricular zone-derived, newly generated neurons populate several olfactory and limbic forebrain regions. *Epilepsy Behav*, 14 Suppl 1:74–80.

Shillingford, J. M., Miyoshi, K., Robinson, G. W., Grimm, S. L., Rosen, J. M., Neubauer, H., Pfeffer, K., and Hennighausen, L. (2002). Jak2 is an essential tyrosine kinase involved in pregnancy-mediated development of mammary secretory epithelium. *Mol Endocrinol*, 16(3):563–70.

Shin, A. C., Townsend, R. L., Patterson, L. M., and Berthoud, H.-R. (2011). "Liking" and "wanting" of sweet and oily food stimuli as affected by high-fat diet-induced obesity, weight loss, leptin, and genetic predisposition. *Am J Physiol Regul Integr Comp Physiol*, 301(5):R1267–80.

Shingo, T. (2003). Pregnancy-Stimulated Neurogenesis in the Adult Female Forebrain Mediated by Prolactin. *Science*, 299(5603):117–120.

Shmela, M. E. and Gicquel, C. F. (2013). Human diseases versus mouse models: insights into the regulation of genomic imprinting at the human 11p15/mouse distal chromosome 7 region. *J Med Genet*, 50(1):11–20.

Shoji, H. and Kato, K. (2006). Maternal behavior of primiparous females in inbred strains of mice: a detailed descriptive analysis. *Physiol Behav*, 89(3):320–8.

Simmons, D. G., Rawn, S., Davies, A., Hughes, M., and Cross, J. C. (2008). Spatial and temporal expression of the 23 murine Prolactin/Placental Lactogen-related genes is not associated with their position in the locus. *BMC Genomics*, 9:352.

Simonin, F., Valverde, O., Smadja, C., Slowe, S., Kitchen, I., Dierich, A., Le Meur, M., Roques, B. P., Maldonado, R., and Kieffer, B. L. (1998). Disruption of the kappa-opioid receptor gene in mice enhances sensitivity to chemical visceral pain, impairs pharmacological actions of the selective kappa-agonist U-50,488H and attenuates morphine withdrawal. *EMBO J*, 17(4):886–97.

Singer, L. T., Salvator, A., Guo, S., Collin, M., Lilien, L., and Baley, J. (1999). Maternal psychological distress and parenting stress after the birth of a very low-birth-weight infant. *Jama*, 281(9):799–805.

Slotkin, R. K. and Martienssen, R. (2007). Transposable elements and the epigenetic regulation of the genome. *Nat Rev Genet*, 8(4):272–85.

Smit, A. F. (1999). Interspersed repeats and other mementos of transposable elements in mammalian genomes. *Curr Opin Genet Dev*, 9(6):657–63.

Smyth, G. K. (2005). Limma: linear models for microarray data. In *Bioinformatics and computational biology solutions using R and Bioconductor*, pages 397–420. Springer.

Snyder, S. K., Wessner, D. H., Wessells, J. L., Waterhouse, R. M., Wahl, L. M., Zimmermann, W., and Dveksler, G. S. (2001). Pregnancy-specific glycoproteins function as immunomodulators by inducing secretion of IL-10, IL-6 and TGF-beta1 by human monocytes. *Am J Reprod Immunol*, 45(4):205–16.

Soares, M. J. (2004). The prolactin and growth hormone families: pregnancy-specific hormones/cytokines at the maternal-fetal interface. *Reprod Biol Endocrinol*, 2:51.

Soares, M. J., Konno, T., and Alam, S. M. K. (2007). The prolactin family: effectors of pregnancy-dependent adaptations. *Trends Endocrinol Metab*, 18(3):114–21.

Soejima, H. and Higashimoto, K. (2013). Epigenetic and genetic alterations of the imprinting disorder Beckwith-Wiedemann syndrome and related disorders. *J Hum Genet*, 58(7):402–9.

Sokoloff, P., Giros, B., Martres, M. P., Bouthenet, M. L., and Schwartz, J. C. (1990). Molecular cloning and characterization of a novel dopamine receptor (D3) as a target for neuroleptics. *Nature*, 347(6289):146–51.

Somers, J. M., Goldner, E. M., Waraich, P., and Hsu, L. (2006). Prevalence and incidence studies of anxiety disorders: a systematic review of the literature. *Can J Psychiatry*, 51(2):100–13.

Sonigo, C., Dray, G., Roche, C., Cédrin-Durnerin, I., and Hugues, J.-N. (2014). Impact of high serum progesterone during the late follicular phase on IVF outcome. *Reprod Biomed Online*, 29(2):177–86.

Sonntag, K. C., Brenhouse, H. C., Freund, N., Thompson, B. S., Puhl, M., and Andersen, S. L. (2014). Viral over-expression of D1 dopamine receptors in the prefrontal cortex increase high-risk behaviors in adults: comparison with adolescents. *Psychopharmacology (Berl)*, 231(8):1615–26.

Stein, A., Gath, D. H., Bucher, J., Bond, A., Day, A., and Cooper, P. (1991). The relationship between post-natal depression and mother-child interaction. *The British Journal of Psychiatry*, 158(1):46–52.

Stein, S. P. and Charles, E. (1971). Emotional factors in juvenile diabetes mellitus: a study of early life experience of adolescent diabetics. *Am J Psychiatry*, 128(6):700–4.

Stewart, D. E., Robertson, E., Dennis, C.-L., Grace, S. L., and Wallington, T. (2003). Postpartum depression: literature review of risk factors and interventions. *Toronto: University Health Network Women's Health Program for Toronto Public Health*.

Surani, M. A., Barton, S. C., and Norris, M. L. (1984). Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature*, 308(5959):548–50.

Surani, M. A., Barton, S. C., and Norris, M. L. (1987). Influence of parental chromosomes on spatial specificity in androgenetic—-parthenogenetic chimaeras in the mouse. *Nature*, 326(6111):395–7.

Surani, M. A., Sasaki, H., Ferguson-Smith, A. C., Allen, N. D., Barton, S. C., Jones, P. A., and Reik, W. (1993). The inheritance of germline-specific epigenetic modifications during development. *Philos Trans R Soc Lond B Biol Sci*, 339(1288):165–72.

Svare, B. and Gandelman, R. (1976). Postpartum aggression in mice: the influence of suckling stimulation. *Horm Behav*, 7(4):407–16.

Svensson-Arvelund, J., Mehta, R. B., Lindau, R., Mirrasekhian, E., Rodriguez-Martinez, H., Berg, G., Lash, G. E., Jenmalm, M. C., and Ernerudh, J. (2015). The human fetal placenta promotes tolerance against the semiallogeneic fetus by inducing regulatory T cells and homeostatic M2 macrophages. *J Immunol*, 194(4):1534–44.

Swerdlow, N. R., Shoemaker, J. M., Bongiovanni, M. J., Neary, A. C., Tochen, L. S., and Saint Marie, R. L. (2005). Reduced startle gating after D1 blockade: effects of concurrent D2 blockade. *Pharmacol Biochem Behav*, 82(2):293–9.

Szyf, M., Weaver, I. C. G., Champagne, F. A., Diorio, J., and Meaney, M. J. (2005). Maternal programming of steroid receptor expression and phenotype through DNA methylation in the rat. *Front Neuroendocrinol*, 26(3-4):139–62.

Szymonowicz, W. and Yu, V. (1987). Severe pre-eclampsia and infants of very low birth weight. *Archives of disease in childhood*, 62(7):712–716.

Takayanagi, Y., Yoshida, M., Bielsky, I. F., Ross, H. E., Kawamata, M., Onaka, T., Yanagisawa, T., Kimura, T., Matzuk, M. M., Young, L. J., and Nishimori, K. (2005). Pervasive social deficits, but normal parturition, in oxytocin receptor-deficient mice. *Proc Natl Acad Sci U S A*, 102(44):16096–101.

Talamantes, F. and Ogren, L. (1988). The placenta as an endocrine organ: polypeptides. *The physiology of reproduction*, 2:2093–144.

Tan, E. K. and Tan, E. L. (2013). Alterations in physiology and anatomy during pregnancy. *Best Pract Res Clin Obstet Gynaecol*, 27(6):791–802.

Tanapat, P., Hastings, N. B., Reeves, A. J., and Gould, E. (1999). Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. *J Neurosci*, 19(14):5792–801.

Taupin, P. (2006). Neurogenesis in the adult central nervous system. *C R Biol*, 329(7):465–75.

Thomson, J. A. and Solter, D. (1988). The developmental fate of androgenetic, parthenogenetic, and gynogenetic cells in chimeric gastrulating mouse embryos. *Genes Dev*, 2(10):1344–51.

Torner, L. (2016). Actions of Prolactin in the Brain: From Physiological Adaptations to Stress and Neurogenesis to Psychopathology. *Front Endocrinol (Lausanne)*, 7:25.

Torner, L. and Neumann, I. D. (2002). The brain prolactin system: involvement in stress response adaptations in lactation. *Stress*, 5(4):249–57.

Torner, L., Toschi, N., Nava, G., Clapp, C., and Neumann, I. D. (2002). Increased hypothalamic expression of prolactin in lactation: involvement in behavioural and neuroendocrine stress responses. *Eur J Neurosci*, 15(8):1381–9.

Tucci, V., Lad, H. V., Parker, A., Polley, S., Brown, S. D. M., and Nolan, P. M. (2006). Gene-environment interactions differentially affect mouse strain behavioral parameters. *Mamm Genome*, 17(11):1113–20.

Tunster, S. J. (2009). *Excess Phlda2 as a mouse model of intrauterine growth restriction.* Cardiff University.

Tunster, S. J., Creeth, H. D. J., and John, R. M. (2015). The imprinted Phlda2 gene modulates a major endocrine compartment of the placenta to regulate placental demands for maternal resources. *Dev Biol*.

Tunster, S. J., Jensen, A. B., and John, R. M. (2013). Imprinted genes in mouse placental development and the regulation of fetal energy stores. *Reproduction*, 145(5):R117–37.

Tunster, S. J., McNamara, G. I., Creeth, H. D. J., and John, R. M. (2016). Increased dosage of the imprinted Ascl2 gene restrains two key endocrine lineages of the mouse Placenta. *Dev Biol*.

Tunster, S. J., Tycko, B., and John, R. M. (2010). The imprinted Phlda2 gene regulates extraembryonic energy stores. *Molecular and cellular biology*, 30(1):295–306.

Tunster, S. J., Van de Pette, M., and John, R. M. (2011). Fetal overgrowth in the Cdkn1c mouse model of Beckwith-Wiedemann syndrome. *Disease Models and Mechanisms*, 4(6):814–821.

Tunster, S. J., Van de Pette, M., and John, R. M. (2012). Impact of genetic background on placental glycogen storage in mice. *Placenta*, 33(2):124–7.

Tunster, S. J., Van De Pette, M., and John, R. M. (2014). Isolating the role of elevated Phlda2 in asymmetric late fetal growth restriction in mice. *Disease Models and Mechanisms*, 7(10):1185–1191.

Tycko, B. and Morison, I. M. (2002). Physiological functions of imprinted genes. *J Cell Physiol*, 192(3):245–58.

Ueki, A., Goto, K., Sato, N., Iso, H., and Morita, Y. (2006). Prepulse inhibition of acoustic startle response in mild cognitive impairment and mild dementia of Alzheimer type. *Psychiatry and clinical neurosciences*, 60(1):55–62.

Ueno, M., Nakayama, H., Kajikawa, S., Katayama, K., Suzuki, K., and Doi, K. (2002). Expression of ribosomal protein L4 (rpL4) during neurogenesis and 5-azacytidine (5AzC)-induced apoptotic process in the rat. *Histol Histopathol*, 17(3):789–98.

Ushikai, M., Asakawa, A., Sakoguchi, T., Tanaka, C., and Inui, A. (2011). Centrally administered urocortin 3 inhibits food intake and gastric emptying in mice. *Endocrine*, 39(2):113–7.

Valero De Bernabé, J., Soriano, T., Albaladejo, R., Juarranz, M., Calle, M. E., Martínez, D., and Domínguez-Rojas, V. (2004). Risk factors for low birth weight: a review. *Eur J Obstet Gynecol Reprod Biol*, 116(1):3–15.

van den Buuse, M. (2010). Modeling the positive symptoms of schizophrenia in genetically modified mice: pharmacology and methodology aspects. *Schizophrenia bulletin*, 36(2):246–270.

van Praag, H., Fleshner, M., Schwartz, M. W., and Mattson, M. P. (2014). Exercise, energy intake, glucose homeostasis, and the brain. *J Neurosci*, 34(46):15139–49.

Varmuza, S. and Mann, M. (1994). Genomic imprinting–defusing the ovarian time bomb. *Trends Genet*, 10(4):118–23.

Vaughan, R. A. and Foster, J. D. (2013). Mechanisms of dopamine transporter regulation in normal and disease states. *Trends Pharmacol Sci*, 34(9):489–96.

Venihaki, M., Sakihara, S., Subramanian, S., Dikkes, P., Weninger, S. C., Liapakis, G., Graf, T., and Majzoub, J. A. (2004). Urocortin III, a brain neuropeptide of the corticotropin-releasing hormone family: modulation by stress and attenuation of some anxiety-like behaviours. *J Neuroendocrinol*, 16(5):411–22.

Vereczkei, A. and Mirnics, K. (2011). Genetic predisposition to schizophrenia: what did we learn and what does the future hold? *Neuropsychopharmacol Hung*, 13(4):205–10.

Vinkenoog, R., Bushell, C., Spielman, M., Adams, S., Dickinson, H. G., and Scott, R. J. (2003). Genomic imprinting and endosperm development in flowering plants. *Mol Biotechnol*, 25(2):149–84.

Visentin, S., Grumolato, F., Nardelli, G. B., Di Camillo, B., Grisan, E., and Cosmi, E. (2014). Early origins of adult disease: low birth weight and vascular remodeling. *Atherosclerosis*, 237(2):391–9.

Voci, V. E. and Carlson, N. R. (1973). Enhancement of maternal behavior and nest building following systemic and diencephalic administration of prolactin and progesterone in the mouse. *J Comp Physiol Psychol*, 83(3):388–93.

Voogt, J. L., Lee, Y., Yang, S., and Arbogast, L. (2001). Regulation of prolactin secretion during pregnancy and lactation. *Prog Brain Res*, 133:173–85.

Vucetic, Z., Totoki, K., Schoch, H., Whitaker, K., Hill-Smith, T., Lucki, I., and Reyes, T. (2010). Early life protein restriction alters dopamine circuitry. *Neuroscience*, 168(2):359–370.

Waddell, B. and Atkinson, H. (1994). Production rate, metabolic clearance rate and uterine extraction of corticosterone during rat pregnancy. *Journal of endocrinology*, 143(1):183–190.

Wadhwa, P. D., Porto, M., Garite, T. J., Chicz-DeMet, A., and Sandman, C. A. (1998). Maternal corticotropin-releasing hormone levels in the early third trimester predict length of gestation in human pregnancy. *American journal of obstetrics and gynecology*, 179(4):1079–1085.

Walf, A. A. and Frye, C. A. (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat Protoc*, 2(2):322–8.

Walker, T. L., Vukovic, J., Koudijs, M. M., Blackmore, D. G., Mackay, E. W., Sykes, A. M., Overall, R. W., Hamlin, A. S., and Bartlett, P. F. (2012). Prolactin stimulates precursor cells in the adult mouse hippocampus. *PLoS One*, 7(9):e44371.

Walker, T. L., Wierick, A., Sykes, A. M., Waldau, B., Corbeil, D., Carmeliet, P., and Kempermann, G. (2013). Prominin-1 allows prospective isolation of neural stem cells from the adult murine hippocampus. *J Neurosci*, 33(7):3010–24.

Walker, W. H., Fitzpatrick, S. L., and Saunders, G. F. (1990). Human placental lactogen transcriptional enhancer. Tissue specificity and binding with specific proteins. *Journal of Biological Chemistry*, 265(22):12940–12948.

Waraich, P., Goldner, E. M., Somers, J. M., and Hsu, L. (2004). Prevalence and incidence studies of mood disorders: a systematic review of the literature. *Can J Psychiatry*, 49(2):124–38.

Watanabe, D. and Barlow, D. P. (1996). Random and imprinted monoallelic expression. *Genes Cells*, 1(9):795–802.

Watkin, H., Richert, M. M., Lewis, A., Terrell, K., McManaman, J. P., and Anderson, S. M. (2008). Lactation failure in Src knockout mice is due to impaired secretory activation. *BMC Dev Biol*, 8:6.

Watson, E. D. and Cross, J. C. (2005). Development of structures and transport functions in the mouse placenta. *Physiology (Bethesda)*, 20:180–93.

Weickert, C. S., Webster, M. J., Colvin, S. M., Herman, M. M., Hyde, T. M., Weinberger, D. R., and Kleinman, J. E. (2000). Localization of epidermal growth factor receptors and putative neuroblasts in human subependymal zone. *J Comp Neurol*, 423(3):359–72.

Weinstock, M. (2008). The long-term behavioural consequences of prenatal stress. *Neurosci Biobehav Rev*, 32(6):1073–86.

Wiemers, D. O., Shao, L.-J., Ain, R., Dai, G., and Soares, M. J. (2003). The mouse prolactin gene family locus. *Endocrinology*, 144(1):313–25.

Wilkins, J. F. and Haig, D. (2003). What good is genomic imprinting: the function of parent-specific gene expression. *Nat Rev Genet*, 4(5):359–68.

Williamson, C. M., Turner, M. D., Ball, S. T., Nottingham, W. T., Glenister, P., Fray, M., Tymowska-Lalanne, Z., Plagge, A., Powles-Glover, N., Kelsey, G., Maconochie, M., and Peters, J. (2006). Identification of an imprinting control region affecting the expression of all transcripts in the Gnas cluster. *Nat Genet*, 38(3):350–5.

Windhorst, U. and Johansson, H. (2012). *Modern techniques in neuroscience research*. Springer Science & Business Media.

Winterhager, E. and Gellhaus, A. (2014). The role of the CCN family of proteins in female reproduction. *Cell Mol Life Sci*, 71(12):2299–311.

Wise, R. A. (1996). Neurobiology of addiction. *Curr Opin Neurobiol*, 6(2):243–51.

Wise, R. A. (2008). Dopamine and reward: the anhedonia hypothesis 30 years on. *Neurotox Res*, 14(2-3):169–83.

Wise, R. A. and Rompre, P. P. (1989). Brain dopamine and reward. *Annu Rev Psychol*, 40:191–225.

Witt, W. P., Litzelman, K., Spear, H. A., Wisk, L. E., Levin, N., McManus, B. M., and Palta, M. (2012a). Health-related quality of life of mothers of very low birth weight children at the age of five: results from the Newborn Lung Project Statewide Cohort Study. *Qual Life Res*, 21(9):1565–76.

Witt, W. P., Wisk, L. E., Cheng, E. R., Hampton, J. M., and Hagen, E. W. (2012b). Preconception mental health predicts pregnancy complications and adverse birth outcomes: a national population-based study. *Matern Child Health J*, 16(7):1525–41.

Woodside, B. (2016). Mood, Food, and Fertility: Adaptations of the Maternal Brain. *Compr Physiol*, 6(3):1493–518.

Wright, K. P. and Johnson, J. V. (2008). Evaluation of extended and continuous use oral contraceptives. *Ther Clin Risk Manag*, 4(5):905–11.

Wright, R. L., Gilmour, G., and Dwyer, D. M. (2013). Microstructural analysis of negative anticipatory contrast: A reconsideration of the devaluation account. *Learn Behav*, 41(4):353–9.

Wu, J. A., Johnson, B. L., Chen, Y., Ha, C. T., and Dveksler, G. S. (2008a). Murine pregnancy-specific glycoprotein 23 induces the proangiogenic factors transforming-growth factor beta 1 and vascular endothelial growth factor a in cell types involved in vascular remodeling in pregnancy. *Biology of reproduction*, 79(6):1054–1061.

Wu, J. A., Johnson, B. L., Chen, Y., Ha, C. T., and Dveksler, G. S. (2008b). Murine pregnancy-specific glycoprotein 23 induces the proangiogenic factors transforming-growth factor beta 1 and vascular endothelial growth factor a in cell types involved in vascular remodeling in pregnancy. *Biol Reprod*, 79(6):1054–61.

Wu, Z., Autry, A. E., Bergan, J. F., Watabe-Uchida, M., and Dulac, C. G. (2014). Galanin neurons in the medial preoptic area govern parental behaviour. *Nature*, 509(7500):325–30.

Wurmbach, E., Yuen, T., and Sealfon, S. C. (2003). Focused microarray analysis. *Methods*, 31(4):306–16.

Yang, Y. H., Dudoit, S., Luu, P., Lin, D. M., Peng, V., Ngai, J., and Speed, T. P. (2002). Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. *Nucleic Acids Res*, 30(4):e15.

Yoneda, T., Saitou, K., Asano, H., Mizushige, T., Matsumura, S., Eguchi, A., Manabe, Y., Tsuzuki, S., Inoue, K., and Fushiki, T. (2009). Assessing palatability of long-chain fatty acids from the licking behavior of BALB/c mice. *Physiol Behav*, 96(4-5):735–41.

Yoon, H., Enquist, L. W., and Dulac, C. (2005). Olfactory inputs to hypothalamic neurons controlling reproduction and fertility. *Cell*, 123(4):669–82.

Yoshihashi, H., Maeyama, K., Kosaki, R., Ogata, T., Tsukahara, M., Goto, Y.-i., Hata, J.-i., Matsuo, N., Smith, R. J., and Kosaki, K. (2000). Imprinting of human GRB10 and its mutations in two patients with Russell-Silver syndrome. *The American Journal of Human Genetics*, 67(2):476–482.

Zeng, Y., Brydges, N. M., Wood, E. R., Drake, A. J., and Hall, J. (2015). Prenatal glucocorticoid exposure in rats: programming effects on stress reactivity and cognition in adult offspring. *Stress*, 18(3):353–361.

Zhang, T. Y., Labonté, B., Wen, X. L., Turecki, G., and Meaney, M. J. (2013). Epigenetic mechanisms for the early environmental regulation of hippocampal glucocorticoid receptor gene expression in rodents and humans. *Neuropsychopharmacology*, 38(1):111–23.

Zhang, X. and Firestein, S. (2002). The olfactory receptor gene superfamily of the mouse. *Nat Neurosci*, 5(2):124–33.

Zhang, Y., Szustakowski, J., and Schinke, M. (2009). Bioinformatics analysis of microarray data. *Methods Mol Biol*, 573:259–84.

Zheng, Y., Zhang, Y.-M., and Ni, X. (2016). Urocortin 2 But Not Urocortin 3 Promotes the Synaptic Formation in Hipppocampal Neurons via Induction of NGF Production by Astrocytes. *Endocrinology*, 157(3):1200–10.

Zhou, Q. Y., Grandy, D. K., Thambi, L., Kushner, J. A., Van Tol, H. H., Cone, R., Pribnow, D., Salon, J., Bunzow, J. R., and Civelli, O. (1990). Cloning and expression of human and rat D1 dopamine receptors. *Nature*, 347(6288):76–80.